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Determination of cytokine and axon guidance molecule profiles in patients with small fiber neuropathy

Bestimmung von Zytokin- und Axon Guidance Molekül-Profilen bei Patienten mit Kleinfaserneuropathie

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1 Introduction

1.1 Small fiber neuropathy (SFN)

1.1.1 Definition and epidemiology of SFN

Small fiber neuropathy (SFN), categorized as a subgroup of painful sensory neuropathies, mostly affects thinly myelinated Aδ and unmyelinated C nerve fibers, which are responsible for pain and thermal perception (Lacomis 2002). Large nerve fibers are not or marginally affected. SFN is characterized by neuropathic pain and sensory disturbance (Voortman et al. 2017). Autonomic disturbance may also be present (Stewart et al. 1992, Thaisetthawatkul et al. 2013). A diagnostic gold standard is missing, hence representative epidemiological data for SFN incidence and prevalence are lacking (Cazzato and Lauria 2017). A recent study reported 53 SFN cases per 100,000 people (Peters et al. 2013) and an incidence of 12 per 100,000 per year (Brouwer et al. 2015) in The Netherlands.

1.1.2 Etiology of SFN

The list of potential etiologies for SFN is long. For classification, three main categories are differentiated: Primary genetic diseases such as Fabry disease (Toyooka 2013) or mutations in sodium channels such as sodium voltage-gated channel α subunit 9 (SCN9A) (Faber et al. 2012) can lead to SFN. Main secondary reasons are listed in Table 1. If the underlying cause of SFN remains unclear, patients are classified as having idiopathic SFN.

Table 1: Etiology of secondary SFN.

Sub-category	Examples
Metabolic	- Diabetes mellitus or impaired glucose tolerance - Vitamin B12 deficiency, hypothyroidism, dyslipidemia
Immune-mediated	- Sarcoidosis - Sjogren´s syndrome

	- Systemic lupus erythematosus
Paraneoplastic	- Small cell lung cancer
Infectious	- HIV infection - Hepatitis C - Lyme disease - Leprosy
Toxic	- Chemotherapeutics (e.g. platinum-containing drugs, vinca alkaloids) - Antibiotics (e.g. isoniazid, nitrofurantion)

Abbreviations: HIV = human immunodeficiency virus.

1.1.3 Clinical presentation of SFN

SFN is characterized by sensory and autonomic disturbance. Patients often describe burning pain accompanied by par- and dysesthesias (Devigili et al. 2008). Pain intensity, duration, and character differ widely between patients. Some patients report pain to be induced by touch (Basantsova et al. 2019) or stimuli such as heat or pressure (Hoeijmakers et al. 2012). Symptoms often start at the lower extremities and spread from distal to proximal (Terkelsen et al. 2017). Pain mostly occurs in feet and hands (Voortman et al. 2017), but may also be generalized (Lauria 2005). Sweating disturbances, dry mucous and skin, erectile dysfunction, decreased intestinal motility, and dysfunction of urinary system are common autonomic symptoms (Basantsova et al. 2019).

1.1.4 Diagnosis of SFN

There is no gold standard for the diagnosis of SFN. In current clinical practice, patients with a typical pain history and after exclusion of large fiber neuropathy undergo clinical examination, quantitative sensory testing (QST), and skin punch biopsy and SFN is assumed, if at least two out of these three tests are pathological (Stewart et al. 1992, Lacomis 2002, Devigili et al. 2008). To increase diagnostic yield, further small fiber tests such as quantitative

sudomotor axon reflex test (QSART) (Thaisetthawatkul et al. 2013), pain related evoked potentials (PREP) (Hoeijmakers et al. 2012), microneurography (Serra et al. 1999), and corneal confocal microscopy (CCM) (Tavakoli and Malik 2011) may be applied.

1.1.5 Therapy of SFN

In secondary SFN, treatment is causal and targets the underlying disease cause (De Greef et al. 2018, Tavee 2018). This may stop progression and may improve symptoms. If no reason can be found for SFN symptoms, patients are treated symptomatically by following the guidelines of neuropathic pain therapy (Cavalli et al. 2019). Drugs of first choice are tricyclic antidepressants (TCA) such as amitriptyline (Attal 2019), serotonin norepinephrine reuptake inhibitors (SNRI) such as duloxetine (Attal 2019), and anticonvulsants such as gabapentanoids (Bates et al. 2019). Non-pharmacological treatment options such as exercise and physiotherapy may be an additional option in the management of neuropathic pain (Pickering et al. 2016, Basantsova et al. 2019).

1.2 Pathophysiological mechanisms of neuropathic pain in SFN

Neuropathic pain is defined as "pain induced by lesions or diseases of the somatosensory nervous system (Treede et al. 2008, Jensen et al. 2011, Colloca et al. 2017)." Neuropathic pain in SFN can be caused genetically or may be acquired by e.g. metabolic imbalance, infectious diseases, and autoimmune disorders or occurs after lesions of the central and peripheral nervous system via toxins or trauma (Hung et al. 2017). Pain is often accompanied by neuropathy symptoms, which encompass positive symptoms (i.e. increase in function) such as mechanic and thermal hyperalgesia, or allodynia and negative symptoms (i.e. loss of function) such as hypoesthesia, and numbness (Austin and Moalem-Taylor 2010). The pathophysiological mechanisms of neuropathic pain in SFN are elaborate (Hoeijmakers et al. 2012).

