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Association between biomarkers of subclinical inflammation and nerve conduction in individuals with recently diagnosed type 1 and type 2 diabetes.

Dissertation

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Abstract

Subclinical inflammation has been implicated in the development of diabetic distal sensorimotor polyneuropathy (DSPN), but the definition of DSPN varied in previous studies focussing on different aspects of DSPN (symptoms, signs or nerve conduction studies (NCS)). Studies using NCS to assess DSPN are scarce and only few studies have been conducted applying recommended composite scores which include a combination of symptoms, signs and NCS as well as sum scores of NCS of different nerves. The aim of the present study is to investigate the association between biomarkers reflecting different aspects of subclinical inflammation and DSPN assessed as motor and sensory nerve conduction velocity (NCV) as well as sum scores of each modality and a composite score combining symptoms, signs and NCS.

513 individuals with recently diagnosed type 2 (n=352) or type 1 (n=161) diabetes were included in a cross-sectional analysis of the baseline population of the observational German Diabetes Study. Biomarkers of subclinical inflammation were assessed using serum samples, including acute-phase proteins (hsCRP), proinflammatory cytokines (IL-6, IL-18), anti-inflammatory adipokines (total, high-molecular-weight [HMW] adiponectin) and soluble adhesion molecules as markers of endothelial dysfunction (sICAM-1, sE-selectin). Motor NCV (median, ulnar, peroneal nerve) and sensory NCV (median, ulnar, sural nerve) were assessed and NCV sum scores of each modality were calculated. Associations between biomarkers of subclinical inflammation and NCV and presence of DSPN (as a combination of symptoms, signs and electrophysiological measures) were estimated using multiple linear and logistic regression models.

High serum IL-6 was associated with the presence of DSPN and reduced motor NCV but not sensory NCV in type 2 but not type 1 diabetes. In addition, high serum total and HMW adiponectin as well as their ratio were associated with the presence of DSPN and decreased motor and sensory NCV in type 2 diabetes. In type 1 diabetes high serum adiponectin was associated with higher motor NCV. Inconsistent associations were observed for hsCRP, IL-18 and adhesion molecules of which IL-18 and sICAM-1 were positively associated with sensory NCV in type 2 diabetes only.

Results indicate an implication of IL-6 in the slowing of motor NCV and DSPN in type 2 diabetes. Reverse associations of adiponectin with DSPN and NCV in type 1 versus type 2 diabetes suggest that the pathogenesis of DSPN may be diverging in these different types of diabetes in the course of which subclinical inflammation seems to play a more prominent role in type 2 diabetes. Lastly results suggest that different nerves are prone differentially to inflammatory-related nerve damage emphasizing the relevance of sum scores.

Zusammenfassung

Subklinische Inflammation steht in Zusammenhang mit der Entwicklung der diabetischen distalen sensomotorischen Polyneuropathie (DSPN). In bisherigen Studien variierten DSPN-Definitionen und fokussierten auf unterschiedliche DSPN-Aspekte (Symptome, Zeichen oder Nervenfunktionsmessungen (NFM)). NFM oder empfohlene Kombinationsmaße als Indikatoren für eine DSPN werden selten genutzt. Letztere kombinieren Symptome, Zeichen und NFM oder beziehen sich auf Summenwerte von NFM verschiedener Nerven. Diese Arbeit untersucht den Zusammenhang verschiedener Aspekte der subklinischen Inflammation mit DSPN, erfasst als sensorische und motorische Nervenleitgeschwindigkeit (NLG), Summenwerte jeder Modalität und als Kombinationsmaß aus Symptomen, Zeichen und NFM.

513 neu diagnostizierte Typ 2 (n=352) und Typ 1 (n=161) Diabetespatienten der Baseline Population der Deutschen Diabetes-Studie gingen in die Querschnittsanalyse ein. Biomarker der subklinischen Inflammation aus Serumproben umfassten: Akut-phase Protein (hsCRP), proinflammatorische Zytokine (IL-6, IL-18), antiinflammatorische Adipokine (total, high-molecular-weight [HMW] Adiponektin) und lösliche Adhäsionsmoleküle (s-ICAM-1, sE-Selektin). Motorische NLG (Nervus medianus, ulnaris, peroneus) und sensorische NLG (Nervus medianus, ulnaris, suralis) wurden gemessen und Summenwerte für jede Modalität berechnet. Zusammenhänge der subklinischen Inflammation mit NLG sowie DSPN-Präsenz (kombiniert aus Symptomen, Zeichen, NFM) wurden mit multiplen linearen und logistischen Regressionsmodellen berechnet.

Höheres IL-6 im Serum war mit DSPN-Präsenz und verminderter motorischer und sensorischer NLG bei Personen mit Typ-2-Diabetes, nicht jedoch Typ-1-Diabetes assoziiert. Höheres Gesamt- und HMW-Adiponektin im Serum sowie ihr Verhältniswert waren mit DSPN-Präsenz und verminderter motorischer und sensorischer NLG in Typ 2 Diabetes assoziiert. Bei Personen mit Typ-1-Diabetes war höheres Serum-Adiponektin mit höherer motorischer NLG verbunden. Inkonsistente Ergebnisse ergaben sich für hsCRP, IL-18 und Adhäsionsmoleküle von denen ausschließlich IL-18 und sICAM-1 positiv mit sensorischer NLG in Typ-2-Diabetespatienten assoziiert waren.

Diese Studie deutet auf eine Relevanz von IL-6 in der Verlangsamung von NLG und DSPN in Typ-2-Diabetes hin. Inverse Zusammenhänge von Adiponektin mit DSPN und NLG in Typ-1- versus Typ-2-Diabetes legen nahe, dass sich die Pathogenese dieser beiden Erkrankungen unterscheidet, wobei subklinische Inflammation eine prominentere Rolle bei Typ-2-Diabetes zu spielen scheint. Außerdem scheinen verschiedene Nerven unterschiedlich anfällig für inflammations-assoziierte Schäden zu sein, was die Relevanz von Summenwerten betont.

Abbreviations

BMI	body mass index
CRP	C-reactive protein
CV	coefficient of variation
d	distance
DSPN	distal sensorimotor polyneuropathy
ELISA	enzyme-linked immunosorbent assay
GDS	German Diabetes Study
HbA1c	glycated haemoglobin
HMW	high-molecular-weight adiponectin
HMW/total adiponectin	HMW-to-total-adiponectin-ratio
hsCRP	high-sensitivity C-reactive protein
IL-6	interleukin-6
IL-18	interleukin-18
m	meter
MI	myocardial infarction
mm	millimeter
MNAP	motor nerve amplitude
MNCV	motor nerve conduction velocity
MNSI	Michigan Neuropathy Screening Instrument
ms	millisecond
μV	microvolt
mV	millivolt
NCS	nerve conduction studies
NCV	nerve conduction velocity
NDS	Neuropathy Disability Score
NFM	Nervenfunktionsmessung
NLG	Nervenleitgeschwindigkeit
NSAID	non-steroidal-anti-inflammatory drugs
NSS	Neuropathy Symptom Score
s	second
SD	standard deviation
sICAM-1	soluble intercellular adhesion molecule-1
SNAP	sensory nerve amplitude
SNCV	sensory nerve conduction velocity
t	time = latency
v	velocity

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1 Introduction

The contribution of an inflammatory component to both type 1 and type 2 diabetes is well established. A mostly metabolic understanding of these diseases is gradually turning into a paradigm evolving around a state of subclinical inflammation (Kolb & Mandrup-Poulsen 2005). Subclinical inflammation refers to chronic low-grade inflammation which becomes evident at the systemic level as indicated by altered levels of circulating pro- and anti-inflammatory cytokines (Kolb & Mandrup-Poulsen 2005).

Accumulating evidence suggests, that subclinical inflammation may also be implicated in the development of distal sensorimotor polyneuropathy (DSPN). DSPN is the most common form of peripheral neuropathy, an important microvascular complication of diabetes mellitus (Boulton et al. 2004a). The association between subclinical inflammation and DSPN remains poorly elucidated as the interpretation of previous studies is limited. First, previous study populations are rather inhomogeneous including either only type 1 or type 2 diabetic patients or a combination of both subgroups. However, evidence suggests a differential pathophysioloy of DSPN in these two types of diabetes (Callaghan et al. 2012). Second, DSPN definition varies between studies as different combinations of *subjective* symptoms and signs and *objective* electrophysiological measures are applied. These different measurements reflect different aspects of the pathophysiology of DSPN and it has been recommended to combine symptoms, signs and electrophysiological measures to diagnose DSPN (England et al. 2005; Tesfaye et al. 2010).

Nevertheless, electrophysiological measures, namely nerve conduction studies (NCS), comprise a central role in the process of diagnosing DSPN. They provide objective and reliable DSPN assessment which is required to confirm DSPN diagnosis (Boulton et al. 2004a; Tesfaye et al. 2010). In addition, abnormalities in nerve conduction appear to be an early indicator of DSPN which may be detected before symptoms and signs evolve (Boulton et al. 2004a). However, evidence suggests that the extent of nerve conduction velocity (NCV) slowing may differ between nerves and that composite nerve conduction test scores may enhance the detection of DSPN in an individual (Charles et al. 2010). Studies investigating the association of subclinical inflammation with NCV as a key parameter of NCS or with composite sum scores in particular are scarce.

The aim of the present study is to investigate the association between systemic levels of biomarkers of subclinical inflammation and DSPN**.** To overcome limitations of previous studies subgroups of type 1 and type 2 diabetes individuals will be

investigated seperately. Furthermore, DSPN will be assessed as recommended using a combination of symptoms, signs and electrophysiological measures as well as sensory and motor NCV and sum scores of each modality.

1.1 Diabetes mellitus: definition and prevalence

Diabetes mellitus refers to a pathological metabolic condition characterized by hyperglycaemia resulting from deficient insulin secretion and/or resistance to insulin action. Diabetes mellitus has reached epidemic levels over the past decades. In 2015 the worldwide diabetic population was estimated to comprise 415 million people. An increase in incidence and prevalence is expected, reaching 642 million by the year 2040 (IDF Diabetes Atlas 2015). Although numerous subtypes of diabetes mellitus have been identified, the vast majority of cases are attributable to either type 1 or type 2 diabetes, which comprise 5-10% and 90-95% of all cases, respectively (Maahs et al. 2010).

Type 2 diabetes is the consequence of resistance to insulin action combined with inadequate compensatory insulin secretion response. This condition results in relative insulin deficiency during early stages of disease eventually progressing to absolute insulin deficiency during the course of disease (ADA 2014). Type 2 diabetes often remains undiagnosed for many years as hyperglycaemia develops gradually and resulting symptoms remain unnoticed by affected individuals (ADA 2014). Numerous factors associated with greater risk of type 2 diabetes have been identified which include increased age, hypertension and dyslipidaemia (e.g. increased triglycerides and low-density lipoprotein cholesterol, decreased high-density lipoprotein cholesterol). In addition, obesity as well as a characteristic body fat accumulation particularly in the abdominal region increase the risk of developing type 2 diabetes (Fletcher et al. 2002; Gress et al. 2000; Qi et al. 2012; Weycker et al. 2009). Furthermore, lifestyle factors namely smoking and a lack of physical activity have been shown to contribute to an increased risk of developing type 2 diabetes (Chang 2012; Cho et al. 2009; Pan et al. 1997; Tuomilehto et al. 2001). Beyond this, a genetic predisposition for type 2 diabetes has been identified, which appears to be even stronger than in type 1 diabetes patients (ADA 2014).

The coexistence of a dysregulated state of glucose metabolism (impaired fasting glucose, impaired glucose tolerance, diabetes mellitus) with abnormally increased cholesterol, hypertension and (abdominal) obesity is referred to as “metabolic syndrome”. The metabolic syndrome is often present in type 2 diabetes and

comprises a substantial risk factor for cardiovascular diseases and strokes (Air & Kissela 2007; Isomaa et al. 2001).

Given a relative rather than an absolute insulin deficiency at least during the early stages of disease, oral medication targeting differential mechanisms of insulin secretion usually precedes insulin substitution (ADA 2014).

Type 1 diabetes is characterized by an immune-mediated destruction of pancreatic beta cells resulting in absolute insulin deficiency. It usually shows an acute onset with symptoms such as polyuria, polydipsia, weight loss, fatigue and ketoacidosis all of which reflect insulin deficiency (Maahs et al. 2010). Type 1 diabetes shows a peak incidence at 10-14 years and therefore comprises the most common diabetes form in children and adolescence (Maahs et al. 2010). Although the incidence in adults is lower, one out of four type 1 diabetic patients is diagnosed as an adult (Haller et al. 2005; Maahs et al. 2010). Usually boys and girls are equally affected, however in regions with a higher incidence (e.g. Europe) an exaggeration of male patients is recorded (Maahs et al. 2010). Beyond age, sex and geographic location as potential risk factors for type 1 diabetes, genetic predisposition seems to play a role in the development of type 1 diabetes. Nevertheless, exact pathophysiological mechanisms remain unknown (Maahs et al. 2010).

One complication commonly found in both type 1 and type 2 diabetes consists in the development of peripheral neuropathy, which will be outlined in greater detail in the following section (Boulton et al. 2005a).

1.2 Diabetic distal sensorimotor polyneuropathy (DSPN)

1.2.1 Definition and classification of DSPN

Diabetic peripheral neuropathies present as a heterogeneous group of syndromes varying in topography, course and clinical signs and symptoms (Boulton et al. 2005a; Dyck et al. 1993). Heterogeneity of this diabetic complication challenges explicit but exhaustive definition and classification and no universally accepted taxonomy has emerged yet (Vinik et al. 2013). Nevertheless, a generally recognized definition refers to diabetic peripheral neuropathy as “the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes” (Boulton et al. 1998). A simple classification further differentiates between peripheral neuropathies which affect the autonomic versus the somatic nervous system. However, “peripheral neuropathy” usually refers to the latter category of which the most common and clinically relevant form is comprised by chronic sensorimotor

polyneuropathy, which accounts for 75% of diabetic neuropathies (Pop-Busui et al. 2017; Shaw et al. 1998). Therefore, the present work will focus on this particular form of neuropathy.

Chronic sensorimotor polyneuropathy starts in the most distal nerves of the lower and upper limbs and is therefore also referred to as “distal sensorimotor polyneuropathy” (DSPN). Although DSPN may be present in the lower as well as the upper limbs it predominantly affects the lower limbs (Boulton et al. 2005a). DSPN occurs symmetrically in both limbs and shows chronic progression, expanding from the feet to distal parts of the lower limbs (Boulton et al. 2005a). If no treatment is introduced, a subclinical state of peripheral sensory and motor nerve dysfunction eventually results in the development of actual symptoms and signs of DSPN (Boulton et al. 1998; Boulton et al. 2004a; Tesfaye et al. 2010).

1.2.2 Symptoms and signs of DSPN

Symptoms subjectively reported by patients are distinguished from signs which are identified during a clinical examination (Boulton et al. 2004a). Symptoms and signs are considered to be either “negative” (representing decreased responsiveness to stimuli and absence of sensation) or “positive” (referring to sensory or motoric experiences arising spontaneously or as response to stimuli often resulting in intensified sensations or pain) (Apfel et al. 2001).

The most frequently experienced “positive” symptoms and signs arise from dysfunction in sensory axons and include burning or deep aching pain, electric or stabbing sensations and paraesthesia (abnormal sensation, which is not necessarily unpleasant e.g. tingling) (Boulton et al. 2004a). Less frequently reported symptoms are hyperaesthesia (overly increased sensitivity to pain), allodynia (perception of a non-painful stimulation as painful) and symptoms that arise from dysfunction of motor axons such as spasms, cramps or fasciculation (Boulton et al. 2004a; Dyck 1988; Vinik et al. 2013).

Typical “negative” symptoms and signs include numbness as well as sensory loss of thermal and pain perception (mediated by small-fibre impairment) and touch and vibration perception (mediated by large-fibre impairment). Moreover, the ankle reflex might be reduced or absent and proprioception (the sense of the relative position of neighbouring parts of the body and strength of effort being employed in movement) may be impaired. This in turn causes sensory ataxia and postural instability, which increases the risk of falls and fractures (Boulton et al. 2004a; Pasnoor et al. 2013).

Symptoms and signs are highly subjective as they are based on the patient´s reports or the examiner´s evaluations during clinical examination. Consequently, diagnosing and estimating severity of DSPN can result in highly subjective descriptions of a patients condition (Tesfaye et al. 2010). To increase objectivity of assessment, numerous instruments have been developed that score subjectively reported symptoms and clinically assessed signs according to a strict protocol (Dyck et al. 1986). Examples of instruments suitable in clinical practice and research include the Neuropathy Symptom Score (NSS), the Neuropathy Disability Score (NDS) and the Michigan Neuropathy Screening Instrument (MNSI) (Asad et al. 2010; Dyck et al. 1993). As the NSS and NDS have been applied in the present study these instruments are presented in Table A-1 and Table A-2 (Appendix) and are described in more detail in section 2.4.1.

1.2.3 Risk factors and pathophysiology of DSPN

Numerous factors have been identified that put diabetic patients at risk to develop DSPN. In type 1 as well as in type 2 diabetes, risk factors most consistently associated with DSPN include increased age, duration of disease and elevated levels of HbA1c indicating poor glycaemic control (Forrest et al. 1997; Harris et al. 1993; Shaw et al. 1998; Tesfaye et al. 1996; Walters et al. 1992). HbA1c is the proportion of glycated haemoglobin of total haemoglobin and reflects glucose levels over the past three months (Sacks 2012). Furthermore, height appears to comprise a risk factor for DSPN, as DSPN is length-dependent, starting in the lower limbs expanding upward during the course of disease (Forrest et al. 1997; Shaw et al. 1998; Tapp et al. 2003). In addition, lifestyle factors such as smoking and physical inactivity have been shown to impact the development of DSPN (Balducci et al. 2006; Forrest et al. 1997; Sands et al. 1997).

The pathophysiological mechanisms implicated in the development of DSPN remain largely uncertain. Nevertheless, research suggests a multifactorial pathogenesis of DSPN in diabetes mellitus. It is suspected that in this state of metabolic dysregulation, oxidative and inflammatory stress is implicated in the development of peripheral nerve damage which again translates into the typical symptoms and signs of DSPN outlined above (Pop-Busui et al. 2017).

In the context of the present understanding of the pathogenesis of DSPN, chronic hyperglycaemia as a result of poor glycaemic control plays a key role. Chronic hyperglycaemia contributes to mitochondrial overproduction of reactive oxygen species and hence oxidative stress. Oxidative stress refers to a situation of imbalance between the production of reactive oxygen species and antioxidant defence (Feldman 2003).

This imbalance appears to be implicated in alterations of mainly five metabolic pathways: activation of the polyol pathway, formation of advanced glycation end products, increased expression of the receptors for advanced glycation end products, increased activation of protein kinase C isoforms and activation of hexosamine pathway (Giacco & Brownlee 2010; Luis-Rodríguez et al. 2012; Sandireddy et al. 2014). A common end point of these pathways is an imbalance of vasoactive agents, which impair microvascular perfusion, causing ischaemia in vasa nervorum that lead to endoneurial hypoxia in peripheral nerves. This condition, also referred to as endothelial dysfunction, results in peripheral nerve damage and subsequent dysfunction of peripheral nerves (Malik et al. 1993; Pasnoor et al. 2013; Sandireddy et al. 2014; Tesfaye et al. 1994).