Animal models of neuropathic pain:

Knowledge of the pathophysiological mechanisms of pain was primarily gained by conducting animal and in vitro experiments. Therefore, different animal models were used to induce neuropathic pain and examine the underlying mechanisms. Symptoms such as spontaneous pain, or thermal and mechanical hyperalgesia are mostly induced by complete or partial ligation of the sciatic nerve (Bennett and Xie 1988, Seltzer et al. 1990). Another variant of partial denervation is sciatic nerve injury with intact sural nerve and lesion of the tibial and common peroneal nerve (Decosterd and Woolf 2000). Also, experimental models leading to drug-induced pain, applying peripheral nerve injuries or inducing metabolic neuropathies were developed (Jaggi et al. 2011, Yalcin et al. 2014).

Pathophysiological mechanisms:

Neuropathic pain is mainly based on central and peripheral sensitization. Central sensitization is caused by ongoing discharges of afferent fibers from the periphery or inhibition of descending pathways (Colloca et al. 2017). Continuing input from peripheral fibers leads to release of neuropeptides and excitatory amino acids, which results in activation of postsynaptic receptors such as N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Baron et al. 2013). Peripheral sensitization is induced by decreased thresholds for stimulation (hyperactivation) and increased firing frequencies of impulses (hyperexcitability), which arises e.g. by dysregulation of ion channels or elevated levels of inflammatory mediators (Cohen and Mao 2014).

Ion channels:

Nerve lesions or diseases provoke expression of neurotransmitters, transcription factors, and growth factors, which lead to increase of gene expression of receptors and enhanced excitability of ion channels (Hung et al.

2017). Thus, ion channels react hypersensitive to regular stimuli or an increased expression of channels results in decreased thresholds for action potential generation (Campbell and Meyer 2006). Voltage-gated sodium and calcium channels are mainly involved in pain transmission (Rolyan et al. 2016, Bennett et al. 2019), whereas ion channels of the transient receptor potential (TRP) family primarily play a role in stimuli detection (Roa-Coria et al. 2019).

Immune system:

Nerve damage by lesion, disease or toxins causes the exposition of inflammatory

mediators and immune cells such as macrophages, T and B lymphocytes via activation of Schwann cells, which are stimulated across extracellular signal related (ERK) mitogen-activated protein (MAP) kinase signalling pathways (Napoli et al. 2012). Imbalance in the expression of pro- and anti-inflammatory cytokines as immune mediators lead to increased nociceptive response in peripheral and central nervous system (Üçeyler et al. 2010, Calvo et al. 2012, Hung et al. 2017).

1.3 Axon guidance molecules and neuropathic pain

1.3.1 Definition and function of axon guidance molecules

Axon guidance molecules are extracellular matrix proteins that modulate axon growth by attraction or repulsion of nerve fibers depending on the respective receptor (Lee et al. 2019). Axon guides are involved in cell development and inflammatory processes (Lee et al. 2019). Axon guidance molecules interact via chemical and mechanical stimuli (Lai Wing Sun et al. 2011). Netrins, semaphorines, slits, Wnt, and ephrines are examples of axon guidance molecules. Their function e.g. cell motility activation, development of white matter structure or reorganization of actin cytoskeleton in the central nervous system is well characterized (Kang et al. 2018, Niftullayev and Lamarche-Vane 2019). In the peripheral nervous system and in skin, function of selected netrins and some of their receptors, e.g. Deleted in Colorectal Cancer (DCC),

neogenin, and the Unc – family (Unc5a-d) and ephrins in epithelial cells have been investigated in recent studies (Lai Wing Sun et al. 2011, Perez White and Getsios 2014).

1.3.2 Netrin 1 (NTN1) and its receptor Unc5b

Netrins are members of the laminin family and interact with receptors of the immunoglobulin family (Kefeli et al. 2017). Netrins were found in five different subtypes and investigated in diverse tissue in humans (Lai Wing Sun et al. 2011, Kefeli et al. 2017). The function of netrins was investigated in animal studies, which showed an increased NTN1 production after nerve lesion and inflammation (Moon et al. 2006, Masuda et al. 2009). Furthermore, NTN1 expression is involved in axon regeneration (Webber et al. 2011). NTN1 operates, depending on the interaction with the respective receptor in two contrasting ways: nerve fiber attraction and repulsion (Boyer and Gupton 2018).

1.3.3 Ephrin A4 (EFNA4) and its receptor Ephrin receptor A4 (EPHA4)

Ephrins interact as ligands of the ephrin receptors (EPH), which are members of the receptor protein-tyrosine kinase (RTK) family (Lisabeth et al. 2013). Ephrins are categorized in two subtypes: the ephrins A, which interact with EPHA1-8 and 10, and the ephrins B, which bind on EPHB 1-4 and 6 (Perez White and Getsios 2014). Apart from being involved in embryonic development, angiogenesis, and synaptic transduction, ephrins play a role in axon guidance and are involved, combined with their receptors, in neuropathic pain development as shown in animal studies (Khangura et al. 2019). Ephrins and their receptors are expressed by neuronal (dorsal root ganglion neurons, spinal dorsal horn) (Khangura et al. 2019), and non-neuronal (breast epithelium, epithelium of the gastrointestinal tract, skin) cells express (Perez White and Getsios 2014). Ephrin A is of particular importance for survival, differentiation, proliferation, and communication of keratinocytes (Perez White and Getsios 2014).

1.4 Cytokines and neuropathic pain

1.4.1 Definition and function of cytokines

Cytokines are small proteins (5-20 kDa), which play a crucial role in cell signalling (Austin and Moalem-Taylor 2010). Apart from immune cells that mainly synthesize and release cytokines, other cells such as fibroblasts and keratinocytes - as major cell types of the skin - also produce and secrete cytokines (Hänel et al. 2013). Cytokines affect the releasing cell by itself as well as neighbouring cells by using autocrine, paracrine, and endocrine action signalling pathways (Thacker et al. 2007). Cytokines can be functionality classified into two groups: pro-inflammatory and algesic, and anti-inflammatory and analgesic cytokines (Austin and Moalem-Taylor 2010). Further classifications are available because some cytokines are acting in both directions – pro- and anti-inflammatory – depending on the respective target location, milieu, and concentration (Liongue et al. 2016).

1.4.2 Cytokines and neuropathic pain

Pro- and anti-inflammatory cytokines are of importance in pathophysiology and development of neuropathic pain (Hung et al. 2017, Kwiatkowski and Mika 2018). Cytokines operate directly and indirectly, i.e. by activation and sensitization of nociceptors via production and release of pro-inflammatory cytokines (direct) and contribution of proteins to nociceptors and fibers such as prostaglandins and neuropeptides (indirect) (Petho and Reeh 2012). Anti-inflammatory cytokine expression is associated with pain relief and inhibition of the production of pro-inflammatory cytokines and their receptors (Hung et al. 2017). Recent studies showed higher expression of pro- and lower expression of anti-inflammatory cytokines in patients with neuropathic pain (Lees et al. 2015, Sommer et al. 2018). Based on these results, pro- and anti-inflammatory cytokine imbalance is involved in neuropathic pain (Hung et al. 2017). In patients with SFN, levels of cytokines are elevated in blood and in whole skin punch biopsies and may cause sensitization of nociceptors (Üçeyler et al. 2010).

1.4.3 Selected algesic, pro-inflammatory cytokines

Several studies gave evidence for the involvement of interleukins on nociceptors leading to neuropathic pain (Fattori et al. 2017). Interleukin-1 beta (IL-1ß) promotes an increase of nerve growth factor (NGF) expression, which may sensitize nociceptors (Vallejo et al. 2010). Increased systemic levels of IL-2 were measured in sera of patients with multifocal motor neuropathy and in white blood cells (WBC) of SFN patients (Gironi et al. 2010, Üçeyler et al. 2010, Furukawa et al. 2014). IL-6 and IL-8 RNA expression was higher in SFN patients compared to healthy controls (Üçeyler et al. 2010). Insulin-like growth factor-1 receptor (IGF-1) may affect nerve formation (Martins et al. 2018), tumor necrosis factor-alpha (TNF) may lead to nociceptor activity (Zhao et al. 2017). Detailed information of the selected pro-inflammatory cytokines and their main functions are summarized in Appendix in overview Table 1.

1.4.4 Selected analgesic, anti-inflammatory cytokines

Recent studies showed lower expression levels of IL-4, IL-10, and transforming growth factor (TGF)-ß1 in patients with neuropathic pain (Üçeyler et al. 2007). IL-4 is reported to decrease pain (Hung et al. 2017), whereas IL-10 and TGFß-1 revealed ambiguous effects on pain development (Hung et al. 2017). Further information is given in Appendix in overview Table 2.

1.5 Skin cells and neuropathic pain

Evidence is increasing on an active role of skin and skin cells in the development of neuropathy and neuropathic pain contributing to the concept of cutaneous nociception (Moehring et al. 2018, Talagas et al. 2018): Close vicinity between keratinocytes, fibroblasts, and axon terminals suggests that these cells influence structure and function of nociceptors (Evdokimov et al. 2019). Furthermore, keratinocytes and fibroblasts are reported to be involved in transmission and transduction of sensory stimuli and in sensitization of

nociceptors (Moehring et al. 2018, Talagas et al. 2018, Evdokimov et al. 2019). Skin cells release algesic mediators such as IL-1 and further cytokines and chemokines, which are expressed by keratinocytes (Feldmeyer et al. 2010, Niebuhr et al. 2010). Expression of nociception-associated ion channels such as voltage-gated sodium channels and TRP channels, as potentially relevant mediators of pain, were also found on skin cell surface (Gouin et al. 2017). Beyond that, skin cells may direct nerve fiber growth through releasing axon guidance cues (Kumamoto et al. 2014).

1.5.1 Keratinocytes

Apart from their barrier function, keratinocytes are secretory active cells, which release cytokines, chemokines, neuropeptides, and adenosin triphosphate (Olah et al. 2012, Hänel et al. 2013). Secretory function and the link between keratinocytes and nerve fibers, e.g. at synapses or chemical and electrical contacts, may inhibit outgrowth of nociceptors (Chateau et al. 2007, Denda et al. 2007, Baumbauer et al. 2015), which may result in a reduction of cutaneous innervation. In turn, proliferation of keratinocytes is influenced by cutaneous nerves (Martinez-Martinez et al. 2011). Keratinocytes also express cytokine receptors, growth factors, and nociceptor-associated ion channels on cell surface, which are involved in the development of neuropathic pain (Olah et al. 2012, Hänel et al. 2013). Thus, pain may be caused directly by stimulation of nociceptors and indirectly by keratinocytes, which perceive stimuli through different receptors, modulate them, and transmit signals to intraepidermal nerve fibers (Denda et al. 2007, Baumbauer et al. 2015).

1.5.2 Fibroblasts

Fibroblasts are the major cell type of the dermis and are in close vicinity to the subepidermal nervous plexus and the proximal part of the intraepidermal nerve fibers. Fibroblasts release mediators such as neuropeptides, cytokines, and pro-enkephalins, which may play a role in generation and maintenance of neuropathic pain (Slominski et al. 2011, Hänel et al. 2013). The dermal skin

cells carry pain-associated receptors and ion channels, which are involved in apoptosis and cell-migration such as voltage-gated sodium channels and TRP-channels (Peier et al. 2002, Zhao et al. 2008, Jain et al. 2011). Due to the close vicinity of fibroblasts to the subepidermal nervous plexus and the dermal part of the nerve fibers, fibroblasts may also be involved in growth modulation and sensitization of cutaneous nerve fibers.