However, it has been shown that intensive glycaemic control is not capable to prevent neuropathy entirely and beneficial effects were larger in type 1 than in type 2 diabetes (Callaghan et al. 2012). This suggests differential mechanisms in the pathogenesis of DSPN in type 1 and type 2 diabetes and emphasizes that especially in type 2 diabetes factors other than hyperglycaemia are of relevance in the development of DSPN (Callaghan et al. 2012). Indeed, research suggests that hypertension, dyslipidaemia (elevated total cholesterol and triglycerides), obesity and a history of cardiovascular disease contribute to an increased risk of DSPN (Callaghan et al. 2012; Sands et al. 1997). The latter variables have been mainly investigated in samples of type 2 diabetic patients, but a few studies indicate that they might be of relevance in type 1 diabetic patients as well (Forrest et al. 1997; Tesfaye et al. 1996).

Above and beyond these well established risk factors for DSPN, increasing evidence points towards a role of inflammatory processes in the development of DSPN in both type 1 and type 2 diabetes (Doupis et al. 2009; Herder et al. 2009; Hussain et al. 2013; Jude et al. 1998). Associations between subclinical inflammation and DSPN will be outlined in greater detail in section 1.4.

1.2.4 Prevalence of DSPN and the diabetic foot syndrome

The prevalence of DSPN reported in diabetic patients throughout the past decades shows great variability ranging from 3.6% up to 50% (Boulton et al. 2005a; Dyck 1988; Young et al. 1993). This variability is in part a consequence of heterogeneity of study populations investigated varying regarding type of diabetes, age or duration of disease all of which have been shown to impact DSPN development. Beyond that, some studies described the prevalence of DSPN in population-based samples while others investigated patients from hospitals or diabetes clinics (Ziegler et al. 2008).

Furthermore, definition criteria and diagnostic tests of DSPN applied differ between studies, focussing on different aspects of DSPN (Boulton et al. 2005a).

For example, in population-based samples of type 2 diabetic patients of different age, neuropathic symptoms and abnormalities in electrophysiological measures were identified in 8.5% of *recently diagnosed* patients and in 41.5% after 10 years of disease onset (Partanen et al. 1995). In a different study, abnormalities in vibration perception threshold indicated the presence of DSPN in 3.6% of *newly* diagnosed type 2 diabetic patients and in 12.7% of patients with known type 2 diabetes (Shaw et al. 1998). A prevalence of 46.3% was identified in a population-based study which applied symptoms and signs to diagnose DSPN after a mean duration of disease of 9 years (Hanley et al. 2005). Estimates of DSPN in type 2 diabetic individuals in the German population are derived from the MONICA/KORA survey, revealing that 28% of patients showed symptoms of DSPN (Ziegler et al. 2008).

In different samples of type 2 diabetic in-patients recruited from diabetic clinics, symptoms and signs were used to assess DSPN. A prevalence of 32.1% was reported in individuals aged between 19 to 90 years and an average disease duration of 6 years and of up to 50% when taking into account only those aged above 60 years (Young et al. 1993). Similarly, a prevalence of 50.8% was identified in patients with an average age of 64 years and a longer disease duartion (Van Acker et al. 2009).

In clinical populations of type 1 diabetic patients different symptoms and signs were used to assess DSPN. Its prevalence ranged from 12.9% to 28.5% after more than 10 years of disease duration in populations displaying a wide age range (Cabezas-Cerrato 1998; Tesfaye et al. 2005; Van Acker et al. 2009; Young et al. 1993). In one clinical trail, signs and NCS were used to identify DSPN in individuals who had received their diagnoses 1 to 15 years ago. In this sample the prevalence increased from 7% to 35% during 13 years after study closeout (Albers et al. 2010).

Although data regarding the prevalence of DSPN vary to a great extent, the careful conclusion might be drawn that at least one manifestation of DSPN is present in at least 20% of diabetic individuals and may reach up to 50% during the course of disease (Boulton et al. 2005a).

In any case, DSPN has to be considered as a common complication in diabetes mellitus. As such it comprises a substantial global health burden as it is associated with considerable adverse health outcomes (Boulton et al. 2005b). The most frequent and severe complication of DSPN is the development of the diabetic foot syndrome (Boulton 2004b; Rathur & Boulton 2007). Loss of protective sensation in DSPN permits minor injury of feet to remain unnoticed. In combination with ischaemia due to atherosclerosis, injuries are less likely to heal. Eventually the dermis is penetrated

which by definition comprises an ulceration, the key feature of the diabetic foot syndrome (Boulton 2004b). The lifetime risk of developing foot ulceration in diabetes patients is 15% (Boulton 2004b). Food ulceration in turn is a precursor in 85% of nontraumatic lower extremity amputations, which are associated with increased morbidity and mortality (Singh et al. 2005). Thus, the estimated mortality rate at one year following amputation is 13% to 40%, and further increases to 39% to 80% at 5 years. These numbers emphasize the severity of complications associated with DSPN as mortality rates following amputation even surpass those of certain kinds of malignant tumours (Singh et al. 2005).

1.3 Diagnostic criteria of DSPN: a central role of nerve conduction studies (NCS)

Although extensive research has been conducted to develop algorithms and diagnostic tools to assess DSPN, no generally applied gold standard has emerged to diagnose DSPN. In accordance with the definition of diabetic peripheral neuropathy stated above, the diagnosis of DSPN first requires the exclusion of causes unrelated to diabetes. These may include chronic inflammatory polyneuropathy, hypothyroidism, uraemia or vitamin B12 deficiency (Boulton et al. 2005a). Furthermore, it is generally agreed on that at least two abnormalities from the following categories have to become evident to diagnose DSPN: neuropathic symptoms, signs upon clinical neurological examination, electrophysiological testing (NCS), quantitative sensory testing and autonomic function tests (Boulton et al. 2005a; Dyck 1988). However, the most accurate diagnosis of DSPN is accomplished in combining subjective symptoms and signs and objective electrophysiological testing (England et al. 2005). These minimal criteria define the following stages of DSPN: possible (presence of symptoms or signs), probable (presence of symptoms and signs), confirmed (presence of abnormal nerve conduction and a symptom or a sign or both) or subclinical DSPN (abnormal nerve conduction without the presence of signs) (Tesfaye et al. 2010).

The aforementioned staging emphasizes the central role of NCS in the process of diagnosing DSPN. First, a confirmed diagnosis of DSPN *requires* abnormalities in NCS as they provide an objective and reliable tool to diagnose DSPN being independent of subjective reports (Boulton et al. 2005a). Second, impaired nerve conduction comprises an early indicator of DSPN, allowing NCS to detect abnormalities in nerve functioning even if symptoms or signs have not yet evolved (Boulton et al. 2004a). Hence, it allows to identify subclinical DSPN which has been estimated to affect 50% of diabetes patients (Boulton et al. 2004a). Given its central

role in the process of diagnosing DSPN in the present study, the following paragraph will first describe NCS in greater detail. Following this, NCV as a measure of DSPN will be emphasized as the present study focuses on this measure in particular.

1.3.1 Performance and parameters of NCS: Latency, nerve conduction velocity (NCV) and amplitude

NCS are electroneurographical measurements involving electrical stimulation of a peripheral nerve at one site and measurement of the evoked response at a different site along the nerve. Specific anatomical landmarks are used for stimulation and recording site, which are described in more detail in section 2.4.2. In *motor* NCS, the peripheral nerve is stimulated in at least two sites and the response is recorded as compound muscle action potential over a muscle supplied by the stimulated nerve. In *sensory* NCS, the peripheral nerve is stimulated in only one site and the evoked response is recorded from purely sensory portions of the stimulated nerve (Conrad & Bischoff 1998; Poeck & Hacke 2001; Stöhr & Pfister 1993). NCS in sensory and motor nerves are mainly characterized by the three parameters latency, NCV and amplitude (Conrad & Bischoff 1998).

Latency

Latency describes the time (measured in milliseconds [ms]) it takes for the electrical impulse to travel from the stimulation to the recording sites in sensory and motor nerves (Conrad & Bischoff 1998).

NCV

NCV quantifies the speed of conduction in nerves and reflects velocity of the fastest axons in a nerve. Sensory NCV is calculated by dividing the distance between stimulation and recording electrode (d in millimetre [mm]) by latency (t in ms), hence v = d/t (in m per s). Sensory NCV may be assessed orthodromically (proximal stimulation site and distal recording site) or antidromically (distal stimulation site and proximal recording site) (Conrad & Bischoff 1998). Motor NCV is calculated by dividing the difference between distal and proximal stimulation site (Δd in mm) by the difference in latency between distal and proximal stimulation site to recording site (Δt in ms), hence v = Δd/Δt (in m per s). Differences are used to minimize the confounding impact of neuromuscular propagation (Conrad & Bischoff 1998; Poeck & Hacke 2001; Stöhr & Pfister 1993). A decrease in NCV can be recorded as a consequence of peripheral nerve dysfunction. It may result from structural damage (e.g. demyelination) or functional alteration (e.g. suppression of sodium ion channels within the nodal region)

in peripheral nerves (Boulton et al. 2005b; Arezzo & Zotova 2002). In contrast, an increase of NCV occurs with increasing temperature (Oh 1993; Todnem et al. 1989).

Amplitude

Amplitude describes the magnitude of the response in sensory and motor nerves (in µV and mV, respectively), reflecting the number of responding fibres and the synchronicity of their activity at maximal stimulation (Conrad & Bischoff 1998). A decrease in amplitude results from a reduced myelinated fibre density which in turn has been shown to result from axonal degeneration (Boulton et al. 2005b; Russell et al. 1996). Changes in both NCV and amplitude have been associated with DSPN (Dyck et al. 1985). The present study will focus on NCV only.

1.3.2 NCV as an outcome measure of DSPN

An abnormality of nerve conduction tests, which is frequently subclinical, appears to be the first objective quantitative indication of diabetic polyneuropathy. Thus, for epidemiologic surveys or controlled clinical trials the use of nerve conduction testing has been suggested as an early and reliable indicator of the occurrence of this condition (Tesfaye 2010). However, evidence suggests that the extent of NCV slowing may differ between nerves (Charles et al. 2010; Partanen et al. 1995). For example, it has been shown that diabetes duration, as a risk factor for DSPN, only affected NCV of the lower limbs, but not the upper limps (Charles et al. 2010). In addition, sensory deficits in the sural nerve appear to represent the earliest electrophysiological changes in DSPN (Arezzo 1997; Partanen et al. 1995). It is feasible to combine NCV of individual nerves to form composite NCV test scores to improve the detection of DSPN in an individual patient. Indeed, this approach has been recommended repeatedly and some studies have shown, that composite nerve conduction test scores provide a better indicator of nerve injury and subsequent functional deficits than single nerve conduction tests (Weisman et al. 2013). Beyond this, composite nerve conduction test scores also appear to be superior to symptoms or clinical signs in detecting DSPN (Dyck et al. 2003; Weisman et al. 2013). In addition, composite nerve conduction test scores best predicted 4-year incidence of DSPN (Weisman et al. 2013). Based on these results it can be concluded that composite nerve conduction test scores may represent a useful indicator of nerve injury which may be applied to stratify risk in individuals with regard to DSPN development in clinical and research protocols (Weisman et al. 2013). Thereby, a lower composite nerve conduction test score indicates more severely impaired peripheral nerve function (Weisman et al. 2013).

1.4 Subclinical Inflammation in type 1 and type 2 diabetes and its association with DSPN

After decades of research the contribution of an inflammatory component to type 1 and type 2 diabetes is well established and evolves around a state of subclinical inflammation (Devaraj et al. 2010; Goldberg 2009; King 2008; Kolb & Mandrup-Poulsen 2005). Subclinical inflammation refers to chronic low-grade inflammation which becomes evident at the systemic level as indicated by altered levels of circulating pro- and anti-inflammatory cytokines (Kolb & Mandrup-Poulsen 2005). In type 1 and type 2 diabetes subclinical inflammation appears to be characterized by alterations of specific immune mediators rather than a uniform upregulation of all inflammatory cytokines (Kolb & Mandrup-Poulsen 2005).

The cause and origins of altered levels of immune mediators in diabetes are diverse. In type 1 diabetes production of proinflammatory cytokines seems to be enhanced by activation of autoimmunity (Goldberg 2009; King 2008). In type 2 diabetes a conjunction of persistent alterations of several mechanisms is assumed, which result in an increased systemic concentration of proinflammatory cytokines. These mechanisms include intracellular changes such as the activation of kinases (Jun N-terminal kinase and IkB kinase-β) and transcription factors (nuclear factor kB), histological changes reflected by the infiltration of immune cells and altered secretion patterns of various tissues, including islets, adipose tissue, liver and muscle cells (Goldberg 2009; Herder et al. 2013b; Navarro & Mora 2006). In type 2 diabetes, visceral adipose tissue in particular, has been shown to be an important determinant of systemic inflammation (Devaraj et al. 2010; Greenfield et al. 2004; Navarro & Mora 2005). It contains activated macrophages that together with adipocytes produce inflammatory adipokines, hence cytokines secreted from adipose tissue (Goldberg 2009; Kang et al. 2005; Lin et al. 2001). However, mechanisms by which chronic inflammation provokes diabetes mellitus are not clear. Beyond this, it is important to point out that subclinical inflammation may only represent one pathophysiological mechanism among others contributing to manifest disease (Herder et al. 2013b; Thorand et al. 2003).

Nevertheless, alterations of immune mediators become evident years before the diagnosis of type 1 or type 2 diabetes and have been further shown to predict the development of these diseases (Devaraj et al. 2010). In addition, anti-inflammatory therapy results in beneficial metabolic effects (Donath & Shoelson 2011). These findings support the assumption that subclinical inflammation contributes to the development of diabetes mellitus and may not be a mere consequence of

hyperglycaemia-triggered cytokine dysregulation in fully established diabetes (Crook 2004; Pickup & Crook 1998).

Despite extensive research conducted regarding the presence of subclinical inflammation in diabetes far less is known regarding its association with diabetic complications. Evidence suggests an implication of subclinical inflammation in macrovascular complications such as cardiovascular disease as well as endothelial dysfunction which is implicated in microvascular complications. Nevertheless, it remains uncertain to what extent subclinical inflammation contributes to DSPN as one out of several microvascular complications. A limited number of previous studies in diabetes patients and population-based samples demonstrated that biomarkers of subclinical inflammation are indeed linked to different aspects of DSPN and NCV. However, underlying pathophysiological mechanisms remain unknown. (Doupis et al. 2009; Herder et al. 2009; Herder et al. 2013a; Herder et al. 2017; Hussain et al. 2013).

A multitude of immune mediators have been investigated in the context of diabetes mellitus. In the present study, the definition of subclinical inflammation comprises the acute-phase protein C-reactive protein (CRP), the proinflammatory cytokines interleukin 6 (IL-6) and interleukin 18 (IL-18), the anti-inflammatory adipokine adiponectin and biomarkers of endothelial dysfunction, namely soluble intercellular adhesion molecule-1 (sICAM-1) and sE-selectin. Previous research strongly suggests that these immune mediators are implicated in the development of diabetes mellitus while only a limited number of studies investigated their implication in DSPN (Devaraj et al. 2010; Doupis et al. 2009; Herder et al. 2017; Nicoletti et al. 2001; Thorand et al. 2005). The following paragraph will outline the implication of these inflammatory mediators in type 1 and type 2 diabetes and their association with DSPN.

Acute-phase protein CRP and proinflammatory cytokines IL-6 and IL-18

IL-6 and CRP were most extensively examined in the context of diabetes and diabetic complications (Devaraj et al. 2010; Pickup & Crook 1998). IL-6 is a proinflammatory cytokine which is produced in different tissues in response to various stimuli. In addition to activated leucocytes and monocytes, endothelial cells, mesenchymal cells, fibroblasts and adipocytes have been shown to be involved in IL-6 production. (Akira et al. 1993; Devaraj et al. 2010; Esposito et al. 2002; Pradhan et al. 2001). IL-6 is a pleiotropic cytokine with various effects on haematopoesis, fibrogenesis, endothelial cell activation, inflammation and stimulation of the acute-phase response (Barnes et al. 2011; Tanaka et al. 2014). CRP is one important mediator of the acute-phase response. It is derived from hepatocytes in which biosynthesis is IL6-dependent and contributes to host defense against acute infection (Pradhan et al. 2001; Tanaka et al.

2014). The proinflammatory cytokine IL-18 is mainly produced by antigen-presenting cells and is a pleiotropic factor involved in inflammation, innate and acquired immune response, artheriosclerotic plaque destabilization as well as apoptosis of endothelial cells and expression of adhesion molecules (Aso et al. 2003; Biet et al. 2002; Mariño & Cardier 2003; Stuyt et al. 2003). Although these immune mediators display many beneficial effects in the immune response, defective and chronic upregulation of these agents has been shown to be implicated in disease.

Systemic levels of IL-6, IL-18 and CRP are found to be elevated in type 2 and type 1 diabetes and further precede and predict the diagnosis of these diseases (Devaraj et al. 2010; Harms et al. 2015; Pickup & Crook 1998; Pradhan et al. 2001; Ryba-Stanislawowska et al. 2014; Thorand et al. 2005). In line with this, elevated levels of these proinflammatory agents correlate with insulin resistance (CRP, IL-6) were shown to induce hyperglycaemia and subsequent hyperinsulinaemia (IL-6) and evoke beta-cell dysfunction and destruction eventually progressing to diabetes (IL-18) (Kolb & Mandrup-Poulsen 2005; Navarro & Mora 2006; Nicoletti et al. 2001; Pradhan et al. 2001).

Furthermore, numerous studies have also demonstrated that IL-6, IL-18 and CRP are related to an increased risk of cardiovascular disease and endothelial dysfunction (Barnes et al. 2011; Blankenberg et al. 2004; Danesh et al. 2004; Danesh et al. 2008; Jefferis et al. 2011).

An increasing body of evidence also suggests that IL-6, IL-18 and CRP are associated with DSPN as a microvascular complication in diabetes mellitus. In type 2 diabetic patients IL-6 and CRP were associated with composite neuropathy scores (MNSI), some specific neuropathic deficits (impaired ankle reflex, vibration perception threshold, pinprick, foot appearance) and food ulceration as a long-term complication of DSPN (Herder et al. 2009; Herder et al. 2013a; Zubair et al. 2012). IL-6 was further related to decreased peroneal NCV, while no association was found between CRP and sural NCV in other samples of type 2 diabetes patients (Kang et al. 2005; Kökoglu et al. 2009; Magrinelli et al. 2015). An inverse association was demonstrated between IL-18 and pain perception as well as impaired ankle reflex in type 2 diabetes (Herder et al. 2009). Associations between neuropathy scores and signs of neuropathy were independent of duration of disease and glycaemic control suggesting that immune activation may not be a mere consequence of hyperglycaemia but contributing independently to neuropathic deficits (Herder et al. 2009; Herder et al. 2013a).

In a population-based sample the MNSI score was positively correlated with IL-6 and IL-18 and not associated at all with CRP (Herder et al. 2013a). Further, a prospective population-based study indicated that higher levels of IL-6 and hsCRP

precede the onset of DSPN (defined using the MNSI) in an age- and sex-adjusted model, although results did not remain significant for hsCRP after adjusting for additional known risk factors for DSPN. Nevertheless, the prospective design of the study underscores a role of inflammation in the pathogenesis of DSPN. IL-18 was not associated with incident DSPN in this study (Herder et al. 2017). As in type 2 diabetes patients IL-6 was related to decreased peroneal NCV (Di Iorio et al. 2006).

Results from a pooled sample of type 1 and type 2 diabetic patients revealed a positive association between CRP and NSS as well as NDS scores (Doupis et al. 2009).

Studies investigating the relationship between biomarkers of subclinical inflammation (IL-6, IL-18, CRP) and different measures of DSPN in samples of type 1 diabetic individuals are not available. Furthermore, the association between IL-18 and NCV has not been investigated so far.