1.6 Aims of the study

Increased levels of pro-inflammatory and decreased levels of anti-inflammatory cytokines measured in whole blood samples were reported to play a role in pain and nerve fiber sensitization in SFN (Üçeyler et al. 2010, Hung et al. 2017). Recent studies emphasized the role of axon guidance molecules in influencing cell development, inflammatory processes, and axon growth direction, e.g. in the spinal cord (Lee et al. 2019). Skin cells, such as fibroblasts and keratinocytes may in turn influence axon guidance cues, participate in axon pathfinding (Kumamoto et al. 2014, Wu et al. 2018), and play a crucial role in the development of neuropathic pain. In a previous study (Üçeyler et al. 2010) a higher gene expression of IL-6 and IL-8 was shown in whole skin samples obtained from SFN patients compared to healthy controls. In patients with length depended SFN, which was defined as a 5-fold higher intraepidermal nerve fiber density (IENFD) in proximal than in distal skin (Üçeyler et al. 2010), gene expression of IL-6, IL-8, and TGF-ß1 was elevated in patient samples compared to the control group. However, increased gene expression levels did not correlate with inflammatory infiltration (Üçeyler et al. 2010).
These findings suppose: 1) systemic and local influence of cytokines in pain development, 2) skin cells to be involved in nociceptor sensitization, and 3) the availability of surrounding skin cells, which secret algesic mediators.
Based on these hypotheses and the evidence of skin cells to be involved in neuronal sensitization and peripheral innervation, further approaches are needed to study underlying pathophysiological mechanisms.

The aims of the present study were:

1) to establish skin cell cultures of keratinocytes and fibroblasts obtained from 3 mm-skin punch biopsies for individual analysis of cell types
2) to study cytokine expression profiles of selected pro- and anti-inflammatory cytokines in WBC and investigate differences between SFN patients and healthy controls for further information of pain development in SFN
3) to analyze gene expression levels of axon guidance molecules, their receptors, and cytokine expression profiles of selected pro- and anti-inflammatory cytokines in isolated primary keratinocytes and fibroblasts of SFN patients and controls.

2 Material and methods

2.1 Patients

From May 2015 to October 2017, 55 patients were prospectively recruited as in- or out-patients at the Department of Neurology, University of Würzburg, Germany. After oral and written information about the study, all patients gave their written consent before inclusion. The ethics committee of the University of Würzburg (#135/15) Medical Faculty approved the study. Inclusion criteria for participation were: ≥18 years of age, typical medical history with neuropathic acral or widespread pain and a clinically assumed SFN. Patients suffering from malignancy within the last five years, severe psychiatric disorder currently requiring treatment, drug or alcohol misuse, inflammatory diseases, diabetes mellitus, polyneuropathy, untreated dysfunction of the thyroid gland or vitamin B12 deficiency were excluded. Further exclusion criteria were pain of different etiology, epilepsy, cardiac pacemaker, eye diseases or surgery, regular usage of hard contact lenses and current legal process.

2.2 Healthy controls

Among family members and friends of the patients, 31 healthy controls were recruited applying the same exclusion criteria. Control subjects also gave their written consent after oral and written information about the study.

2.3 Clinical examination and questionnaires

2.3.1 Medical and pain history

Detailed medical history was recorded with particular focus on pain, sensory disturbance, and autonomic dysfunction. Pain history covered pain character, localization, duration, and intensity estimated on a numeric rating scale (NRS) with 0 “no pain” and 10 “worst pain imaginable”. Factors alleviating and enhancing pain were also recorded. Study participants were further asked to fill in the following questionnaires: Graded Chronic Pain Scale (GCPS; six months recall) (Von Korff et al. 1992), Pain Catastrophizing Scale (PCS) (Meyer et al. 2008), and “Allgemeine Depressionsskala” (ADS) for depressive symptoms.

2.3.2 Clinical examination, nerve conduction studies, and small fiber tests

All patients underwent a complete neurological examination. Extensive laboratory tests were conducted as described under 2.5. Nerve conduction studies were performed on the right sural (sensory) and tibial (motor) nerves to exclude large fiber polyneuropathy following a standard procedure (Kimura 2001). Data were compared with normative values of the laboratory. For small fiber assessment, six small fiber tests were performed including QST to determine sensory profiles by following recommendations of the German Research Network of Neuropathic Pain (Rolke et al. 2006). PREP were recorded to investigate A-delta fiber conductance (Üçeyler et al. 2013). As a method to study single C-fiber afferents, microneurography was performed in selected patients (Schmelz et al. 1995, Hagbarth 2002). Autonomic small nerve fiber function was evaluated by QSART. CCM was conducted to non-invasively

analyze corneal innervation (Tavakoli and Malik 2011). Skin punch biopsies from the right lateral calf, 10 cm above the malleolus and the upper thigh, 20 cm below the spina iliaca anterior were obtained to quantify the IENFD (Üçeyler et al. 2010). Length-dependency (i.e. ≥5-fold higher proximal than distal IENFD (Üçeyler et al. 2010)) was determined.

2.4 Routine and extended laboratory blood tests

2.4.1 Laboratory tests

All patients underwent venous blood drawing. For standardization, peripheral blood was taken between 8:00 am and 9:00 am after overnight fasting. Patients were asked to avoid heavy physical activity, heavy meals or alcohol on the previous day. Current infection was an exclusion criterion for taking part. Laboratory tests included a full blood count, electrolytes, liver and kidney function, thyroid stimulating hormone (TSH), vitamin B12 and Haemoglobin A1c (HbA1c).

2.4.2 Genetic analysis

Nine ml venous blood was additionally taken for genetic analysis, which was performed by the Institute of Human Genetics at the University of Aachen, Germany. A gene panel including the core genes AAAS, ARL6IP1, ATL1, ATL3, CLTCL1, DNMT1, DST, FAM134B, FLVCR1, GLA, GMPPA, IKBKAP, KIF1A, NAGLU, NGF, NTRK1, PRDM12, RAB7A, SCN9A, SCN10A, SCN11A, SPTLC1, SPTLC2, TRPA1, TTR, WNK1 was sequenced.

2.4.3 Oral glucose tolerance test (OGTT)

An oral glucose tolerance test (OGTT) (Accu-Chek Dextro O.G.-T., Roche Diabetes Care Deutschland) was performed on capillary blood to determine fasting blood sugar. Blood sugar levels were measured at 60 min and 120 min after sugar challenge with 300 ml glucose sirup. Two-hours values between

140-199 g/dl were classified as impaired glucose tolerance, ≥200 g/dl as diabetes (Kerner and Bruckel 2014).

2.5 White blood cell (WBC) isolation

Two nine ml ethylenediaminetetraacetic acid (ETDA) monovettes were used for WBC isolation. Blood was withdrawn during routine blood drawn by following the same pre-conditions. During 25 min incubation time, nine 15 ml falcon tubes (greiner bio-one, Kremsmünster, Austria) were prepared with 7.5 ml erythrocyte lysis (EL)-Buffer (Quiagen, Hilden, Germany), stored on ice and vortexed two times after 7 min and 15 min. After centrifugation (400 g, 10 min, 4°C) and discarding of the supernatant fluid, cell pellet was re-suspended in 3 ml EL-buffer. Subsequently, centrifugation (400 g, 10 min, 4°C) was repeated, the supernatant was removed, and the cell pellet was re-suspended in 1.5 ml RNA protect cell reagent (Quiagen, Hilden, Germany), aliquoted 500 µl each, and frozen at -80°C for RNA-isolation, which is described in detail under 2.8.1.

2.6 Skin punch biopsy

Six-mm skin punch biopsies were obtained from the right lateral calf and the upper thigh by using a biopsy punch (Biopsy Punch 6 mm, Stiefel, Offenbach am Main, Germany) and under local anaesthesia (1-2 ml scandicain 1%, Aspen Pharma, München, Germany) (Üçeyler et al. 2010). After dissection of subcutis, tissue was divided into two pieces: one piece was used for analyzing the IENFD, the second piece was utilized to obtain primary fibroblast and keratinocyte cell cultures. To determine the IENFD, tissue was incubated in 4% paraformaldehyde at 4°C for 2 h and then sliced into 50 µm sections. For visualization of the IENFD, skin sections were immunoreacted with antibodies against the pan-axonal marker protein-gene product 9.5 (PGP9.5) 1:1000 (Zytomed, Berlin, Germany). Nerve fibers were quantified using a fluorescence microscope (Axiophot 2, Zeiss, Oberkochen, Germany) by following defined criteria (Lauria et al. 2005). Results were compared with the normative values of

the laboratory, which are based on 199 healthy controls (lower leg: 125 women, 73 men; upper thigh: 105 women, 51 men).

2.7 Cell culture experiments

2.7.1 Isolation of human dermal fibroblasts

After mechanical separation of epidermis and dermis by using a sterile forceps and scalpel, the dermis was cut into three pieces which were placed in a cell culture flask, CELLSTAR ®, 25 ml (Greiner bio-one, Kremsmünster, Austria). After adding few drops Dulbecco`s Modified Eagle Medium F-12 Nutrient Mixture (Ham), (+) L-Glutamine (DMEM/F-12) culture medium (Life Technologies, Carlsbad, CA, USA), which was supplemented with antibiotics penicillin/streptomycin (1%) (Life Technologies, Carlsbad, CA, USA) and fetal calf serum (FCS) (10%), the skin samples were incubated at 37°C, 5% CO_2 for 60 min. To avoid dehydration or floating, another 2 ml of complete DMEM/F-12 culture medium were added to the skin samples.

2.7.2 Isolation of human epidermal keratinocytes

The epidermis was cut into 12 pieces, which were divided into three cell culture flasks with the epidermal side on bottom. Cells were treated as described in 6.2.1 for the first three days but were monitored daily to watch the time point when keratinocytes grow out beyond the original sample. With detection of the first keratinocytes, the switch to EpiLife medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA), which was supplemented with 10 000 U/ml penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA) and EpiLife Defined Growth Supplement (Life Technologies, Carlsbad, CA, USA) was performed.

2.7.3 Cultivation of skin cells

2.7.3.1 Cultivation and splitting procedure of fibroblasts

Fibroblasts were cultivated under stable conditions (at 37°C, 5% CO_2), regular visual control, and changing the medium every three days for about two to three weeks. Changing the medium included removing the consumed medium and feeding cells with 5 ml fresh and pre-warmed (37°C) complete DMEM culture medium. It may take three to seven days until fibroblasts are visible under the microscope and two to three weeks to get 90% confluence for passaging them at a ratio of 1:3. For trypsinizing fibroblasts, TrypLE™ Express Enzyme (-) phenol red (TrypLE Express) (Life Technologies, Carlsbad, CA, USA) was used. After aspirating DMEM culture medium, 1 ml of TrypLE Express was pipetted to each cell culture flask, which was subsequently incubated (1 min at 37°C, CO_2 5%) to round up the fibroblasts and detach them from the cell culture flask. Enzymatic digestion was stopped by adding 10 ml of DMEM culture medium and removing the supernatant liquid after centrifugation. After resuspending the cell pellet by adding 10 ml of DMEM culture medium, cells were transferred to a 75 ml cell culture flask, CELLSTAR ® (Greiner bio-one, Kremsmünster, Austria) for further cultivation.