Adiponectin as an anti-inflammatory factor

Adiponectin belongs to the adipokines as it is a protein mostly secreted by adipocytes of white adipose tissue (Berg et al. 2001; Kershaw & Flier 2004). Nevertheless, adiponectin levels fall with increased adiposity and are decreased in obesity (Nigro et al. 2014). In contrast, adiponectin levels increase with age and women show approximately 30% to 50% higher levels than men (Hotta et al. 2000; Nigro et al. 2014). Although the exact underlying regulatory mechanisms remain unclear several effects of adiponectin are well described. Adiponectin has been found to act as insulin-sensitizing, anti-inflammatory and antiatherogenic protein (Nigro et al. 2014; Singer et al. 2012). It is further inversely correlated with systemic levels of total cholesterol, low-density lipoprotein cholesterol and triglycerides while positively associated with high-density lipoprotein cholesterol (Ljubic et al. 2015; Ouchi & Walsh 2007; Yamamoto et al. 2002). Different isoforms of adiponectin have been identified in serum and plasma (Hara et al. 2006; Kizer et al. 2012; Nigro et al. 2014). Some authors suggested that high-molecula-weight (HMW) adiponectin may be the biologically most active form, as it correlates most closely with insulin sensitivity and metabolic abnormalities (Hara et al. 2006; Nakashima et al. 2006). Furthermore, the HMW-to-total-adiponectin-ratio (HMW/total adiponectin) has been suggested to better reflect the biological activity of adiponectin (Hara et al. 2006; Nakashima et al. 2006). However, others were not able to confirm an advanced utility of HMW or the HMW-to–total-adiponectin-ratio compared to total adiponectin (Almeda-Valdes et al. 2010; Singer et al. 2012; Zhu et al. 2010)

Given its anti-inflammatory, vasoprotective and metabolic effects, it seems feasible to assume that adiponectin may be implicated in a wide array of diseases. In

deed, research strongly supports an implication of adiponectin in diabetes mellitus and cardiovascular disease and some research also points towards an implication of adiponectin in diabetic microvascular complications (Fantuzzi 2008; Lieb et al. 2012; Ljubic et al. 2015; Nigro et al. 2014). However, the role of adiponectin in these diseases is little understood as findings remain conflicting.

Lower adiponectin levels are observed in type 2 diabetes patients and even predict disease, while increased levels are consistently found in type 1 diabetes patients (Fantuzzi 2008; Hadjadj et al. 2005; Krakoff et al. 2003; Saraheimo et al. 2005). The inverse relationship between adiponectin and incidence of type 2 diabetes is thought to reflect a widely agreed paradigm according to which, obesity is associated with inflammation in adipose tissue and that proinflammatory factors suppress adiponectin production. Lower levels of adiponectin in turn promote inflammation in a self-sustaining manner as its own anti-inflammatory capacity is reduced and further contribute to increased insulin resistance (Fantuzzi 2008). Supporting this paradigm, first lower adiponectin levels in type 2 diabetes and non-diabetic individuals are associated with higher body mass index (BMI) and greater central obesity in particular and weight loss was shown to increase adiponectin levels (Engeli et al. 2003; Hotta et al. 2000; Mather et al. 2008). Second, IL-6 as a proinflammatory cytokine was shown to suppress adiponectin production (Fantuzzi 2008; Saraheimo et al. 2005). Third, animal studies have shown that application of exogenous adiponectin reversed insulin resistance and protected against diet-induced insulin resistance (Maeda et al. 2002; Yamauchi et al. 2001). However, in humans the inverse association between adiponectin and type 2 diabetes leveled off above approximately 20 μg/ml and experimental data of adiponectin infusion are lacking (Herder et al. 2013b; Kizer et al. 2012). Thus, it remains uncertain whether changes in adiponectin are a determinant or consequence of insulin resistance (Herder et al. 2013b).

Similary, mechanisms underlying the upregulation of adiponectin and its physiological implication in type 1 diabetes are poorly understood (Fantuzzi 2008; Hadjadj et al. 2005; Saraheimo et al. 2005). In this inflammatory condition in the context of autoimmune disease, adiponectin levels are not related to BMI (Fantuzzi 2008; Saraheimo et al. 2005). However, as in type 2 diabetes, adiponectin levels are positively associated with insulin sensitivity, although insulin sensitivity has been shown to be lower at any given adiponectin level compared with healthy controls (Pereira et al. 2012). Taken together, there is some evidence pointing towards differential functions of adiponectin in obesity-associated versus classical inflammatory conditions (Kolb & Mandrup-Poulsen 2005).

Regarding the association between adiponectin and cardiovascular disease one might expect a protective role of adiponectin, as it exerts antiatherogenic effects and has beneficial effects on important risk factors for cardiovascular disease such as dyslipidaemia (Lubjic et al. 2015; Singer et al. 2012). However, only few studies demonstrated this inverse association between adiponectin levels and risk for cardiovascular disease in the general population and type 2 diabetes (Pischon et al. 2004; Schulze et al. 2005). Most studies that investigated this matter reported contrasting results. Thus, positive associations between adiponectin levels and cardiovascular disease and all-cause mortality were reported in individuals with prevalent cardiovascular disease (Dekker et al. 2008; Wu et al. 2014), chronic kidney disease (Menon et al. 2006), patients with chronic heart failure (Kistorp et al. 2006) as well as in the general population (Laughlin et al. 2007) and type 2 diabetic patients (Singer et al. 2012). Furthermore, no association was found between adiponectin and risk for cardiovascular disease in the general population (Satter et al. 2006), in patients with pre-existing cardiovascular disease (Satter et al. 2006) and type 2 diabetes patients (Krzyzanowski et al. 2009). In addition to that, type 1 diabetic individuals display increased risk of cardiovascular disease despite increased adiponectin levels (Fantuzzi 2008; Frystyk et al. 2005). Potential explanations for confliciting results have been proposed and include adiponectin resistance and reverse causality (Satter 2011; Singer et al. 2012). The latter implicates that elevated adiponectin may reflect underlying cardiovascular disease as adiponectin release is increased by atrial natriuretic peptide and brain natriuretic peptide (Sattar 2011; Singer et al. 2012).

Little research has been conducted regarding the association between adiponectin and diabetic microvascular complications. For example, higher adiponectin levels were found in type 2 diabetic individuals with retinopathy compared to those without retinopathy (Jung et al. 2014; Kato et al. 2008; Pradeepa et al. 2015). With regard to DSPN, adiponectin levels were found to be related to increased odds for the presence of neuropathy (defined using the MNSI) in type 2 diabetic individuals (Jung et al. 2014). However, adiponectin was also found to be related to a lower incidence of food ulceration while no association was found with NCV (Kökoglu et al. 2009; Matsuda et al. 2004; Zubair et al. 2012). In a population-based sample adiponectin was not found to be related to neuropathy (defined using the MNSI) (Herder et al. 2013a). However, in a prospective population-based sample lower systemic levels of adiponectin were associated with a higher risk of DSPN (MNSI) over 6.5 years (Herder et al. 2017).

Soluble adhesion molecules sICAM-1 and sE-selectin

Adhesion molecules expressed on endothelial cells interact with leukocytes and trigger transmigration of these cells into the subendothelial space. In healthy organisms this interaction targets inflammation to appropriate sites (Gearing & Newman 1993; Goldberg 2009). However, endothelial dysfunction indicates a general proinflammatory and procoagulative state in the vasculature and comprises a first step towards artherosclerosis (Gearing & Newman 1993). Adhesion molecules shedded from the surface of activated endothelium appear in the circulation as measurable levels of soluble adhesion molecules and indicate endothelial activation and comprise markers of endothelial dysfunction and vascular inflammation (Devaraj et al. 2006). The present study focuses on the following two adhesion molecules. The integrin sICAM-1 is produced by endothelial cells and other cells including epithelial cells, fibroblasts, leukocytes, and tumour cells while the selectin sE-selectin is exclusively produced by endothelial cells providing a more specific marker of endothelial dysfunction (Fasching et al. 1996; Gearing & Newman 1993).

A great body of research has shown that diabetes mellitus is associated with endothelial dysfunction evidenced by increased levels of sICAM-1 and sE-selectin in type 2 as well as type 1 diabetes (Blüher et al. 2002; Devaraj et al. 2006, Pham et al. 2012). Both sICAM-1 and sE-selectin are thought to be induced by proinflammatory cytokines and chronic as well as acute hyperglycaemia (Blüher et al. 2002; Ceriello et al. 1998; Esposito et al. 2002; Peschel & Niebauer 2003.). However, research regarding the association between adhesion molecules and DSPN is scarce. In pooled samples of type 1 and type 2 diabetic patients sICAM-1 and sE-selectin were found to be higher in neuropathic compared to non-neuropathic patients. Furthermore, they were associated with composite neuropathy scores (NSS and NDS) as well as decreased peroneal NCV after 5 years of follow-up (Doupis et al. 2009; Jude et al. 1998). In a population-based sample sICAM-1 was found to be positively related to the MNSI score ans impaired ankle reflex. Nevertheless, this association did not remain significant after adjusting for potentially confounding variables including risk factors for diabetes and the metabolic syndrome (Herder et al. 2013a). In a prospective population-based study sICAM-1 was associated with higher risk of DSPN (MNSI) over 6.5 years and with progression of DSPN (defined as change in DSPN) in an age- and sex-adjusted model (Herder et al. 2017). While the association between sICAM-1 and *incidence* of DSPN did not remain significant after adjusting for further anthropometric and metabolic variables, the association between sICAM-1 and *progression* of DSPN strengthened with further adjustment and indicates that vascular inflammation and endothelial activation promotes the progression of DSPN (Herder et al. 2017).

Taken together, proinflammatory cytokines (CRP, IL-6, IL-18) and adhesion molecules (sICAM-1, sE-selectin) are elevated in type 1 and type 2 diabetes. The anti-inflammatory adipokine adiponectin is found to be decreased in type 2 diabetes while elevated in type 1 diabetes. These findings support the notion of an implication of subclinical inflammation in diabetes mellitus. Given this enhanced understanding of diabetes mellitus as an inflammation-related condition, it seems reasonable to investigate if subclinical inflammation may as well be involved in the development of diabetic microvascular complications such as DSPN. A growing body of evidence indeed suggests that subclinical inflammation is related to DSPN in diabetes mellitus (Devaraj et al. 2010; Goldberg 2009; King 2008; Kolb & Mandrup-Poulsen 2005). However, the exact nature of the association between different biomarkers of subclinical inflammation and DSPN remains poorly characterized as existing results are inconsistent and even diverging in some cases. Inconsistency of results may in part be a consequence of different DSPN measures being used in different studies, which usually assess only single aspects (e.g. only symptoms or only signs) of DSPN. In addition, study populations are rather inhomogeneous as some studies included both, type 1 and type 2 diabetic individuals (Doupis et al. 2009) while others focused on type 2 diabetic individuals only (Magrinelli et al. 2015) or on the general population comprising a substantial proportion of individuals with prediabetes or type 2 diabetes (Herder et al. 2013a).

Only limited research has been conducted regarding the association between different biomarkers of subclinical inflammation and NCV in particular. Nevertheless, NCV is of increasing relevance in the diagnosis of DSPN. It is an early indicator of this condition and provides a highly reliable measure that above and beyond the aforementioned facts is required to accomplish a confirmed diagnosis of DSPN (Boulton et al. 2005a). Moreover, NCV sum scores have been shown to detect nerve damage much better than nerve conduction of single nerves (Dyck et al. 2003; Weisman et al. 2013). However, evidence regarding the association between sum scores as a sensitive marker of DSPN and subclinical inflammation is lacking. Up to date only a single study in type 2 diabetic patients investigated this association and reported a positive association between the macrophage biomarker CD 163 (as indicator of inflammatory processes) and a neuropathy sum score combining several parameters of nerve function of different nerves (Kallestrup et al. 2015). Nevertheless, similar analyses have not yet been performed for other biomarkers of subclinical inflammation.

1.5 Study aims and hypothesis

Given a lack of consistent data elucidating how subclinical inflammation is related to DSPN, the general aim of the present study is to systematically investigate the association between biomarkers reflecting different aspects of subclinical inflammation (acute-phase proteins, proinflammatory cytokines, adiponectin and soluble adhesion molecules) and DSPN. To overcome limitations of previous studies this analysis will carefully stratify by diabetes type. Furthermore, only patients with recently diagnosed type 1 or type 2 diabetes are included (duration since diagnosis ≤ 12 month). This approach was chosen to reduce the impact of potential confounding comorbidities, which usually develop throughout disease. Beyond this, DSPN will be operationalized through a recommended combination of symptoms, signs and electrophysiological measures (England et al. 2005; Weisman et al. 2013). As the latter play a central role in diagnosing DSPN, the present study will focus on NCV as a measure of DSPN.

Previous studies suggest that NCV may be affected differentially in different nerves. Hence, slowing of NCV appears to occur to a greater extent in the lower (peroneal and sural nerve) than in the upper limbs (median and ulnar nerve) (Weisman et al. 2013). In the present study NCV will be assessed in each of the aforementioned nerves to capture a wide array of possible effects of subclinical inflammation on NCV. In line with this, NCV measures will be taken of sensory (median, ulnar and sural nerve) and motor (median, ulnar and peroneal nerve) properties of these nerves. Furthermore, NCV sum scores combining NCV measures of different single nerves of the same modality will be used as they have been recommended to more accurately detect and estimate the severity of peripheral nerve damage in an individual (Dyck et al. 2003; Weisman et al. 2013).

Based on current research it is hypothesized that IL-6, CRP, IL-18, sICAM-1, sE-selectin and adiponectin are associated with DSPN in type 1 and type 2 diabetes. Different isoforms of adiponectin have been identified of which HMW adiponectin and the HMW-to-total-adiponectin-ratio have been suggested to be the biologically most active form in the context of insulin resistance. (Hara et al. 2006; Kizer et al.2012; Nigro et al. 2014). Therefore, total and HMW adiponectin as well as their ratio will be considered. DSPN will be assessed as motor and sensory NCV as well as sum scores of each modality and as a combined score of symptoms, signs and electrophysiological measures according to modified Toronto Consensus criteria (Dyck et al. 2011).

2 Study population, material and methods

2.1 Study population

The German Diabetes Study (GDS) is a prospective observational study which investigates the natural history of recent-onset diabetes, the progression of the disease and the development of diabetes-associated complications (Szendroedi et al. 2016). The aim of the GDS is to identify prognostic factors that influence the course of disease and the development of disease-related complications. All procedures performed meet the standards of the Declaration of Helsinki and were approved by the ethics committee of Heinrich Heine University, Düsseldorf (current reference number 4508, previous reference number 2478). The GDS is registered at ClinicalTrail. gov (identifier number: NCT01055093). Informed written consent was obtained from all participants.

Participants were recruited through advertisement in local newspapers and on the institutional homepage and through flyers at local general practitioners. Potential participants were screened for eligibility during recruitment via a telephone interview, during which a trained research assistant determined whether all inclusion and exclusion criteria were met.

Inclusion criteria for entry into the study population of GDS comprised diagnosis of either type 1 or type 2 diabetes, duration since diagnosis of diabetes ≤1 year and participants had to be aged between 18-69 years. Exclusion criteria included acute infection, current pregnancy or immunosuppressive therapy, symptomatic peripheral arterial disease, history of malignancies, severe congestive heart failure, renal or liver disease or secondary diabetes due to other conditions than type 1 or type 2 diabetes mellitus. Participants were also not eligible when suffering from any severe psychiatric or addictive disease.

The GDS was started in 2005, follow-up data are being collected at 5-year-intervals for up to 20 years. The present cross-sectional analysis is based on baseline data obtained from participants who entered the study between its start in September 2005 and December 2011 (n=513).

Participants arrived at the clinical research centre after overnight fasting (≥8h). They were further asked to refrain from taking glucose-lowering medication 3 days prior to their visit and if insulin-treated, the last dose had to be applied the night before the visit.

Figure 1 gives an overview on baseline data collection to assess sociodemographic, clinical and behavioural data of relevance in the present study and for metabolic and immunological phenotyping.

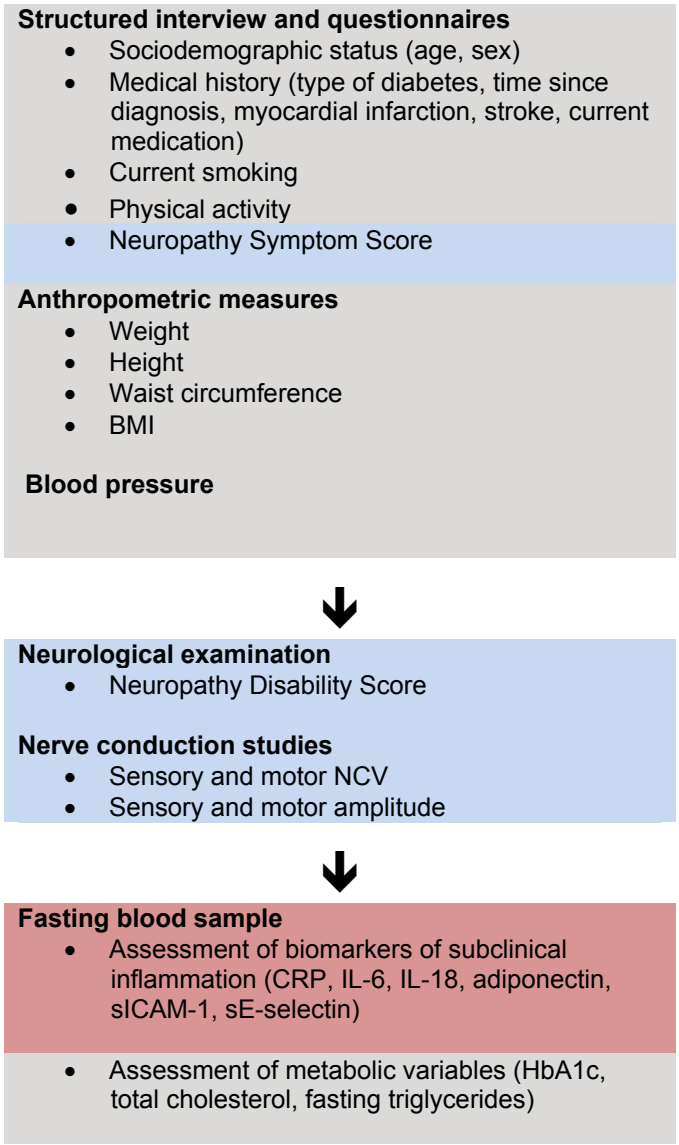

Figure 1: Baseline data collection in the present study for detailed clinical, metabolic and immunological phenotyping. Variables are arranged according to their role in the present study. Variables assessed as potential confounding variables appear in gray, outcome measures in blue, and exposures appear in red.

2.2 Sociodemographic, clinical and behavioural data collection

Information on age, diabetes type, diabetes duration, medical history (myocardial infarction [MI], stroke) and medication (non-steroidal-anti-inflammatory drugs [NSAID], antihypertensive and lipid-lowering medication) was obtained by self-report during the standardized interview. If necessary, a self-reported diabetes diagnosis was confirmed by records of fasting plasma glucose level of ≥7.0 mmol/L or ≥11.1 mmol/L at 120 min

during a standardized 75-g oral glucose tolerance test in accordance with diagnostic criteria introduced by the American Diabetes Association (ADA 2014). Type 1 diabetes was confirmed by presence of autoantibodies, classical symptoms, and/or ketoacidosis at onset of disease. Physical activity and smoking were assessed via questionnaires and dichotomous variables were created. A participant was classified as “physical active” if participating regularly in sports for at least one hour per week during leisure time in summer and winter. Current smoking was defined as smoking at least one cigarette (or cigar, pipe) per day. Blood pressure was measured following a 15-minute resting-period in a sitting position three times on the right arm using a validated automated device (OMROM 705IT, Mannheim, Germany). The mean of the second and third measurement were used for analysis.