2.7.3.2 Cultivation and splitting procedure for keratinocytes

Except for using EpiLife medium, keratinocytes were cultivated as described for fibroblasts above. Cells were splitted at 75% confluence, usually after two weeks. To detach the initially very adhesive and tightly packed keratinocytes, cells were incubated for up to 20 min at 37°C, CO_2 5% with StemPro Accutase (Life Technologies, Carlsbad, CA, USA) to release them from the cell culture flask and from each other. In subsequent passages when cells were spread over the whole cell culture flask and less tightly to each other, incubation for up to 12 min at 37°C, CO_2 5% with StemPro Accutase was sufficient. StemPro Accutase was diluted by adding 10 ml EpiLife medium. After centrifugation and resuspension by adding 15 ml EpiLife medium, the cell solution was transferred to three 25 ml cell culture flasks for further cultivation.

2.7.4 Freezing of skin cells

After two passages, cells were splitted as described under 2.6.3.2. After centrifugation and decanting the supernatant, the cell pellet was resuspended in 2 ml pre-cooled (+8°C) freezing medium. Freezing medium contained the respective cell culture medium which was supplemented by 5% Dimetyl Sulfoxide (DMSO) (Carl Roth, Karlsruhe, Germany) as a cryoprotective agent. The homogenous cell suspension was then aliquoted into two Cryovials (Biozym Scientific, Hessisch Oldendorf, Germany) which were placed in Mr Frosty (Thermo Fisher Scientific, Waltham, Massachusetts, USA), an isopropanol chamber, and stored at -80°C overnight. Frozen cells were then transferred to liquid nitrogen and stored before further processing.

2.8 Gene expression analysis

2.8.1 Ribonucleic acid (RNA) extraction

After two passages, ribunocleic acid (RNA) was extracted from fibroblasts and keratinocytes using the miRNeasy Mini Kit #217004 (Quiagen, Hilden, Germany) and following manufacturer’s recommendations. The cell pellet was suspended in 700 µl QIAzol Lysis reagent (containing guanidine thiocyanate and phenol) and incubated for 5 min at room temperature (RT). After adding 140 µl chloroform (Carl Roth, Karlsruhe, Germany) and shaking the solution vigorously for 15 s, the sample was again incubated for up to 3 min at RT and centrifuged for 15 min at 12.000 g at +4°C. Subsequently, three phases appeared, an upper aqueous phase, the interphase consisting of the cell debris, and the discardable flow-through. After pipetting 525 µl ethanol 100% to the upper aqueous phase, several centrifugation steps (each 8.000 g, 20 sec, RT) were performed using a silica-membrane RNeasy spin column and distinct buffers (RWT and RPE buffer) to eliminate contaminants according to the manufacturer`s protocol. Finally, messenger-RNA (mRNA) was eluted in 30 µl RNAse-free-water. The RNA concentration was measured using a Nanodrop®

spectrophotometer (Peqlab, Erlangen, Germany). Samples were stored at -80°C before further processing.

2.8.2 Reverse transcription PCR

Two hundred and fifty ng RNA was necessary for reverse transcription PCR to gain cDNA from mRNA. For receiving a constant total volume of 32.8 µl, sterile, distilled water (Braun, Melsungen, Germany) was added to the RNA according to the following formula:

$$Volume(RNA)\mu l = 32.8\ \mu l - \left(\frac{concentration\ (RNA\ sample)\frac{ng}{\mu l}}{250\ ng} \right)$$

Utilized reagents (TaqMan Reverse Transcription Reagents) and cyclers were purchased from Applied Biosystems (Darmstadt, Germany). After supplementing 5 µl Random Hexamer, samples were incubated (3 min, 85°C) on a heating block (Liebisch, Bielefeld, Germany) for denaturation process. 2 µl Oligo-DT and 60.2 µl Master Mix (containing 10 µl 10x PCR buffer, 6.25 µl multiscribe reverse transcriptase, 2 µl RNase inhibitor, 22 µl $MgCl_2$, and 20 µl dNTPs) were added and polymerase chain reaction (PCR) was run in an PCR-Cycler Advanced Primus 96-PCR (Peqlab Biotechnology, Erlangen, Germany) at the following conditions: annealing (25°C, 10 min), reverse transcription (48°C, 60 min), and enzyme inactivation (95°C, 5 min). The acquired copy-desoxyribonucleic acid (cDNA) was stored at -20°C before further processing.