2.3 Anthropometric measurements

BMI was calculated as weight in kilograms divided by height in meters squared. Body weight and height were measured on a calibrated scale with statiometer with participants in light clothing and without shoes to the nearest of 0.5 kg and 0.1 cm, respectively (seca 674, Hamburg, Germany). Waist circumference was measured midway between the lower rib margin and the superior anterior iliac spine.

2.4 Neuropathy assessment and definition of stages of DSPN

2.4.1 Clinical assessment of DSPN

The NSS and the NDS were applied to assess neuropathy as proposed by Young (Young et al. 1993). Both instruments are presented in the appendix. Symptoms and signs were included in clinical neuropathy scores only if considered to be most likely due to DSPN but not other neurological diseases by an experienced investigator. Vitamin B6 or B12 deficiency as potential cause of polyneuropathy cannot be ruled out with certainty, as their concentrations were not assessed. However, individuals with clinical symptoms or signs of manifest Vitamine B6 or B12 deficiency were excluded from the present analysis.

NSS: Subjective reported symptoms of DSPN, namely burning/ numbness/ paraesthesia (score 2) and weakness/ cramping/ pain (score 1) are assessed. Moreover, the location of these symptoms is assessed: feet (score 2), lower leg (score 1), other location (score 0). Furthermore, exacerbation (during the night only/ during day and night time/ during the day only) and improvement of symptoms (walk/ stand/

sit or lay down) are scored anticlimactic from 2 to 0. One point is added, if a patient is awakened from sleep by symptoms. Scores range from 0 to 10, any score greater than one or one indicates abnormality (Dyck et al. 1993). In more detail, 3 to 4 points are categorized as mild symptoms, 5 to 6 points as moderate symptoms and 7 to 10 as severe symptoms (Young et al. 1993).

NDS: Signs of DSPN are assessed as a clinical composite score based on the following four tests performed by trained physicians during a neurological examination: First, the ankle reflex is elicited in a sitting position using an appropriate hammer and the response is graded as normal (score 0), decreased/elicited only after reinforcement (score 1), absent (score 2). Second, vibration perception is measured with a Rydel-Seiffert AB-125c-64 Hz tuning fork (Barthelmes, Tuttlingen, Germany) at the great toe, three times on each side. Scoring is based on the time period for which the participant reports to be able to sense the vibration from the tuning fork. Vibration perception is considered as being present (score 0) if vibration is sensed relatively long (5/8 to 8/8), as being diminished or absent (score 1) if vibration is sensed shorter than normal (0.5/8 to 4.5/8) or is not perceived at all (0/8). Values were age-adjusted (Martina et al. 1998). Third, pain perception is assessed via pin-prick test three times on the dorsal side of each foot and is scored as normal (3/3, score 0), reduced (2/3 or 1/3, score1) or absent (0/3, score 1). Fourth, temperature perception is measured using a TipTherm at the dorsal side of the foot (Gesellschaft für neurologische Diagnostik, Düsseldorf, Germany). TipTherm is a pen-like device with a metal cylinder on one end capable of causing a cold sensation at room temperature and a plastic cylinder on the other side, in contrast eliciting a warm sensation at room temperature. Each cylinder is applied to the skin for one second in a random order and the participant has to decide which of the two touches feels colder. Three trials are conducted on each side. Thermal perception was classified as normal (score 0) if two out of three trials were rated correctly and as abnormal (score 1) if at least two out of three trials were rated incorrectly. Both feet are tested and scored independently and scores of each foot are added to obtain the final score, which ranges from 0 to 10, with the maximum score indicating a complete loss of sensory modalities and absent reflexes.

2.4.2 Electrophysiological measurements

Motor and sensory nerve conduction velocities (MNCV, SNCV) and amplitudes (MNAP, SNAP) were assessed with a Nicolet nerve stimulator S403 and a Nicolet VikingQuest electromyograph (Nicolet Biomedical, Madison, Wisconsin, USA) using Nicolet VikingQuest surface electrodes (Natus medical, San Carlos, California, USA). In all cases the measurements were carried out in the right arm and the right leg at a

skin temperature of 33-34°C. Limbs were warmed if necessary to maintain local skin temperature during the entire period of measurement. Specific anatomical landmarks were used for both stimulation and recording site as reported before (Ziegler et al. 1988): MNCV (in m/s) and MNAP (in mV) were measured in the median nerve (antecubital fossa-wrist; recording electrodes over the musculus abductor pollicis brevis), ulnar nerve (sulcus N. ulnaris-wrist; recording electrodes over the musculus abductor digiti minimi) and peroneal nerve (head of the fibula-ankle joint; recording electrodes over the musculus extensor digitorum brevis). SNCV (in m/s) and SNAP (in µV) were measured orthodromically in the median nerve (digit II-wrist) and ulnar nerve (digit V-wrist) and antidromically in the sural nerve (distal part of musculus gastrocnemiums-lateral malleolus).

2.4.3 Definition of neuropathy stages

Neuropathy stages were defined according to modified Toronto Consensus criteria (Dyck et al. 2011). Stages of DSPN comprised subclinical DSPN (stage 1a: NDS≤2, NSS≤2, peroneal MNCV<2.5th percentile and sural SNCV<2.5th percentile and/or sural SNAP<2.5th percentile), confirmed asymptomatic DSPN (stage 1b: NDS≥3, NSS≤2, peroneal MNCV<2.5th percentile and sural SNCV<2.5th percentile and/or sural SNAP<2.5th percentile) and confirmed symptomatic DSPN (stage 2a: NSS≥3, peroneal MNCV<2.5th percentileand sural SNCV<2.5th percentile and/or sural SNAP<2.5th percentile). For this study the presence of DSPN was defined as present if criteria for the stages of subclinical, confirmed asymptomatic or confirmed symptomatic DSPN were met and a dichotomous variable was created (DSPN absent/ DSPN present).

2.5 Laboratory measurements

Table 1 gives an overview on laboratory measurement (assays applied, inter- and intra-coefficients of variation [CV]) of metabolic parameters (HbA1c, total cholesterol, triglycerides), acute-phase proteins and proinflammatory cytokines (hsCRP, IL-6, IL-18), anti-inflammatory adipokines (total and HMW adiponectin) and biomarkers of endothelial dysfunction (sE-selectin, sICAM-1). Detailed information on biospecimen type and handling (storage duration and temperature, freeze-thaw cycles, assays applied) is given in Table A-3 (Appendix), as reported before (Weber et al. 2015).

Briefly, total cholesterol, triglycerides and biomarkers of inflammation were assayed from serum, and HbA1c from EDTA whole blood. All blood samples were drawn after overnight fasting.

IL-6, IL-18, total and HMW adiponectin and adhesion molecules were assessed using commercially available enzyme-linked immunosorbent assay (ELISA) kits. As the ratio of HMW to total adiponectin has been suggested to be a particular sensitive marker of the biological activity of adiponectin this variable was calculated by dividing HMW adiponectin by total adiponectin (HMW-to-total-adiponectin-ratio). hsCRP was analysed using a Roche/Hitachi c311 analyzer. A high-sensitivity assay was used which is able to detect very low levels of CRP, which is therefore referred to as high-sensitivity CRP (hsCRP).

Total cholesterol and triglycerides were analysed using an enzymatic assay on Hitchi 912 or Modular P system. Hb1Ac was determined via high-performance liquid chromatography on the Variant-II analyzer (Bio-rad, München, Germany). Hb1Ac values were adjusted according to the Diabetes Control and Complication Trial and appear as percent values from which subsequently mmol/mol values were calculated (Sacks 2012). Analyses were carried out in the central laboratory of the teaching Hospital of Heinrich-Heine University, Düsseldorf and at the Institute of Clinical Diabetology at the German Diabetes Center in Düsseldorf.

Table 1: Laboratory measurement of metabolic parameters and biomarkers of subclinical inflammation

Variables	Assay	Intra-assay CV (%)	Inter-assay CV (%)
HbA1c (percent and mmol/mol)	Variant-II (Bio-rad, Munich, Germany)	<1	<1
Total cholesterol (mmol/l)	Enzymatic assay on Hitachi 912 or Modular P system (both Rocher Diagnostics, Mannheim, Germany)	<1/1.9	2.2/3.5
Triglycerides (mmol/l)	Enzymatic assay on Hitachi 912 or Modular P system (Roche Diagnostics, Mannheim, Germany)	<1/1.1	2.2/4.2
hsCRP (mg/L)	Roche/Hitachi c 311 analyzer (Basel, Switzerland)	1.9	3.9
IL-6 (pg/mL)	Quantikine HS ELISA (R&D Systems, Wiesbaden, Germany)	6.0	12.2
IL-18 (pg/mL)	IL-18 ELISA (MBL, Nagoya, Japan)	6.5	12.1
Total adiponectin (ng/mL)	Adiponectin (Multimeric) ELISA (ALPCO Diagnostics, Salem, NH, USA)	2.34	7.88
HMW adiponectin (ng/mL)	Adiponectin (Multimeric) ELISA (ALPCO Diagnostics, Salem, NH, USA)	3.56	10.82
sE-selectin (ng/mL)	Quantikine HS ELISA (R&D Systems, Wiesbaden, Germany)	3.3	5.0
sICAM-1 (ng/mL)	Quantikine HS ELISA (R&D Systems, Wiesbaden, Germany)	2.0	3.3

The table gives an overview on laboratory measurements used to assay metabolic parameters and biomarkers of subclinical inflammation. CV, coefficient of variation; HMW, high-molecular-weight adiponectin (adapted from Weber et al. 2015).

2.6 Statistical analysis

A cross-sectional analysis from the baseline visit of the GDS is presented. All analyses were stratified by diabetes type because there is evidence that risk factors of DSPN differ between type 1 and type 2 diabetes (Callaghan et al. 2012). Characteristics of the study populations (participants with type 1 or type 2 diabetes) are presented as

proportions (%) for categorical variables, as mean ± standard deviation (SD) for continuous variables with Gaussian distribution and as median (25^{th} and 75^{th} percentiles) for continuous variables with non-Gaussian distribution. The Kolmogorov-Smirnov test was applied to test for Gaussian distribution.

Differences between the subsamples of type 1 and type 2 diabetes participants in categorical variables with 2 classes were tested using Fisher´s exact test. Differences in continuous variables with Gaussian distribution were tested using Student´s t–test (unpaired, two-tailed). Differences in continuous variables without Gaussian distribution were tested with the Mann-Whitney U-test. Variables which did not show a Gaussian distribution (triglycerides, all biomarkers of subclinical inflammation) were log-transformed (log: natural logarithm) before entering statistical analysis. Pearson correlation coefficients were calculated to assess associations between log-transformed biomarkers of subclinical inflammation with each other and with sociodemographic, metabolic and anthropometric variables.

Multivariable logistic regression models increasing in complexity were applied to assess associations between biomarkers of subclinical inflammation as independent variables (separate models for each biomarker) and presence of DSPN, as dependent variable. Covariables were selected based on potential risk factors for DSPN, which have been described before (see section 1.2.3). Model 1 adjusted for age and sex. Model 2 additionally included time since diagnosis of diabetes, HbA1c, waist circumference, height, total cholesterol and hypertension (defined as blood pressure ≥140/90 mmHg or use of antihypertensive medication) as covariates. Model 3 additionally adjusted for current smoking (yes/no), physical activity (yes/no), use of lipid-lowering medication (yes/no) and NSAID (yes/no) as well as for history of myocardial infarction and/or stroke (yes/no). The latter variable was not included in the statistical analysis in the subsample of type 1 diabetic participants, because only a single participant displayed this risk factor.

Multivariable linear regression models were applied to assess the associations between biomarkers of subclinical inflammation as independent variables (separate models for each biomarker) and MNCV and SNCV as continuous dependent variables. Furthermore, MNCV and SNCV sum scores were calculated and entered multivariable linear regression models as dependent variables. To obtain sum scores, individual z-scores of median, ulnar and peroneal MNCV and of median, ulnar and sural SNCV were added to provide a MNCV sum score and a SNCV sum score, respectively. As outlined earlier, sum scores combine NCV of different nerves. In using z-scores equal weight is given to each nerve and sum scores result in a more sensitive assessment of nerve function in an individual with low sum scores reflecting a more severe

impairment of peripheral nerve function. The same covariates as in the multivariable logistic regression models were included.

Statistical analyses were performed using SPSS version 22 (IBM, Ehningen, Germany) and R version 3.2.4 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria). A p value of <0.05 was chosen and considered to indicate statistical significance of the investigated associations. Statistical analyses were not adjusted for multiple testing.

3 Results

3.1 Description of the study population

The following paragraph will provide an overview of the demographic, anthropometric and metabolic characteristics of the study population. Following this, the prevalence of DSPN and the distribution of inflammatory mediators will be described.

3.1.1 Characteristics of the study population

The study sample consisted of 513 individuals with recently diagnosed type 2 diabetes (n=352) and type 1 diabetes (n=161). Table 2 shows the demographic, anthropometric and metabolic characteristics of the study population stratified by type of diabetes. Significant differences between these subsamples were present for most characteristics. The subsample of type 2 diabetic participants was comprised of a higher percentage of male participants, on average displayed a higher BMI and waist circumference and was older and of shorter height compared to the subsample of type 1 diabetic participants. In addition, type 2 diabetic participants showed better glycaemic control as indicated by lower HbA1c, higher lipid levels and higher blood pressure compared to type 1 diabetic participants. Beyond this, less physical activity and a higher prevalence of myocardial infarction and/or stroke were reported in the subsample of type 2 compared to type 1 diabetes participants. Lastly, a higher percentage of type 2 compared to type 1 diabetes participants reported the intake of lipid-lowering and antihypertensive medication. Type 1 and type 2 diabetic participants did not differ significantly regarding NSAID intake, current smoking status or time since diagnosis.

Table 2: Demographic, anthropometric and metabolic characteristics of the study population stratified by type of diabetes

Variables	Type 2 diabetes	Type 1 diabetes	p
n	352	161	
Sex (% male)	66.2	60.9	<0.001
Age (years)	52.6 ± 10,6	35.9 ± 12,5	<0.001
18-19	0.6%	8.7%	
20-29	2.8%	31.1%	
30-39	9.7%	20.5%	
40-49	23.0%	23.6%	
50-59	38.0%	11.2%	
60-69	25.0%	5.0%	
Body mass index (kg/m²)	31.7 ± 6.1	24.9 ± 4.3	<0.001
Waist circumference (cm)	106 ± 14	87 ± 14	<0.001
Height (cm)	172 ± 9	176 ± 10	0.001
Time since diagnosis of diabetes (days)	181 ± 96	195 ± 100	0.125
HbA1c (%)	6.53 ± 1.09	6.91 ± 1.70	0.008
HbA1c (mmol/mol)	48 ± 12	52 ± 19	0.008
Total cholesterol (mmol/l)	5.2 ± 1.1	4.7 ± 0.9	<0.001
Fasting triglycerides (mmol/l)	1.4 (1.0; 2.1)	0.8 (0.6; 1.2)	<0.001
Systolic blood pressure (mmHg)	142 ± 18	131 ± 15	<0.001
Diastolic blood pressure (mmHg)	85 ± 11	78 ± 10	<0.001
Hypertension (%)†	60.1	16.1	<0.001
Current smoking (%)	24.6	25.6	0.82
Physically inactive (%)††	39.9	23.0	<0.001
History of myocardial infarction and/or stroke (%)	5.1	0.6	0.010
Lipid-lowering medication (%)	23.6	2.5	<0.001
Antihypertensive medication (%)	52.1	8.1	<0.001
Non-steroidal-anti-inflammatory drugs (%)	4.5	1.9	0.961

Data are presented as percent value, median (25th, 75th percentile), mean ± SD. The maximum number of missing data is 19 for type 2 diabetes and 7 for type 1 diabetes. †Hypertension is defined as blood pressure ≥140/90 mmHg or use of antihypertensive medication. ††Physical inactivity is defined as absence of regular physical training at the time of the examination.

3.1.2 Prevalence of DSPN

The prevalence of DSPN as well as measures of motor and sensory NCV are shown in Table 3. In the present study the prevalence of DSPN was higher in type 2 diabetes (25.6%) than in type 1 diabetes (19.3%). Furthermore, motor and sensory NCV were lower in type 2 than in type 1 diabetes.

Table 3: Prevalence of DSPN, MNCV and SNCV stratified by type of diabetes

	Type 2 diabetes	Type 1 diabetes	p
n	352	161	
DSPN (%)	25.6	19.3	<0.001
NDS	1.7 ± 2.2	0.6 ± 1.3	<0.001
	1 (0; 2)	0 (0; 0)	<0.001
NSS	1.2 ± 2.4	0.5 ± 1.6	<0.001
	0 (0; 0)	0 (0; 0)	<0.001
Motor nerve conduction velocity (MNCV)			
Median MNCV (m/s)	53.4 ± 3.2	55.5 ± 3.9	<0.001
Ulnar MNCV (m/s)	55.8 ± 5.7	56.7 ± 5.3	0.097
Peroneal MNCV (m/s)	44.7 ± 5.1	46.1 ± 4.0	0.002
Sum score MNCV (m/s)	153.94 ± 12.36	158.30 ± 10.50	<0.001
Sensory nerve conduction velocity (SNCV)			
Median SNCV (m/s)	51.7 ± 6.8	55.3 ± 6.0	<0.001
Ulnar SNCV (m/s)	53.3 ± 5.8	54.1 ± 4.4	0.100
Sural SNCV (m/s)	44.6 ± 5.8	45.6 ± 5.0	0.066
Sum score SNCV (m/s)	149.78 ± 13.70	155.03 ± 11.90	<0.001

Data are presented as percent value, median (25^{th}, 75^{th} percentile), mean ± SD. The maximum number of missing data is 19 for type 2 diabetes and 7 for type 1 diabetes. DSPN, distal sensorimotor polyneuropathy; NDS, Neuropathy Disability Score; NSS, Neuropathy Syptom Score; MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity.

3.1.3 Distribution of biomarkers of subclinical inflammation

Distributions of biomarkers of subclinical inflammation are presented in Table 4, stratified by type of diabetes. Higher levels of hsCRP, IL-6, IL-18, sICAM-1 and sE-selectin were observed in the subsample of type 2 diabetic participants compared to the subsample of type 1 diabetic participants. In contrast, levels of total and HMW adiponectin were lower in the subsample of type 2 diabetic participants compared to type 1 diabetic participants.

Table 4: Distribution of biomarkers of subclinical inflammation stratified by type of diabetes

	Type 2 diabetes	Type 1 diabetes	p
n	352	161	
hsCRP (mg/L)	3.0 (1.6; 5.6)	1.1 (0.6; 2.0)	<0.001
IL-6 (pg/mL)	1.94 (1.32; 2.65)	0.88 (0.65; 1.38)	<0.001
IL-18 (pg/mL)	287 (227; 384)	260 (183; 322)	<0.001
Total adiponectin (ng/mL)	4008 (2983; 5355)	6101 (4112; 8095)	<0.001
HMW adiponectin (ng/mL)	1636 (995; 2440)	2751 (1557; 4280)	<0.001
HMW/total adiponectin	0.40 (0.33; 0.48)	0.45 (0.36; 0.54)	0.003
sE-selectin (ng/mL)	244 (205; 288)	217 (182; 253)	<0.001
sICAM-1 (ng/mL)	40.9 (30.7; 55.2)	34.5 (24.8; 44.9)	<0.001

Data are presented as median (25^{th}, 75^{th} percentile). The maximum number of missing data is 19 for type 2 diabetes and 7 for type 1 diabetes. HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio.