2.8.3 Quantitative real-time PCR

The following target genes were analyzed: pro-inflammatory cytokines interleukin(IL-)1ß (Hs00174097_m1), IL-6 (Hs00174131_m1), IL-8 (Hs00174103_m1), TNF-α (Hs00174128_m1), and the insulin like growth factor (IGF)-1 (Hs01547656_m1); anti-inflammatory cytokines: IL-4 (Hs00174122_m1), IL-10 (Hs00174086_m1), and transforming growth factor (TGF)-ß1 (Hs99999918_m1); axon guidance molecules and their main receptor

ephrin-A4 (EFNA4) (Hs00193299_m1) and ephrin-receptor-A4 (EPHA4) (Hs00953178_m1), netrin1 (NTN1) (Hs00924151_m1) and Unc5b (Hs00900710_m1). Samples were measured by using a Micro Amp Optical 96-Well Reaction Plate (Applied Biosystems, Darmstadt, Germany). Each plate contained a negative control without cDNA to exclude potential desoxyribonucleic acid (DNA) contamination and a calibrator sample, which was determined individually for each target gene, cell type and localization of biopsy to allow inter-plate comparability. As calibrator sample, the sample whose threshold cycle (Ct)-values was next to the respective control group mean Ct values was utilized. Reaction mixture of the targets consisted out of 5 µl cDNA, 2 µl of TaqMan Universal Master Mix (Applied Biosystems, Darmstadt, Germany), 1.75 µl sterile, distilled water (Braun, Melsungen, Germany), and 0.25 µl of the respective primer. Target genes were measured as triplicates. As housekeeping gene and endogenous control eukaryotic, 18sRNA (Hs99999901_s1) was used. Reaction mixture of 18sRNA, which was measured as duplicates, included 2.5 µl cDNA, 2 µl of TaqMan Universal Master Mix (Applied Biosystems, Darmstadt, Germany), 4.25 µl sterile, distilled water (Braun, Melsungen, Germany), and 0.25 µl 18sRNA. For the measurement StepOnePlus™ Cycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA) run on the following conditions: first incubation (50°C, 2 min), second incubation (95°C, 10 min), 40 cycles (95°C, 15 sec and 60°C, 1 min). Data evaluation was performed by using the $2^{-\Delta\Delta Ct}$ method in comparison to a calibrator sample.

2.9 Statistical analysis

SPSS 25 (IBM Deutschland GmbH, Ehningen, Germany) was used for statistical analysis. The non-parametric Mann-Whitney-U-test was applied, since data were not-normally distributed. Categorical variables were analyzed by using the Chi²-test, correlations were determined Spearman-test. P values <0.05 were considered statistically significant. Data were not normally distributed.

3 Results

3.1 Clinical and laboratory data

3.1.1 Study population

Fifty-five patients, 34 women and 21 men were recruited. Median age was 54 years (range 19-73). In 17/55 (31%) patients impaired glucose tolerance or a pathological HbA1c was found. 8/55 (15%) patients had variations in pain associated genes of unknown pathogenicity. Hypothyroidism, vitamin B12 deficiency, and a combination of psoriasis, Hashimoto thyroiditis, and vitiligo was found in 3/55 (5%) patients. In 33/55 (60%) patients, no apparent etiology was found, and they were classified as having idiopathic SFN. 31 healthy controls (19 women, 12 men) with a median age of 53 years (range 21-66) took part in the study.

3.1.2 Clinical characteristics

Table 2 summarizes the main characteristics of the study cohort.

Table 2: Main characteristics of study cohort.

	patients, n=55
Age [years]	54 (19-73)
Gender (F/M)	34/21
BMI [kg/m^2]	26 (19-42)
Time since diagnosis [years]	0.4 (<1 month-12)
Employment - regularly working - retired due to pain	 29 (53%) 2 (6%)
Family history positive for - chronic pain - neurological disorders - affective disorders	 15 (27%) 16 (29%) 2 (6%)
Autonomic dysfunction (patient report)	

- voiding problems	6 (11%)
- sexual dysfunction	7 (13%)
- repetitive syncopes	none
- dyshidrosis	30 (55%)
Trophic changes (patient report)	
- skin	15 (27%)
- hair	13 (24%)
- nails	4 (7%)

Data are given as median and range in brackets.

Abbreviations: BMI = body mass index; F = female; M = male.

Pain characteristics

Table 3 gives details about patients` pain characteristics. Pain history provided the following results: 54/55 (98%) patients described focal to widespread pain with a median current pain intensity of 4/10 NRS (range 0-8). Pain was reported as burning (44/55, 80%) and stabbing (33/55, 60%), sometimes in combination, hence the numbers do not sum up to 100%. 51/55 (93%) patients reported symmetric pain. Most of the patients suffered from permanent pain with intermitted increase in intensity (43/55, 78%).

Table 3: Pain characteristics of study cohort.

	patients, n=55
Pain duration [years]	4 (<1 month-24)
Pain distribution	
- acral	17 (31%)
- generalized	22 (40%)

Pain dynamics - permanent pain with intermittent increases in pain intensity - permanent - attacks	 27 (49%) 16 (29%) 11 (20%)
Pain descriptors - burning - stabbing	 44 (80%) 33 (60%)
Paresthesias in painful area	45 (82%)
Analgesic medication - none - monotherapy - combination of ≥2	 18 (33%) 22 (40%) 16 (29%)
Pain intensity - current pain intensity (NRS) - maximal pain intensity (NRS) - mean pain intensity (NRS)	 4 (0-8) 8 (3-10) 5 (1-8)

Data are given as median and range in brackets.

Abbreviations: NRS = numeric rating scale.

Clinical examination

In 30/55 (55%) patients, signs of small fiber impairment were found in neurological examination: thermal hypoesthesia 13/55 (24%), hypoalgesia 8/55 (15%), hyperalgesia 7/55 (13%), allodynia 4/55 (7%), dysesthesia upon touch 4/55 (7%), pinprick hypoesthesia 3/55 (5%), hyperesthesia 2/55 (4%), paresthesia upon touch 1/55 (2%). In some patients ≥1 pathological finding was present; thus, the sum exceeds 100%.