Correlations of immune mediators with each other and with demographic, anthropometric and metabolic risk factors in type 1 and type 2 diabetic participants are presented in Table A-4, A-5 and A-6 (Appendix). Briefly, in both subsamples proinflammatory mediators and adhesion molecules were positively associated among themselves and with each other. IL-18 was inversely associated with different isoforms of adiponectin in type 1 and 2 diabetes. sE-selectin was inversely associated with different isoforms of adiponectin in type1 diabetes.

Proinflammatory mediators (hsCRP, IL-6, IL-18) and adhesion molecules (sICAM-1, sE-selectin) were significantly associated with higher BMI and waist circumference and with a less favourable metabolic profile (e.g. increased triglycerides, total cholesterol, HbA1c) in type 1 and type 2 diabetes. In both subsamples different isoforms of adiponectin were associated with lower BMI, waist circumference, height and triglyceride levels and with female sex. In type 2 but not type 1 diabetic participants, different measures of adiponectin were further associated with higher age, reduced HbA1c, increased total cholesterol and NSAID intake.

3.2 Association between biomarkers of subclinical inflammation and DSPN

In the following paragraph, associations between biomarkers of subclinical inflammation and DSPN will be presented, stratified by type of diabetes. First, associations between biomarkers of subclinical inflammation and MNCV will be presented for single nerves (Table 5 and 6) and for the sum score (Table 7). Second,

associations between biomarkers of subclinical inflammation and SNCV will be shown for single nerves (Table 8 and 9) and for the sum score (Table 10). Lastly, results regarding the association between biomarkers of subclinical inflammation and presence of DSPN are presented (Table 11).

3.2.1 Association between biomarkers of subclinical inflammation and MNCV in patients with type 1 and type 2 diabetes

In type 2 diabetic individuals IL-6 was inversely associated with the MNCV sum score (Table 7). This association was mostly driven by associations between IL-6 and median and peroneal NCV (Table 5). In addition, total and HMW adioponectin as well as their ratio were inversely associated with the MNCV sum score (Table 7). Associations between different measures of adiponectin and MNCV sum score were mostly driven by associations between adiponectin measures and peroneal NCV (Table 5). Furthermore, hsCRP levels were inversely associated with the MNCV sum score in model 1 but failed to reach statistical significance after further adjustment (Table 7, models 2 and 3).

In type 1 diabetic individuals hsCRP, IL-6 and sICAM-1 levels were inversely associated with the MNCV sum score. However, statistical significance did not remain after further adjustment (Table 7). In contrast, positive associations were found between total as well as HMW adiponectin and the sum score for motor NCV, which were strengthened after further adjustment and reached statistical significance after full adjustment (Table 7, model 3). This association was mainly driven by positive associations between total as well as HMW adiponectin and ulnar MNCV (Table 6).

To emphasize differences between type 1 and type 2 diabetic patients, effect sizes of significant associations and their 95% confidence interval are summarized in direct comparison of both subsamples in Figure 2 (MNCV in single nerves) and 3 (MNCV sum score). Inverse associations between IL-6 and motor NCV were found in both types of diabetes but after full adjustment they remained significant only in type 2 diabetes participants. Contradicting results became evident in these subsamples of different diabetes types regarding adiponectin´s association with MNCV. While a positive association was found between adiponectin and MNCV in type 1 diabetes, adiponectin measures were inversely associated in type 2 diabetes. Beyond this, wider confidence intervals in the smaller subsample of type 1 diabetes indicate reduced statistical power.

Table 5: Association between biomarkers of subclinical inflammation and MNCV of single nerves in patients with type 2 diabetes

Nerve type	Immune mediator	Model 1		Model 2		Model 3	
		β	p	β	p	β	p
Median MNCV	hsCRP	**-0.161**	**0.004**	-0.073	0.242	-0.075	0.250
	IL-6	**-0.227**	**<0.001**	**-0.161**	**0.005**	**-0.159**	**0.009**
	IL-18	0.001	0.979	0.079	0.137	0.093	0.089
	Total adiponectin	0.036	0.543	-0.009	0.875	-0.024	0.680
	HMW adiponectin	0.044	0.456	-0.003	0.954	-0.023	0.690
	HMW/total adiponectin	0.046	0.420	0.005	0.927	-0.018	0.756
	sE-selectin	-0.088	0.098	0.006	0.916	0.030	0.592
	sICAM-1	**-0.129**	**0.016**	-0.065	0.229	-0.044	0.447
Ulnar MNCV	hsCRP	-0.085	0.129	-0.075	0.243	-0.082	0.209
	IL-6	-0.083	0.117	-0.068	0.243	-0.066	0.282
	IL-18	0.004	0.939	0.032	0.559	0.031	0.575
	Total adiponectin	-0.057	0.328	-0.081	0.166	-0.088	0.134
	HMW adiponectin	-0.054	0.349	-0.082	0.159	-0.094	0.110
	HMW/total adiponectin	-0.040	0.471	-0.068	0.232	-0.084	0.142
	sE-selectin	0.025	0.639	0.063	0.261	0.072	0.206
	sICAM-1	-0.033	0.535	-0.008	0.886	0.024	0.685
Peroneal MNCV	hsCRP	-0.071	0.185	-0.097	0.096	**-0.116**	**0.054**
	IL-6	**-0.146**	**0.004**	**-0.146**	**0.005**	**-0.174**	**0.002**
	IL-18	0.070	0.165	0.091	0.067	0.098	0.052
	Total adiponectin	**-0.118**	**0.033**	**-0.140**	**0.009**	**-0.158**	**0.004**
	HMW adiponectin	**-0.115**	**0.038**	**-0.147**	**0.005**	**-0.168**	**0.002**
	HMW/total adiponectin	-0.088	0.102	**-0.129**	**0.012**	**-0.149**	**0.004**
	sE-selectin	-0.018	0.723	0.000	1.000	0.009	0.861
	sICAM-1	-0.006	0.910	0.015	0.763	0.022	0.688

The table gives regression coefficients (β) and corresponding p values from linear regression models. Immune mediators entered the models as log-transformed levels in the units given in Table 1. Model 1: adjusted for age and sex. Model 2: model 1 + time since diagnosis of diabetes, HbA1c, waist circumference, height, total cholesterol, hypertension. Model 3: model 2 + current smoking, physical activity, use of lipid-lowering medication, use of NSAIDs, history of myocardial infarction and/or stroke. HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio; MNCV, motor nerve conduction velocity.

Table 6: Association between biomarkers of subclinical inflammation and MNCV of single nerves in patients with type 1 diabetes

Nerve type	Immune mediator	Model 1		Model 2		Model 3	
		β	p	β	p	β	p
Median MNCV	hsCRP	**-0.205**	**0.009**	-0.105	0.234	-0.078	0.396
	IL-6	**-0.229**	**0.003**	-0.167	0.052	-0.124	0.188
	IL-18	-0.072	0.352	-0.013	0.866	0.003	0.970
	Total adiponectin	0.058	0.497	0.038	0.666	0.081	0.373
	HMW adiponectin	0.044	0.615	0.027	0.759	0.074	0.418
	HMW/total adiponectin	0.010	0.905	0.005	0.954	0.046	0.598
	sE-selectin	-0.075	0.335	0.016	0.842	0.049	0.565
	sICAM-1	**-0.217**	**0.004**	**-0.170**	**0.028**	-0.126	0.167
Ulnar MNCV	hsCRP	-0.096	0.245	-0.154	0.110	-0.084	0.382
	IL-6	**-0.200**	**0.014**	**-0.259**	**0.005**	-0.139	0.157
	IL-18	-0.037	0.645	-0.056	0.508	-0.044	0.599
	Total adiponectin	0.127	0.150	**0.190**	**0.049**	**0.252**	**0.007**
	HMW adiponectin	0.129	0.155	0.179	0.063	**0.254**	**0.007**
	HMW/total adiponectin	0.099	0.269	0.122	0.185	**0.198**	**0.028**
	sE-selectin	0.009	0.909	0.009	0.920	0.075	0.402
	sICAM-1	-0.157	0.051	**-0.193**	**0.022**	-0.095	0.321
Peroneal MNCV	hsCRP	-0.120	0.137	-0.127	0.151	-0.095	0.288
	IL-6	-0.121	0.127	-0.060	0.488	-0.012	0.897
	IL-18	0.087	0.266	0.091	0.243	0.092	0.239
	Total adiponectin	0.081	0.344	0.111	0.209	0.170	0.054
	HMW adiponectin	0.096	0.277	0.109	0.218	0.165	0.061
	HMW/total adiponectin	0.096	0.274	0.081	0.343	0.121	0.156
	sE-selectin	0.042	0.596	0.083	0.308	0.087	0.295
	sICAM-1	-0.043	0.581	-0.055	0.483	-0.054	0.549

The table gives regression coefficients (β) and corresponding p values from linear regression models. Immune mediators entered the models as log-transformed levels in the units given in Table 1. Model 1: adjusted for age and sex. Model 2: model 1 + time since diagnosis of diabetes, HbA1c, waist circumference, height, total cholesterol, hypertension. Model 3: model 2 + current smoking, physical activity, use of lipid-lowering medication, use of NSAIDs. HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio; MNCV, motor nerve conduction velocity.

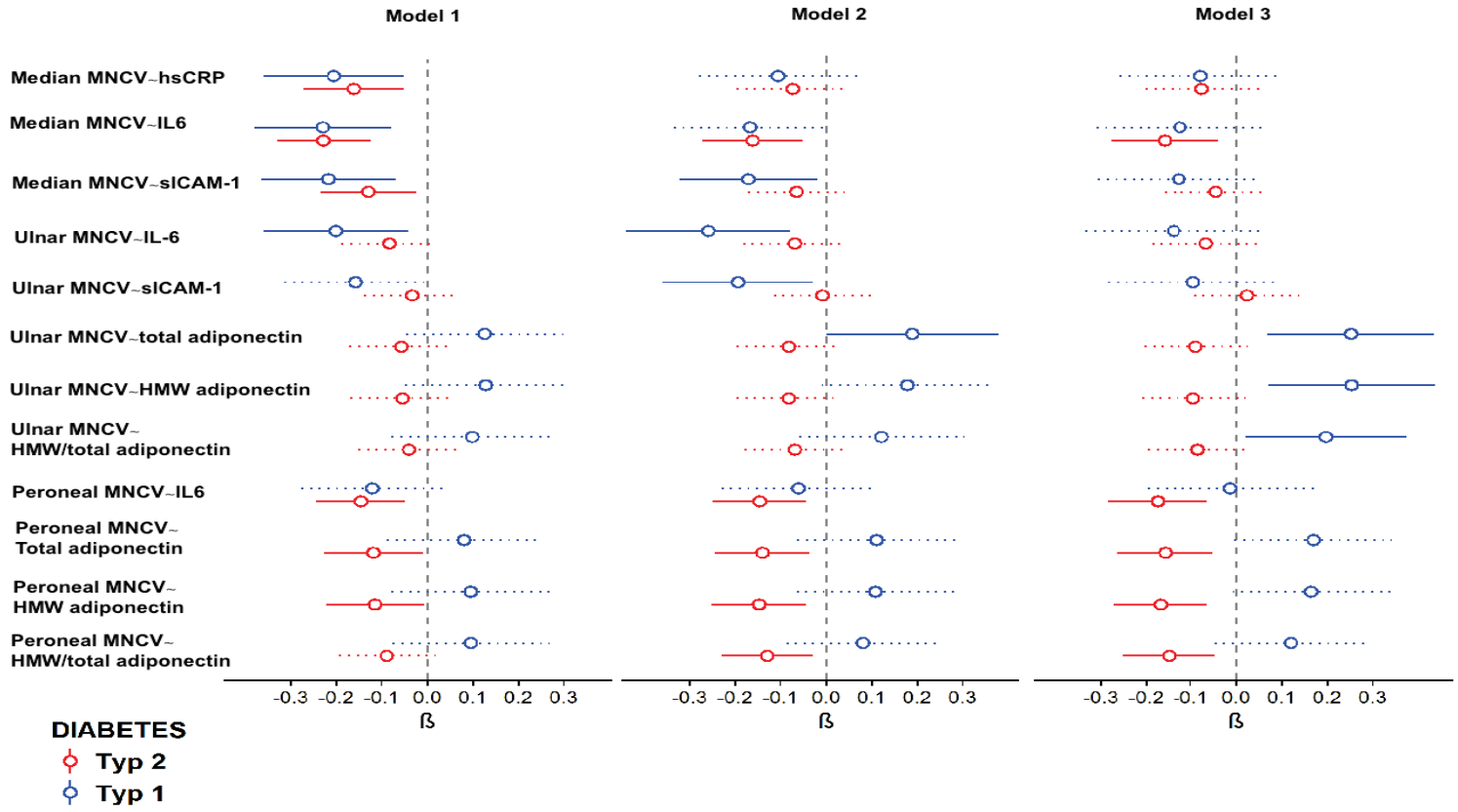

Figure 2: Comparison of regression coefficients (β) and 95% confidence intervals of significant associations between biomarkers of subclinical inflammation and MNCV of single nerves in patients with type 1 and type 2 diabetes. Dotted lines indicate non-significant associations (p>0.05); continuous lines indicate significant associations (p<0.05). HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio; MNCV, motor nerve conduction velocity.

Table 7: Association between biomarkers of subclinical inflammation and MNCV sum score in patients with type 1 and type 2 diabetes

Diabetes Type	Immune mediator	Model 1		Model 2		Model 3	
		β	p	β	p	β	p
Type 2 Diabetes	hsCRP	**-0.128**	**0.019**	-0.097	0.107	-0.109	0.080
	IL-6	**-0.184**	**0.001**	**-0.152**	**0.005**	**-0.161**	**0.006**
	IL-18	0.032	0.531	0.081	0.117	0.088	0.091
	Total adiponectin	-0.060	0.289	-0.094	0.088	**-0.111**	**0.048**
	HMW adiponectin	-0.054	0.339	-0.096	0.083	**-0.117**	**0.037**
	HMW/total adiponectin	-0.035	0.517	-0.079	0.141	-0.103	0.058
	sE-selectin	-0.032	0.530	0.028	0.604	0.045	0.406
	sICAM-1	-0.067	0.197	-0.023	0.652	0.001	0.985
Type 1 Diabetes	hsCRP	**-0.176**	**0.027**	-0.162	0.073	-0.107	0.238
	IL-6	**-0.233**	**0.003**	**-0.209**	**0.017**	-0.120	0.199
	IL-18	-0.014	0.859	0.005	0.954	0.017	0.830
	Total adiponectin	0.112	0.188	0.143	0.113	**0.211**	**0.017**
	HMW adiponectin	0.113	0.198	0.133	0.141	**0.208**	**0.019**
	HMW/total adiponectin	0.085	0.326	0.087	0.314	0.154	0.072
	sE-selectin	-0.012	0.878	0.043	0.606	0.088	0.298
	sICAM-1	**-0.179**	**0.020**	**-0.180**	**0.023**	-0.117	0.195

The table gives regression coefficients (β) and corresponding p values from linear regression models. Immune mediators entered the models as log-transformed levels in the units given in Table 1. Model 1: adjusted for age and sex. Model 2: model 1 + time since diagnosis of diabetes, HbA1c, waist circumference, height, total cholesterol, hypertension. Model 3: model 2 + current smoking, physical activity, use of lipid-lowering medication, use of NSAIDs, history of myocardial infarction and/or stroke. HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio; MNCV, motor nerve conduction velocity.

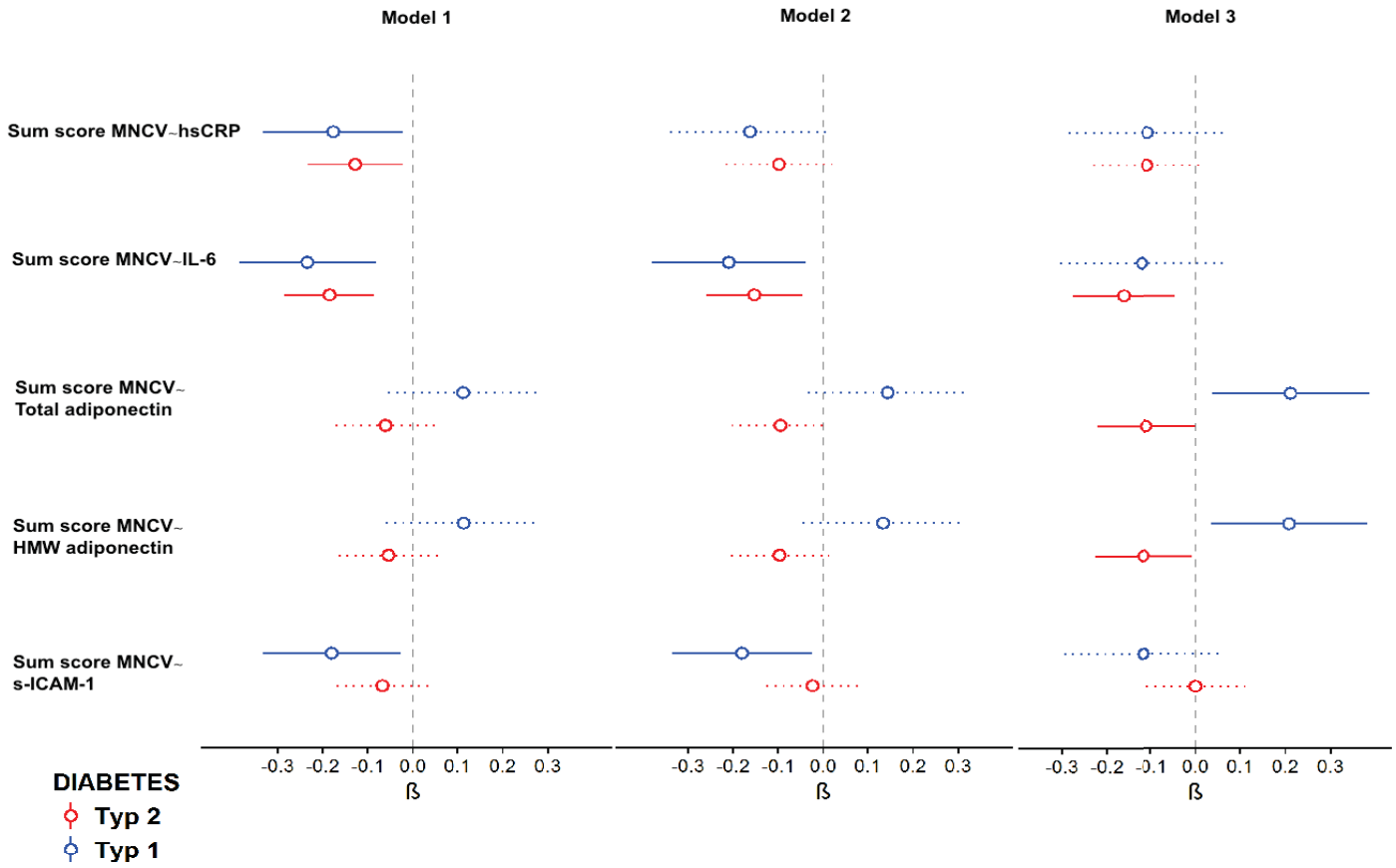

Figure 3: Comparison of regression coefficients (β) and 95% confidence intervals of significant associations between biomarkers of subclinical inflammation and MNCV sum score in patients with type 1 and type 2 diabetes. Dotted lines indicate non-significant associations (p>0.05); continuous lines indicate significant associations (p<0.05) HMW, high-molecular-weight adiponectin; MNCV, motor nerve conduction velocity.

3.2.2 Association between biomarkers of subclinical inflammation and SNCV in patients with type 1 and type 2 diabetes

In type 2 diabetic individuals total and HMW adiponectin as well as their ratio were inversely associated with the SNCV sum score (Table 10). These associations were similar for median, ulnar and sural NCV (Table 8). Furthemore, IL-18 was positively associated with the SNCV sum score after full adjustment (Table 10, model 3). Lastly, sICAM-1 levels were positively associated with median SNCV after adjustment (Table 8, model 2 and 3).

In type 1 diabetic individuals, inverse associations between HMW adiponectin as well as the ratio of HMW-to-total-adiponectin with ulnar SNCV were significantly associated and remained statistically significant after full adjustment (Table 9, model 3).

To emphasize differences between type 1 and type 2 diabetic patients, effect sizes of significant associations and their 95% confidence interval are summarized in direct comparison of both subsamples in Figure 4 (SNCV in single nerves) and Figure 5 (SNCV sum score). Inverse associations between different measures of adiponectin and SNCV were found in both subsamples of type 1 and type 2 diabetes. However, in type 1 diabetes patients these associations did not reach statistical significance with the exception of ulnar SNCV. Wider confidence intervals of effect sizes in the smaller subsample of type 1 diabetes patients indicate reduced statistical power.

Table 8: Association between biomarkers of subclinical inflammation and SNCV of single nerves in patients with type 2 diabetes

Nerve type	Immune mediator	Model 1		Model 2		Model 3	
		β	p	β	p	β	p
Median SNCV	hsCRP	-0.013	0.822	0.084	0.184	0.082	0.214
	IL-6	-0.049	0.363	0.030	0.601	0.032	0.607
	IL-18	-0.030	0.568	0.025	0.646	0.033	0.552
	Total adiponectin	-0.110	0.062	**-0.138**	**0.016**	**-0.145**	**0.014**
	HMW adiponectin	-0.097	0.096	**-0.123**	**0.033**	**-0.135**	**0.021**
	HMW/total adiponectin	-0.062	0.275	-0.078	0.165	-0.096	0.092
	sE-selectin	-0.042	0.432	0.053	0.343	0.061	0.282
	sICAM-1	0.061	0.259	**0.123**	**0.022**	**0.138**	**0.018**
Ulnar SNCV	hsCRP	-0.063	0.258	-0.019	0.764	-0.007	0.910
	IL-6	-0.064	0.227	-0.041	0.478	-0.020	0.740
	IL-18	0.086	0.098	**0.122**	**0.022**	**0.122**	**0.025**
	Total adiponectin	**-0.115**	**0.045**	**-0.130**	**0.024**	**-0.142**	**0.016**
	HMW adiponectin	**-0.116**	**0.043**	**-0.134**	**0.019**	**-0.146**	**0.021**
	HMW/total adiponectin	-0.095	0.088	**-0.115**	**0.040**	**-0.125**	**0.027**
	sE-selectin	-0.085	0.105	-0.037	0.507	-0.032	0.577
	sICAM-1	-0.055	0.298	-0.021	0.691	0.006	0.922
Sural SNCV	hsCRP	-0.043	0.432	-0.083	0.176	-0.098	0.123
	IL-6	-0.037	0.470	-0.060	0.280	-0.068	0.253
	IL-18	0.077	0.134	0.078	0.134	0.087	0.103
	Total adiponectin	**-0.131**	**0.020**	**-0.136**	**0.014**	**-0.143**	**0.013**
	HMW adiponectin	**-0.128**	**0.023**	**-0.140**	**0.012**	**-0.149**	**0.009**
	HMW/total adiponectin	-0.100	0.069	**-0.118**	**0.029**	**-0.130**	**0.019**
	sE-selectin	-0.050	0.329	-0.064	0.234	-0.059	0.285
	sICAM-1	-0.046	0.379	-0.052	0.320	-0.063	0.272

The table gives regression coefficients (β) and corresponding p values from logistic regression models. Immune mediators entered the models as log-transformed levels in the units given in Table 1. Model 1: adjusted for age and sex. Model 2: model 1 + time since diagnosis of diabetes, HbA1c, waist circumference, height, total cholesterol, hypertension. Model 3: model 2 + current smoking, physical activity, use of lipid-lowering medication, use of NSAIDs, history of myocardial infarction and/or stroke. HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio; SNCV, sensory nerve conduction velocity.

Table 9: Association between biomarkers of subclinical inflammation and SNCV of single nerves in patients with type 1 diabetes

Nerve type	Immune mediator	Model 1		Model 2		Model 3	
		β	p	β	p	β	p
Median SNCV	hsCRP	-0.101	0.199	-0.062	0.496	-0.065	0.488
	IL-6	-0.067	0.392	-0.052	0.550	-0.078	0.418
	IL-18	0.018	0.815	0.043	0.592	0.040	0.625
	Total adiponectin	-0.039	0.645	-0.062	0.489	-0.076	0.410
	HMW adiponectin	-0.083	0.336	-0.097	0.279	-0.113	0.220
	HMW/total adiponectin	-0.135	0.116	-0.125	0.145	-0.141	0.112
	sE-selectin	**-0.158**	**0.041**	-0.137	0.094	-0.124	0.154
	sICAM-1	-0.123	0.108	-0.114	0.152	-0.145	0.115
Ulnar SNCV	hsCRP	-0.061	0.459	-0.141	0.141	-0.156	0.116
	IL-6	-0.001	0.987	-0.036	0.697	-0.049	0.633
	IL-18	-0.002	0.983	-0.027	0.748	-0.002	0.978
	Total adiponectin	**-0.171**	**0.049**	-0.147	0.118	-0.147	0.129
	HMW adiponectin	**-0.218**	**0.014**	**-0.195**	**0.038**	**-0.191**	**0.049**
	HMW/total adiponectin	**-0.240**	**0.006**	**-0.219**	**0.015**	**-0.211**	**0.024**
	sE-selectin	-0.057	0.484	-0.085	0.328	-0.068	0.460
	sICAM-1	-0.115	0.148	**-0.175**	**0.037**	**-0.211**	**0.030**
Sural SNCV	hsCRP	-0.014	0.864	-0.080	0.385	-0.090	0.339
	IL-6	0.154	0.053	0.122	0.169	0.112	0.243
	IL-18	0.053	0.507	-0.004	0.964	-0.016	0.844
	Total adiponectin	0.026	0.766	0.088	0.334	0.120	0.194
	HMW adiponectin	-0.012	0.889	0.034	0.710	0.062	0.501
	HMW/total adiponectin	-0.072	0.413	-0.052	0.550	-0.036	0.687
	sE-selectin	**0.160**	**0.045**	0.129	0.121	0.137	0.113
	sICAM-1	0.048	0.546	0.009	0.913	-0.023	0.805

The table gives regression coefficients (β) and corresponding p values from logistic regression models. Immune mediators entered the models as log-transformed levels in the units given in Table 1. Model 1: adjusted for age and sex. Model 2: model 1 + time since diagnosis of diabetes, HbA1c, waist circumference, height, total cholesterol, hypertension. Model 3: model 2 + current smoking, physical activity, use of lipid-lowering medication, use of NSAIDs. HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio. SNCV, sensory nerve conduction velocity.

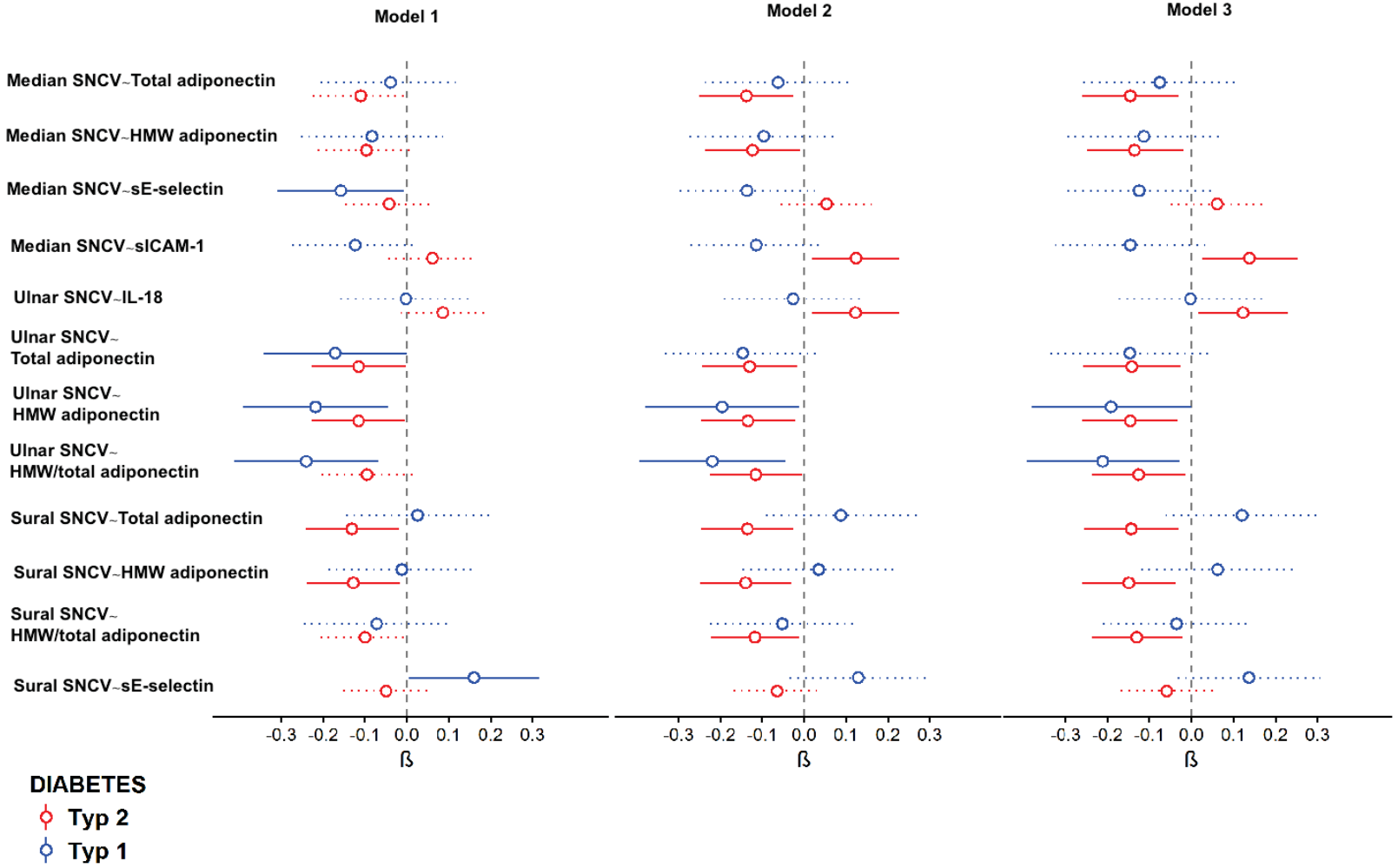

Figure 4: Comparison of regression coefficients (β) and 95% confidence intervals of significant associations between biomarkers of subclinical inflammation and SNCV of single nerves in patients with type 1 and type 2 diabetes. Dotted lines indicate non-significant associations (p>0.05); continuous lines indicate significant associations (p<0.05). HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio; SNCV, sensory nerve conduction velocity.

Table 10: Association between biomarkers of subclinical inflammation and SNCV sum score in patients with type 1 and type 2 diabetes

Diabates type	Immune mediator	Model 1		Model 2		Model 3	
		β	*p*	β	*p*	β	*p*
Type 2 diabetes	hsCRP	-0.047	0.389	-0.019	0.758	-0.026	0.689
	IL-6	-0.062	0.236	-0.036	0.524	-0.034	0.575
	IL-18	0.075	0.146	**0.103**	**0.0496**	**0.110**	**0.040**
	Total adiponectin	**-0.163**	**0.004**	**-0.180**	**0.001**	**-0.193**	**0.001**
	HMW adiponectin	**-0.160**	**0.004**	**-0.180**	**0.001**	**-0.196**	**0.001**
	HMW/total adiponectin	**-0.126**	**0.021**	**-0.143**	**0.009**	**-0.162**	**0.003**
	sE-selectin	-0.066	0.201	-0.017	0.753	-0.010	0.863
	sICAM-1	-0.003	0.954	0.027	0.610	0.038	0.509
Type 1 diabetes	hsCRP	-0.088	0.264	-0.131	0.152	-0.141	0.139
	IL-6	0.035	0.650	0.015	0.866	-0.001	0.991
	IL-18	0.028	0.721	0.005	0.951	0.010	0.900
	Total adiponectin	-0.078	0.353	-0.055	0.541	-0.045	0.631
	HMW adiponectin	-0.132	0.126	-0.111	0.221	-0.101	0.280
	HMW/total adiponectin	**-0.187**	**0.028**	-0.165	0.055	-0.160	0.075
	sE-selectin	-0.015	0.850	-0.024	0.771	-0.008	0.928
	sICAM-1	-0.078	0.313	-0.116	0.150	-0.146	0.118

The table gives regression coefficients (β) and corresponding p values from linear regression models. Immune mediators entered the models as log-transformed levels in the units given in Table 1. Model 1: adjusted for age and sex. Model 2: model 1 + time since diagnosis of diabetes, HbA1c, waist circumference, height, total cholesterol, hypertension. Model 3: model 2 + current smoking, physical activity, use of lipid-lowering medication and use of NSAIDs. HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio; SNCV, sensory nerve conduction velocity.

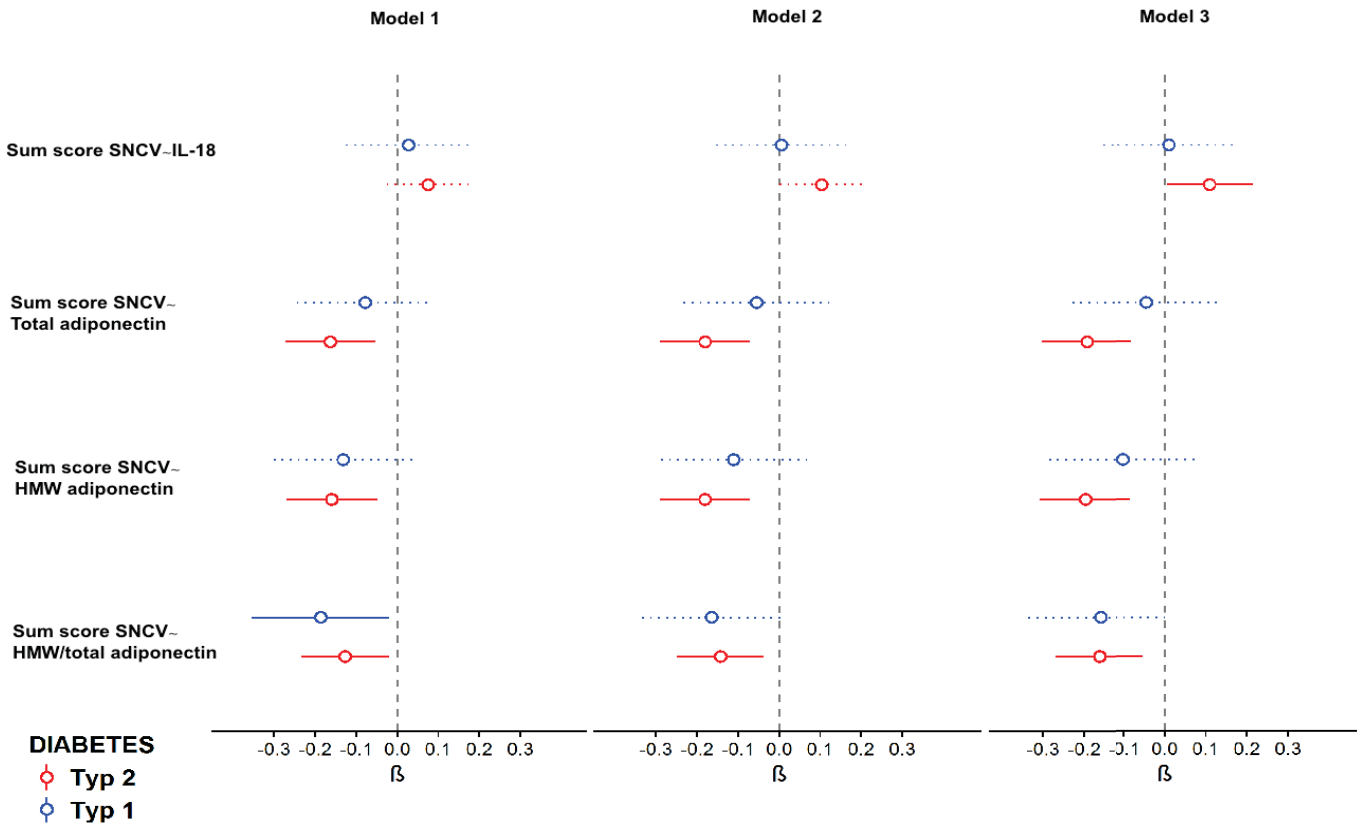

Figure 5: Comparison of regression coefficients (β) and 95% confidence intervals of significant associations between biomarkers of subclinical inflammation and SNCV sum score in patients with type 1 and type 2 diabetes. Dotted lines indicate non-significant associations (p>0.05); continuous lines indicate significant associations (p<0.05) HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio; SNCV, sensory nerve conduction velocity.

3.2.3 Association between biomarkers of subclinical inflammation and presence of DSPN in patients with type 1 and type 2 diabetes

As shown in Table 11 IL-6, total and HMW adiponectin were positively associated with the presence of DSPN in the subsample of type 2 diabetic participants and remained statistically significant after full adjustment (models 2 and 3). The association between the HMW-to-total-adiponectin-ratio and presence of DSPN reached statistical significance only after further adjustment (model 2 and 3). In contrast, hsCRP, IL-18, sE-selectin and sICAM-1 were not associated with the presence of DSPN in type 2 diabetic participants. In the subgroup of type 1 diabetic participants, no association was found between biomarkers of subclinical inflammation and presence of DSPN.

Significant associations in type 2 diabetes patients are summarized in Figure 6. Effect sizes of these associations and their 95% confidence intervals are presented in direct comparison to corresponding associations in type 1 diabetes. Effect sizes were larger in type 2 diabetes, increased with further adjustment and displayed narrower confidence intervals than corresponding associations in type 1 diabetes.

Table 11: Association between biomarkers of subclinical inflammation and presence of DSPN in patients with type 1 and type 2 diabetes

Diabetes type	Immune mediator	Model 1		Model 2		Model 3	
		β	p	β	p	β	p
Type 2 diabetes	hsCRP	0.207	0.136	0.179	0.261	0.175	0.216
	IL-6	**0.513**	**0.028**	0.486	0.058	**0.575**	**0.039**
	IL-18	-0.282	0.352	-0.515	0.114	-0.484	0.152
	Total adiponectin	**0.784**	**0.016**	**0.949**	**0.006**	**1.007**	**0.005**
	HMW adiponectin	**0.478**	**0.021**	**0.579**	**0.005**	**0.615**	**0.007**
	HMW/total adiponectin	0.812	0.077	**0.974**	**0.045**	**1.057**	**0.032**
	sE-selectin	0.241	0.416	0.008	0.980	0.005	0.988
	sICAM-1	0.417	0.329	0.151	0.734	0.149	0.760
Type 1 diabetes	hsCRP	0.197	0.327	0.249	0.301	0.244	0.337
	IL-6	0.201	0.573	0.155	0.707	0.118	0.796
	IL-18	0.025	0.953	-0.003	0.994	-0.032	0.946
	Total adiponectin	-0.477	0.271	-0.692	0.166	-0.883	0.097
	HMW adiponectin	-0.333	0.248	-0.457	0.154	-0.578	0.083
	HMW/total adiponectin	-0.682	0.323	-0.838	0.248	-1.086	0.142
	sE-selectin	0.135	0.778	0.036	0.945	0.125	0.827
	sICAM-1	0.385	0.607	0.340	0.675	0.879	0.386

The table gives regression coefficients (β) and corresponding p values from logistic regression models. Immune mediators entered the models as log-transformed levels in the units given in Table 1. Model 1: adjusted for age and sex. Model 2: model 1 + time since diagnosis of diabetes, HbA1c, waist circumference, height, total cholesterol, hypertension. Model 3: model 2 + current smoking, physical activity, use of lipid-lowering medication, use of NSAIDs, history of myocardial infarction and/or stroke (the latter variable for type 2 diabetes only). HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio.

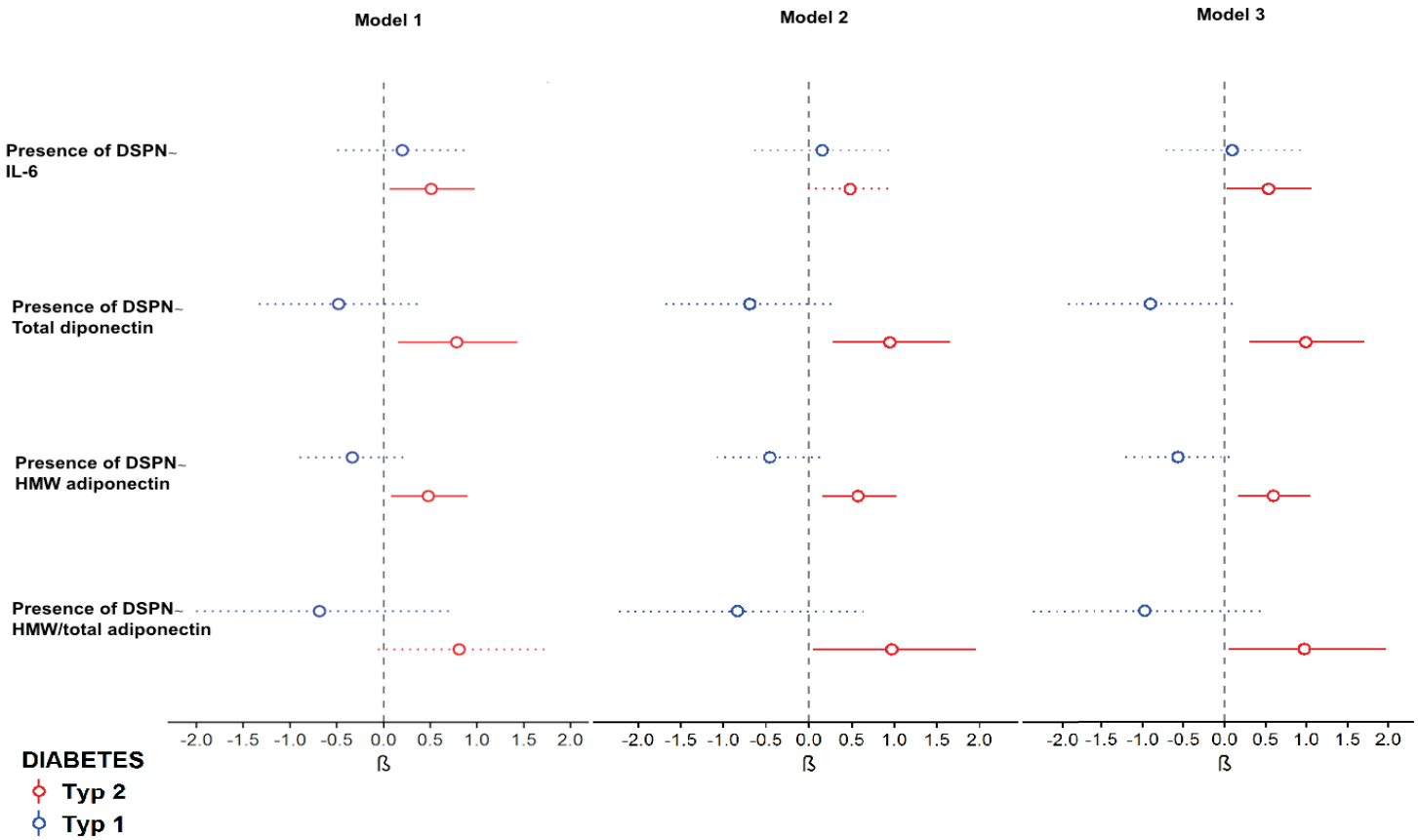

Figure 6: Comparison of regression coefficients (β) and 95% confidence intervals of significant associations between biomarkers of subclinical inflammation and presence of DSPN in patients with type 1 and type 2 diabetes. Dotted lines indicate non-significant associations ($p>0.05$); continuous lines indicate significant associations ($p<0.05$). HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio.

4 Discussion

The present study identified associations between multiple biomarkers of subclinical inflammation and NCV in patients with recently diagnosed type 1 or type 2 diabetes. Taken together, the study has the following three main findings. First, in individuals with type 2 diabetes, higher serum IL-6 was associated with the presence of DSPN and reduced motor NCV, whereas no consistent associations were observed for hsCRP, IL-18, sICAM-1 and sE-selectin. Second, higher levels of HMW and total adiponectin were consistently associated with the presence of DSPN and both reduced MNCV and SNCV in individuals with type 2 diabetes. Third, in patients with type 1 diabetes higher HMW and total adiponectin were associated with higher MNCV.

4.1 Study population

4.1.1 Characteristics of the study population

The present study consists of subsamples of type 1 and type 2 diabetic participants. The average time since patients had received their diagnosis of diabetes mellitus was 181 days in type 2 diabetic individuals and 195 days in type 1 diabetic individuals. Time since diagnosis did not differ significantly between both subsamples. This is of particular importance as this design allows to investigate the association between biomarkers of subclinical inflammation and DSPN before the onset of potentially confounding complications which usually emerge in the course of disease. However, subsamples of type 1 and type 2 diabetes in the present study were recruited as part of an ongoing prospective observational study which is not designed as a population-based study. Therefore, the present study population cannot claim to represent the population of diabetes patients in Germany.

Significant differences between type 1 and type 2 diabetic participants regarding anthropometric, metabolic and demographic characteristics nevertheless reflect typical features commonly associated with type 1 and type 2 diabetes. Thus, in line with the previous literature type 2 diabetic participants were older and features of the metabolic syndrome were more pronounced in this subsample (Air & Kissela 2007; Isomaa et al. 2001). Type 2 diabetic participants on average were obese, showed greater abdominal distribution of fat mass (as indicated by waist circumference), hypertension and increased levels of cholesterol and triglycerides. Consequently, the intake of lipid-lowering and antihypertensive medication and prevalence of myocardial infarction and strokes were reported to be higher in type 2 diabetes patients.

A higher percentage of male participants in the subgroup of type 1 diabetes is in line with the current literature which reports a greater percentage of male patients at least in the European population (Maahs et al. 2010). In the subsample of type 2 diabetic patients, a higher percentage of male than female participants became evident. The percentage of male type 2 diabetic patients was also higher than in other cohorts which may be a consequence of a more frequent manifestation of disease in men under the age of 65 (Bonetti et al. 2011; Davies et al. 2008, Gatling et al. 2001). In line with this a total of 75% of the type 2 diabetic participants in the present study was younger than 65 years. This selection bias results from the inclusion criteria based on an age range of 18-69 years, whereas older patients with newly diagnosed type 2 diabetes are not recruited (Szendroedi et al. 2016).

Taken together, the sample may not be representative of the german diabetic population but nevertheless it seems well suited to investigate the hypothesis outlined above as it appears to reflect differences commonly found between type 1 and type 2 diabetes.

4.1.2 Prevalence of DSPN

In the present sample the prevalence of DSPN assessed as a combined measure of symptoms, signs and NCS was 25.6% in type 2 diabetes and 19.3% in type 1 diabetes. At first sight, these results are comparable with previous studies. A prevalence of 28% of DSPN was reported in type 2 diabetic patients in the German population and in 12.9% to 28.5% of type 1 diabetic individuals in clinic-based sample (Albers et al. 2010; Cabezas-Cerrato 1998; Tesfaye et al. 2005; Van Acker et al. 2009; Young et al.1993; Ziegler et al. 2008). Beyond that, a higher prevalence of DSPN in type 2 compared to type 1 diabetes is consistenly supported by previous research (Cabezas-Cerrato 1998; Van Acker et al. 2009; Young et al. 1993). However, in the present study diabetic individuals with *recent* onset of disease were investigated, in which the prevalence has been reported to be lower (Partanen et al. 1995; Shaw et al. 1998). Therefore, results of the present study suggest that the prevalence of DSPN in newly diagnosed diabetic individuals might be higher than expected and may be identified when taking into account symptoms, signs and NCV.

4.1.3 Biomarkers of subclinical inflammation

In the present study type 1 and type 2 diabetic participants exhibited a different serum immune profile with significant higher levels of proinflammatory cytokines and soluble

adhesion molecules in type 2 than in type 1 diabetes while levels of adiponectin were significantly lower in type 2 compared to type 1 diabetes.

In previous studies increased levels of inflammatory mediators were consistently reported in the diabetic population when compared to healthy controls (Devaraj et al. 2010). However, given that subsamples of the present study were recruited as part of a larger observational study, data of a healthy control group were not available. Nevertheless, the present study extends current knowledge, as levels of proinflammatory mediators were shown to differ significantly between type 1 and type 2 diabetes patients and were higher in type 2 diabetes. Lower levels of adiponectin in type 2 compared to type 1 diabetes in the present study reflect the current literature which consistently reports adiponectin to be decreased in type 2 diabetes while increased in type 1 diabetes (Krakoff et al. 2003; Nigro et al. 2014; Fantuzzi 2008; Hadjadj et al. 2005). However, exact regulatory mechanisms of adiponectin and their pathophysiological implications in these two diseases remain uncertain. Given its insulin-sensitizing effect in type 1 and type 2 diabetes it might be speculated that differences in adiponectin between these two types of diabetes are a consequence of a more complex interplay between insulin and adiponectin. Accordingly, higher adiponectin levels in type 1 diabetes may be due to a lack of endogenous insulin production while decreased adiponectin levels in type 2 diabetes may result as a response to chronic hyperinsulinaemia. Previous research also links adiponectin to cardiovascular disease and some authors suggested that adiponectin may act as an indicator of cardiovascular diseases. In this context atrial natriuretic peptide and brain natriuretic peptide which both indicate cardiovascular stress, have been shown to increase adiponectin release (Sattar 2011). Therefore, adiponectin might reflect underlying cardiovascular disease which is the most important macrovascular complication of diabetes mellitus. Nevertheless, this approach cannot explain the robust finding of higher adiponectin levels in type 1 compared to type 2 diabetes. Moreover, in the present sample the presence of cardiovascular disease was very low as only recently diagnosed diabetes patients were included.

4.2 Subclinical inflammation and DSPN in type 1 and type 2 diabetes

Regarding the association between subclinical inflammation and NCV it was hypothesized that acute-phase proteins (hsCRP) and proinflammatory mediators (IL-6, IL-18), adhesion molecules (sICAM-1, sE-selectin) and adiponectin are associated with MNCV and SNCV and with presence of DSPN in type 1 and type 2 diabetes.

Acute-phase protein hsCRP and proinflammatory cytokines IL-6 and IL-18

In type 2 diabetes participants, higher IL-6 levels were associated with reduced MNCV and with the presence of DSPN. However, no association was found between IL-6 and SNCV. These findings suggest a differential role of IL-6 in NCV slowing in motor versus sensory nerves, at least in type 2 diabetes. The association between IL-6 and the MNCV sum score was mostly driven by associations between IL-6 and peroneal and median NCV. This may indicate that different nerves are prone differentially to inflammation-related NCV slowing. Similarly, it has been suggested by others that the extent of NCV slowing differs between different nerves (Charles et al. 2010; Partanen et al. 1995). However, present results do not support the notion that nerves of the upper versus the lower limbs are affected differently as associations between IL-6 and peroneal and median NCV were comparable (Chrales et al. 2010). As the exact mechanisms linking biomarkers of subclinical inflammation to NCV slowing are unknown, potential differential effects on different nerves and nervemodality remain descriptive. Nevertheless, present findings are supported by previous studies in type 2 diabetes and population-based samples which demonstrated an inverse association between higher IL-6 levels and decreased motor NCV and positive associations between IL-6 and numerous symptoms and signs of DSPN (Herder et al. 2009.; Herder et al. 2013a; Kökoglu et al. 2009; Magrinelli et al. 2015).

In type 1 diabetic patients of the present study IL-6 was not associated with MNCV or SNCV or presence of DSPN. At first sight, this may suggest a differential role of IL-6 in the slowing of NCV in type 1 versus type 2 diabetes patients. However, wider confidence intervals of effect sizes in type 1 diabetes patients suggest reduced statistical power in this subsample. This is most likely a consequence of a smaller sample size of type 1 diabetic participants in the present study. After all, direct comparison of effect sizes and confidence intervals of the association between IL-6 and motor NCV in type 1 and type 2 diabetes patients suggest similar inverse associations in both subsamples. Nevertheless, the latter assumption remains speculative as results in the subsample of type 1 diabetes participants did not reach statistical significance. However, from a mechanistic perspective it is biologically plausible that IL-6 which has various proinflammatory properties, may be implicated in structural and/or functional deficits of nerve fibres by direct or indirect effects in both diabetes types.

In the present study no association was found between hsCRP and MNCV or SNCV or presence of DSPN in type 1 or type 2 diabetes patients. Previous research regarding the association between CRP and DSPN is ambiguous. While some studies did

demonstrate an association of CRP with symptoms and signs of DSPN in type 1 and type 2 diabetes (Herder 2009.; Herder 2013a), others did not find an association between CRP and sural NCV in type 2 diabetic individuals (Kang et al. 2005; Kökoglu et al. 2009; Magrinelli et al. 2015). As mentioned above, in the present study insufficient statistical power in the subsample of type 1 diabetes individuals may have been of relevance. Nevertheless, present results support the assumption that CRP is not associated with NCV in recently diagnosed type 2 diabetic individuals. Taken together, even though previous research provides some evidence that CRP may be implicated in the development of DSPN, there is considerable more evidence for a causal role of the IL-6 system in the development of DSPN which is supported by results of the present study (Devaraj et al. 2010). However, a temporal relationship cannot be concluded based on the present cross-sectional design. Nevertheless the aforementioned assumption is supported by a prospective population-based study which reported increased systemic levels of IL-6 to be associated with higher risk of DSPN over 6.5 years (Herder et al. 2017).

This is the first study to demonstrate an association between IL-18 and NCV. In the present study a positive association was found between IL-18 and ulnar SNCV in type 2 diabetes participants. Results indicate that higher levels of IL-18 are associated with less NCV slowing. However, no association was found between IL-18 and presence of DSPN or MNCV in type 1 or type 2 diabetes patients. Although previous findings regarding the association between IL-18 and DSPN are inconsistent, some results according to which IL-18 was inversely associated to some measures of DSPN in type 2 diabetes, point into the same direction as present results (Herder et al. 2009). Taken together previous results and results of the present study suggest that higher IL-18 levels may be associated with less symptoms, signs and SNCV slowing. Although at first sight it appears that results suggest a rather protective role of IL-18 in DSPN, an association between IL-18 and symptoms and signs is not supported by results of the present study as no association became evident between IL-18 and presence of DSPN. Beyond that, IL-18 was previously found to be associated with a higher MNSI score in a population-based sample (Herder et al. 2013a), while no association was found between IL-18 and DSPN in a prospective population-based study (Herder et al. 2017). Given the cross-sectional design of the present study in which furthermore only recently diagnosed diabetic individuals were investigated, the exact role of IL-18 in the development of DSPN remains unresolved as a more complex interplay between IL-18, NCV and symptoms and signs of DSPN cannot be ruled out with certainty.

Adiponectin as an anti-inflammatory factor

In type 2 diabetic individuals, higher levels of HMW and total adiponectin as well as their ratio were associated with decreased MNCV and SNCV and presence of DSPN. Hence, results suggest that higher adiponectin levels are associated with greater risk of DSPN. Up to date no other study has demonstrated an independent inverse association between adiponectin levels and NCV as an indicator of DSPN. Previous studies in various populations either showed a positive association between adiponectin and different measures of DSPN or were not able to show any association at all (Herder et al. 2009; Herder et al. 2013a; Jung et al. 2014). In a prospective population-based study lower systemic adiponectin levels were linked to a higher risk of DSPN over 6.5 years (Herder et al. 2017). Results of the present study are counterintuitive as adiponectin has been shown to act anti-inflammatory and atheroprotective and higher adiponectin levels are associated with decreased risk to develop type 2 diabetes (Nigro et al. 2014). One possible explanation might be that findings are a result of confounding by underlying cardiovascular disease. As mentioned earlier release of adiponectin is increased by atrial natriuretic peptide as well as brain natriuretic peptide which both indicate cardiac stress (Sattar 2011). However, in the present study extensive adjustment for the history of myocardial infarction and/or stroke as well as for numerous cardiovascular risk factors diminishes the risk of confounding by cardiovascular disease. Beyond this, the presence of this macrovascular diabetic complication is unlikely in the present study sample, as only recently diagnosed type 1 and type 2 diabetic individuals were included. Interestingly, some studies have shown that higher adiponectin levels are also associated with retinopathy which is a different manifestation of microvascular complications in diabetes. Higher levels of adiponectin were found in type 2 diabetic patients with retinopathy compared to those without retinopathy (Jung et al. 2014; Kato et al. 2008; Pradeepa et al. 2015). These findings support results of the present study and accordingly point towards an implication of higher adiponectin in microvascular complications in type 2 diabetes. Nevertheless, exact mechanisms linking adiponectin to microvascular complications and DSPN in particular remain uncertain. Despite its anti-inflammatory and atheroprotective functions and its beneficial effects on the risk for incidence of type 2 diabetes, data of the present study suggest that at least during recent onset of disease higher adiponectin may be associated with DSPN development. However, as analyses were cross-sectional conclusions regarding causal associations cannot be drawn. Therefore it remains uncertain whether adiponectin directly impacts nerve function or whether adiponectin is upregulated, eventually in a protective approach, as a consequence of nerve damage.

In type 1 diabetic patients higher total and HMW adiponectin levels were associated with higher MNCV. These results strongly contrast findings in type 2 diabetes and suggest differential associations between adiponectin and DSPN in type 1 versus type 2 diabetes. It is possible that the upregulation of adiponectin reflects a protective mechanism sustaining motor nerve function in type 1 diabetes. Nevertheless, this interpretation of results remains speculative as exact underlaying regulatory mechanisms of adiponectin are uncertain and it is not known how adiponectin exerts effects on nerve function. The presented positive association may as well be a consequence of confounding conditions which however is highly unlikely as extensive adjustment for potential confounders was performed. Previous studies have indeed suggested differential roles of adiponectin in type1 and type 2 diabetes which supports the emerging notion of the present study (Fantuzzi 2008; Frystyk et al. 2005).

Interestingly, associations between adiponectin and NCV sum scores were mostly driven by adiponectins association with peroneal NCV in type 2 diabetes and with ulnar NCV in type 1 diabetes. Results suggest that different nerves might be affected differentially by inflammation-associated NCV slowing, underscoring the relevance of sum scores.

Lastly, in the present study associations between HMW and total adiponectin and their ratio on the one hand and DSPN on the other hand did not show considerable differences. It has been suggested by some authors that HMW adiponectin might be biologically more active and of greater relevance in the context of insulin-sensitivity (Hara et al. 2006; Nakashima et al. 2006). Closer correlations have been reported between HMW adiponectin and insulin-resistance and other metabolic abnormalities than between total adiponectin and these variables (Singer et al. 2012). However, others were not able to attribute this advantage to HMW adiponectin (Almeda-Valdes et al. 2010; Singer et al. 2012; Zhu et al. 2010). The present study extends current knowledge indicating that HMW does not appear to be of additional value compared to total adiponectin.

Soluble adhesion molecules sICAM-1 and sE-selectin

In the present study adhesion molecules as marker of endothelial dysfunction were neither associated with presence of DSPN nor motor NCV in type 1 or type 2 diabetes. However, increased sICAM-1 levels were found to be associated with higher SNCV in type 2 diabetes but not type 1 diabetes, although only after full adjustment.

Present findings contrast previous findings which on the one hand demonstrated positive associations between adhesion molecules and symptoms and

signs of DSPN and on the other hand demonstrated inverse associations between sICAM-1 levels and peroneal NCV in pooled samples (Doupis et al. 2009; Herder et al. 2013a; Jude et al. 1998). In addition, a positive association was reported between sICAM-1 and the progression of DSPN in a prospective population-based study (Herder et al. 2017). However, associations of adhesion molecules with DSPN and NCV in particular are highly inconsistent in the present study and mostly fail statistical significance. Therefore, results suggest that an implication of these biomarkers in DSPN at least in recently diagnosed diabetic patients is rather unlikely. Nevertheless, as mentioned before, reduced statistical power in the subsample of type 1 diabetes limits interpretation of results. Diverging results might also be a consequence of different chracteristics of investigated study populations. In the present study recently diagnosed diabetic patients were investigated while previous results were conducted in samples of diabetic patients who had received their diagnosis up to 20 years before the study (Doupis et al. 2009; Jude et al. 1998). Beyond that, the present cross-sectional study investigated the association between sICAM-1 and incidence of DSPN but not progression of DSPN. Given that exact pathways underlying the association between subclinical inflammation and alterations in NCV in general are largely unknown, the exact cause and nature of these particular associations remain uncertain.

4.3 Strength and limitations

The present study is characterized by numerous strengths although some limitations became evident, all of which will be discussed briefly in the following paragraph. Associations between biomarkers of subclinical inflammation and DSPN were investigated in separate samples of type 1 and type 2 diabetes. This allows comparison of subsamples and detection of differences between these different types of diabetes regarding the association of subclinical inflammation and DSPN. Therefore, the present study design contributes substantially to the extension of current knowledge regarding this matter as studies in both subsamples, but particularly in type 1 diabetes are very limited.

Short duration of disease in the study sample comprises a strength of the present study as it allowed to investigate associations between biomarkers of subclinical inflammation and DSPN before the onset of potentially confounding complications usually emerging in the course of diabetes mellitus. In addition to that, extensive adjustment for potential confounding variables comprises a strength of the present study.

Nerve conduction studies were carried out applying gold-standard methods and validated criteria and further differentiating between MNCV versus SNCV allowing to detect differential effects of subclinical inflammation on different nerve modalities. The inclusion of NCV sum scores as a recommended but yet novel measure of DSPN extends current knowledge, providing evidence for their utility as an indicator for DSPN. In addition to that different isoforms of adiponectin were included extending the data conducted in previous studies, however indicating that these different measures of adiponectin do not differ substantially from each other.

Limitations arose mostly as a consequence of the study design applied. First and foremost, the cross-sectional design of the present study does not allow to draw conclusions regarding the temporal association between biomarkers of subclinical inflammation and DSPN or NCV or regarding the causality in these associations. In addition, the study did not include a healthy control group, which limits the interpretation of results. For example, the conclusion of an absolute increase of inflammatory agents in type 1 or type 2 diabetes cannot be drawn. However, data of a healthy control group were not available as the present study was conducted in the framework of a larger study that was originally designed as an observational study investigating the natural history of diabetes. Furthermore, a smaller sample size of type 1 diabetic compared to type 2 diabetic participants resulted in reduced statistical power in the subsample of type 1 diabetes increasing the risk of type II errors. Differences in sample size were a consequence of consecutive inclusion of participants into the larger study which was inevitable due to the study design. As the larger study was not designed as a population-based study and given that only recently diagnosed type 1 and type 2 diabetic participants were included, results of the present study are not generalizable to the German population of diabetes patients or to patients with longer disease duration or other forms of diabetes mellitus.

Vitamin B12 deficiency as potential cause of DSPN was not ruled out, as data on this parameter were not available. Lastly, analyses were not adjusted for multiple testing, as immune mediators and outcome variables were not independent of each other and Bonferroni correction which is commonly used for adjustment would have been a too conservative approach. As a consequence, the risk for type I error is increased.

4.4 Conclusion and outlook

This is the first study that systematically investigated the association between biomarkers of subclinical inflammation, NCV and DSPN comparing subsamples of type 1 and type 2 diabetes.

The present study indicates that increased serum IL-6 is associated with reduced NCV in type 2 diabetes and suggests that the IL-6 system may contribute to peripheral large nerve fibre dysfunction and DSPN. Furthermore, opposite associations between adiponectin and NCV in type 1 versus type 2 diabetes support the notion that the pathogenesis of DSPN may be diverging in these different types of diabetes: First, in type 2 diabetes, beyond metabolic risk factors, subclinical inflammation may play a more prominent role than in type 1 diabetes. Second, some biomarkers of subclinical inflammation appear to exert differential effects in type 1 compared to type 2 diabetes. Lastly, results of the present study provide some limited evidence that different nerves and different nerve modalities may be differentially prone to inflammation-related nerve damage supporting the notion of sum scores to evaluate an individual´s condition.

Further research is needed to investigate the association between subclinical inflammation and DSPN and NCV in type 1 diabetes to overcome limitations due to reduced statistical power of the present study. Beyond this, longitudinal studies are needed to draw causal conclusions and get more insight in potential underlying pathophysiological mechanisms linking subclinical inflammation to DSPN and NCV. Potential differential effects on different nerves and different nerve modality need further exploration. Amplitude as another important measure in NCS indicating DSPN should be considered as an outcome measure in future studies investigating the association between subclinical inflammation and DSPN.

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6 Appendix

Table A-1: Neuropathy Symptom Score (NSS)

	Yes (points)*	No (points)	
Symptomatology: Foot/lower leg			
Burning sensation	2	0	
Numbness	2	0	
Paraesthesia	2	0	Total points:
Feeling of weakness (fatigue, exhaustion)	1	0	
Cramps	1	0	
Pain	1	0	Total points:
Location			
Feet	2	0	
Lower leg	1	0	
Elsewhere	0	0	Total points:
Exacerbation			
Present at night	2	0	
Present during day and night	1	0	
Only present during the day	0	0	
Patient is awakened from sleep by the symptoms	Add 1		Total points:
Symptoms improvement when			
Walking	2	0	
Standing	1	0	
Sitting or lying down	0	0	Total points:

Scoring of the NSS: 3-4 = mild symptoms; 5-6 = moderate symptoms; 7-10 = severe neuropathic symptoms. *In each point column, the maximum score can be given only once.

Table A-2: Neuropathy Disability Score (NDS)

	Right side	Left side
Ankle reflex		
Normal	0	0
Diminished	1	1
Absent	2	2
Vibration perception		
Normal	0	0
Diminished/absent	1	1
Pain perception		
Normal	0	0
Diminished/absent	1	1
Temperature perception		
normal	0	0
Diminished/absent	1	1

Scoring of the NDS: 3-5 = mild neuropathic deficits; 6-8 = moderate neuropathic deficits; 9-10 = severe neuropathic deficits.

Table A-3: Biospecimen type and handling and laboratory measurement of metabolic parameters and biomarkers of subclinical inflammation

Variables	Biospecimen type	Storage duration until analysis	Storage temperature	Freeze-thaw cycles before analysis	Assay	Intra-assay CV (%)	Inter-assay CV (%)	Measurement range (for undiluted samples	Comments
HbA1c (Percent and mmol/mol)	EDTA whole blood	0-6h	RT	n/a	Variant-II (Bio-rad, Munich, Germany)	<1	<1	n/a as analyser has automated dilution system at certain range	
Total cholesterol (mmol/l)	Serum from whole blood with clot activator	0-6h	RT	n/a	Enzymatic assay on Hitachi 912 or Modular P system (both Rocher Diagnostics, Mannheim, Germany)	<1/1.9	2.2/3.5	n/a as analyser has automated dilution system at certain range	
Triglycerides (mmol/l)	Serum from whole blood with clot activator	0-6h	RT	n/a	Enzymatic assay on Hitachi 912 or Modular P system (Roche Diagnostics, Mannheim, Germany)	<1/1.1	2.2/4.2	n/a as analyser has automated dilution system at certain range	
hsCRP (mg/L)	Serum from whole blood with clot activator	3.25-6.5 years	-80°C	0	Roche/Hitachi c 311 analyzer (Basel, Switzerland)	1.9	3.9	n/a as analyser has automated dilution system at certain range	
IL-6 (pg/mL)	Serum from whole blood with clot activator	3.25-6.5 years	-80°C	1	Quantikine HS ELISA (R&D Systems, Wiesbaden, Germany)	6.0	12.2	0.156-10 pg/ml	All samples were measured undiluted and yielded values within the measurement range
IL-18 (pg/mL)	Serum from whole blood with clot activator	3.25-6.5 years	-80°C	1	IL-18 ELISA (MBL, Nagoya, Japan)	6.5	12.1	10.24-1000 pg/ml	All samples were measured at 1:5 dilution and yielded values within the measurement range

Total adiponectin (ng/mL)	Serum from whole blood with clot activator	0.5-6.5 years	-80°C	1	Adiponectin (Multimeric) ELISA, (ALPCO Diagnostics, Salem, NH, USA)	2.34	7.88	Range of standards 0.075-4.8 ng/ml	All samples were pretreated and measured according to the manufacturer´s instructions (final dilution factor 1:5151)
HMW adiponectin (ng/mL)	Serum from whole blood with clot activator	0.5-6.5 years	-80°C	1	Adiponectin (Multimeric) ELISA, (ALPCO Diagnostics, Salem, NH, USA)	3.56	10.82	Range of standards 0.075-4.8 ng/ml	All samples were pretreated and measured according to the manufacturer´s instructions (final dilution factor 1:5151)
sE-selectin (ng/mL)	Serum from whole blood with clot activator	3.25-6.5 years	-80°C	1	Quantikine HS ELISA (R&D Systems, Wiesbaden, Germany)	3.3	5.0	0.125-8 ng/ml	All samples were measured at 1:10 dilution and yielded values above the lower limit of detection. Samples exceeding the upper limit of detection were measured at 1:20 dilution.
sICAM- (ng/mL)	Serum from whole blood with clot activator	3.25-6.5 years	-80°C	1	Quantikine HS ELISA (R&D Systems, Wiesbaden, Germany)	2.0	3.3	1.56-50 ng/ml	All samples were measured at 1:20 dilution and yielded values within the measurement range

n/a, not applicable; RT, room temperature (20-25°C); CV, coefficient of variation; HMW, high-molecular-weight adiponectin; hsCRP, high-sensitivity C-reactive protein; IL, interleukin; sE-selectin, solouble E-selectin; sICAM, solouble intercellular adhesion molecule-1 (adapted from Weber et al. 2015).

Table A-4: Pearson correlations between biomarkers of subclinical inflammation and demographic, anthropometric and metabolic risk factors in patients with type 1 diabetes

Variable	hsCRP	IL-6	IL-18	Total adiponectin	HMW adiponectin	HMW/total adiponectin	sE-selectin	sICAM-1
Sex (female/ male)	0.090	0.068	-0.082	**0.436*****	**0.476*****	**0.464*****	**-0.219****	0.019
Age (years)	-0.009	**0.194***	0.147	-0.090	-0.081	-0.054	-0.007	0.143
Body mass index (kg/m²)	**0.372*****	**0.320*****	**0.296*****	**-0.281*****	**-0.251*****	**-0.162***	**0.344*****	**0.253*****
Waist circumference (cm)	**0.363*****	**0.368*****	**0.296*****	**-0.396*****	**-0.374*****	**-0.278*****	**0.335*****	**0.229****
Height (cm)	0.110	-0.034	0.019	**-0.252*****	**-0.303*****	**-0.336*****	0.147	-0.067
Time since diagnosis of diabetes (days)	-0.139	-0.113	-0.050	**0.193***	**0.187***	0.146	-0.097	-0.033
HbA_{1c} (%)	**0.197***	**0.237****	**0.161***	0.004	-0.013	-0.038	**0.184***	0.110
Total cholesterol (mmol/l)	0.156	-0.029	0.119	0.002	0.005	0.010	-0.025	0.138
Fasting triglycerides (mmol/l)	**0.298*****	**0.233****	**0.296*****	**-0.412*****	**-0.405*****	**-0.328*****	**0.271*****	**0.246****
Systolic blood pressure (mmHg)	0.069	0.055	0.073	**-0.204****	**-0.189***	-0.135	0.138	0.147
Diastolic blood pressure (mmHg)	**0.217***	**0.178***	0.117	-0.147	-0.085	0.028	0.118	0.095
Hypertension†	**0.214****	0.105	0.000	-0.092	-0.058	0.006	0.144	**0.178***
Current smoking	**0.260*****	**0.411*****	**0.163***	-0.060	-0.015	0.059	**0.230****	**0.463*****

Physically inactive††	0.140	0.120	0.107	-0.097	-0.048	0.039	0.009	0.125
History of myocardial infarction and/or stroke	0.032	0.079	0.054	0.134	0.115	0.065	-0.099	**0.179***
Lipid-lowering medication	0.064	0.051	0.067	-0.012	-0.014	-0.016	-0.069	0.092
Antihypertensive medication	**0.233****	0.146	0.063	**-0.173***	**-0.170***	-0.138	0.082	0.101
Non-steroidal anti-inflammatory drugs	0.063	0.062	0.001	-0.013	0.004	0.032	0.106	0.106

Correlations are assessed by Pearson correlation coefficients r. Concentration of immune markers were log-transformed. *p<0.05; **p<0.01; ***p<0.001 Hypertension, physical inactivity, history of myocardial infarction and/or stroke, and medication use were dichotomized. †Hypertension is defined as blood pressure ≥ 140/90 mmHg or use of antihypertensive medication. ††Physical inactivity is defined as absence of regular physical training at the time of the examination. HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio.

Table A-5: Pearson correlations between biomarkers of subclinical inflammation and demographic, anthropometric and metabolic risk factors in patients with type 2 diabetes

Variable	hsCRP	IL-6	IL-18	Total adiponectin	HMW adiponectin	HMW/total adiponectin	sE-selectin	sICAM-1
Sex (% male)	**0.211*** **	0.097	-0.030	**0.352*** **	**0.356*** **	**0.308*** **	0.020	**0.134***
Age (years)	-0.089	0.066	0.016	**0.244*** **	**0.223*** **	**0.160** **	-0.033	-0.057
Body mass index (kg/m²)	**0.465*** **	**0.361*** **	**0.223*** **	-0.085	**-0.109***	**-0.127***	**0.295*** **	**0.174*** **
Waist circumference (cm)	**0.395*** **	**0.352*** **	**0.248*** **	**-0.178*** **	**-0.200*** **	**-0.202*** **	**0.260*** **	**0.154** **
Height (cm)	**-0.206*** **	-0.103	-0.032	**-0.300*** **	**-0.312*** **	**-0.282*** **	-0.083	**-0.131***
Time since diagnosis of diabetes (days)	-0.034	-0.105	-0.029	0.057	0.082	**0.107***	0.023	-0.027
HbA_{1c} (%)	**0.144** **	**0.173*** **	**0.152** **	-0.095	**-0.111***	**-0.117***	**0.174*** **	**0.172*** **
Total cholesterol (mmol/l)	0.105	0.021	0.013	**0.152** **	**0.130** **	0.080	**0.132***	0.076
Fasting triglycerides (mmol/l)	**0.142** **	0.074	**0.193*** **	**-0.301*** **	**-0.303*** **	**-0.261*** **	**0.230*** **	**0.179*** **
Systolic blood pressure	0.039	0.066	0.034	0.018	0.010	-0.003	**0.189*** **	0.002

(mmHg)								
Diastolic blood pressure (mmHg)	**0.162****	0.089	0.083	-0.016	-0.023	-0.029	**0.186*****	0.049
Hypertension†	**0.115***	0.047	0.030	-0.032	-0.037	-0.040	**0.176*****	0.059
Current smoking	0.104	**0.199*****	0.025	-0.039	-0.011	0.031	0.080	**0.340*****
Physically inactive††	**0.158****	**0.200*****	**0.123***	-0.010	0.010	0.038	0.093	**0.171*****
History of myocardial infarction and/or stroke	0.002	0.019	-0.068	-0.007	0.023	0.063	-0.019	-0.018
Lipid-lowering medication	**-0.135***	-0.029	0.057	0.014	0.029	0.047	-0.042	0.027
Antihypertensive medication	-0.003	0.006	-0.001	-0.047	-0.052	-0.053	0.042	0.003
Non-steroidal anti-inflammatory drugs	**0.114***	0.093	0.039	**0.107***	0.073	0.015	0.012	0.072

Correlations are assessed by Pearson correlation coefficients r. Concentration of immune markers were log-transformed. *p<0.05; **p<0.01; ***p<0.001Hypertension, physical inactivity history of myocardial infarction and/or stroke, and medication use were dichotomized. †Hypertension is defined as blood pressure ≥ 140/90 mmHg or use of antihypertensive medication. ††Physical inactivity is defined as absence of regular physical training at the time of the examination. HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio.

Table A-6: Pearson correlations between biomarkers of subclinical inflammation in patients with type 1 (grey) and type 2 (white) diabetes

Variable	hsCRP	IL-6	IL-18	Total adiponectin	HMW adiponectin	HMW/total adiponectin	sE-selectin	sICAM-1
hsCRP	1	**0.50*** **	**0.33*** **	-0.07	-0.04	-0.01	**0.17** **	**0.33*** **
IL-6	**0.58*** **	1	**0.28** **	-0.14	-0.12	-0.07	**0.30*** **	**0.38*** **
IL-18	**0.16** **	**0.19*** **	1	-0.15	-0.15	**-0.16***	**0.30*** **	**0.32*** **
Total adiponectin	-0.08	**-0.08**	**-0.11***	1	**0.96*** **	**0.73*** **	**-0.22** **	-0.07
HMW adiponectin	-0.07	-0.07	**-0.13***	**0.96*** **	1	**0.90*** **	**-0.23** **	-0.04
HMW/total adiponectin	-0.04	-0.04	**-0.15***	**0.76*** **	**0.91*** **	1	**-0.22***	0.02
sE-selectin	**0.24*** **	**0.27*** **	**0.27*** **	-0.09	-0.10	-0.10	1	**0.51*** **
sICAM-1	**0.38*** **	**0.37*** **	**0.26*** **	-0.03	-0.03	-0.02	**0.47*** **	1

Correlations are assessed by Pearson correlation coefficients r. Concentration of immune mediators of subclinical inflammation were log-transformed. *p<0.05; **p<0.01; ***p<0.001. HMW, high-molecular-weight adiponectin; HMW/total adiponectin, HMW-to-total-adiponectin-ratio.

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