Nerve conduction studies

Nerve conduction studies of the sural and tibial nerve gave normal results in all patients. Nerve conduction velocity (NCV), distal motor latency (dmL), sensory

nerve action potential (SNAP), and compound muscle action potential (CMAP) were within normal ranges when compared with normative values of the laboratory. Table 4 lists main results of the nerve conduction studies:

Table 4: Results of nerve conduction studies.

		patients, n=55	
		women, n=34	**men, n=21**
sural nerve	Sensory nerve action potential (μV)	16.7 (4.1-50.2)	13.5 (3.4-39.8)
	Nerve conduction velocity (m/s)	47.3 (41.7-54.2)	46.5 (41.0-54.2)
tibial nerve	Proximal CMAP (mV)	15.9 (1.5-28.0)	12.9 (6.0-28.5)
	Distal CMAP (mV)	21.0 (4.5-30.7)	18.2 (9.0-34.3)
	Distal motor latency (ms)	3.5 (2.8-5.7)	3.9 (2.8-4.9)
	Nerve conduction velocity (m/s)	47.6 (34.9-53.8)	44.7 (39.5-50.5)

Abbreviations: CMAP = compound muscle action potential.

Small fiber characteristics

In the clinical examination, 30/55 (55%) patients showed sings typical for SFN. Distal IENFD was reduced in 34/55 (62%) patients. Small fiber impairment was found in QST 13/55 (24%), PREP 31/55 (56%), CCM 29/55 (53%), and QSART 7/55 (13%). Table 5 gives a synopsis of all small fiber test results.

Table 5: Small fiber characteristics of study cohort.

	patients, n=55	**controls***
Clinical examination pathological (N)	30/55 (55%)	NA
QST pathological (N)	13/55 (24%)	41/296 (14%)
Distal IENFD pathological (N)	34/55 (62%)	39/179 (22%)
Proximal IENFD pathological (N)	19/55 (35%)	19/139 (14%)

PREP pathological (N)	31/55 (56%)	38/149 (26%)
CCM pathological (N)	29/55 (53%)	16/67 (24%)
QSART pathological (N)	7/55 (13%)	10/27 (37%)

Abbreviations: CCM = corneal confocal microscopy; IENFD = intraepidermal nerve fiber density; N = number; NA = not applicable; PREP = pain related evoked potentials; QSART= quantitative sudomotor axon reflex test; QST = quantitative sensory testing;
* n varies depending on the small fiber test. Control cohort include historical healthy control samples.

3.1.3 Laboratory test results

3.1.3.1 Laboratory data

Laboratory tests were normal except for the following: elevated HbA1c (n=7; normative value ≤ 6.1%), impaired glucose tolerance (n=15; second hour value > 140 mg/dl), degraded vitamine B12 (n=2; normative value ≤ 197 pg/ml, and pathological TSH results (n=6; normative values (0.3-4.0 mlU/l)).

3.1.3.2 Genetic alterations in SFN patients

Variations in pain associated genes were detected in 5/55 (9%) patients. Two patients carried potentially pathogenic mutations: one patient a gain-of-function mutation in the gene encoding the voltage-gated sodium channel SCN10A, the second patient in SCN11A. Genetic variations in transient receptor potential vanilloid 3 (TRPV3) and in transient receptor potential subfamily M member 3 (TRPM3) genes were found in the further two patients. Clinical pathogenicity and significance remain unclear. In one further patient, a heterozygote variant of the NGF gene was detected.

3.2 Gene expression levels of WBC

Comparing gene expression of the investigated pro- and anti-inflammatory cytokines in WBC, gene expression of the pro-inflammatory cytokines IL-2, IL-8

and TNF was higher in SFN patients compared to healthy controls ($p<0.05$ each, Fig. 1). No differences were found in the expression of anti-inflammatory cytokines between WBC of patients and controls (Fig. 2).

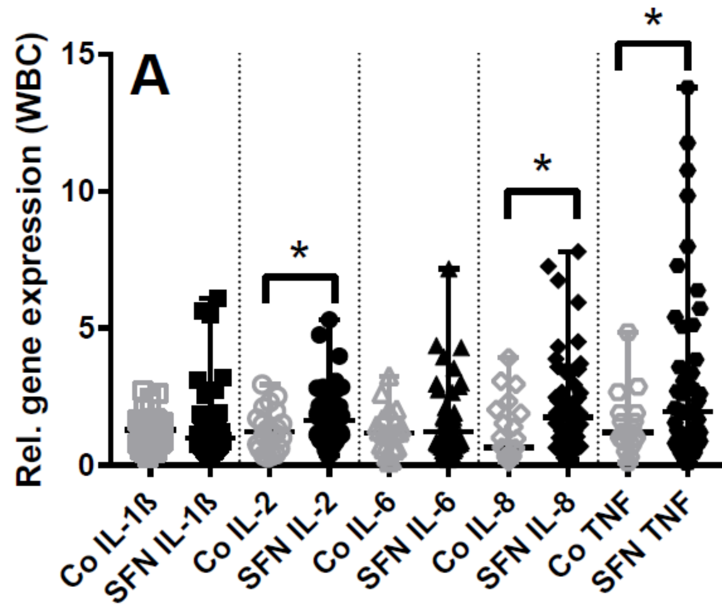


Figure 1: Gene expression of pro-inflammatory cytokines in WBC of SFN patients compared to healthy controls. Number of samples investigated: n (SFN) = 55; n (Co) = 24.

Abbreviations: Co = controls; IL- = interleukin; rel. = relative; SFN = small fiber neuropathy; TNF = tumor necrosis factor; WBC = white blood cells.

*$p<0.05$.

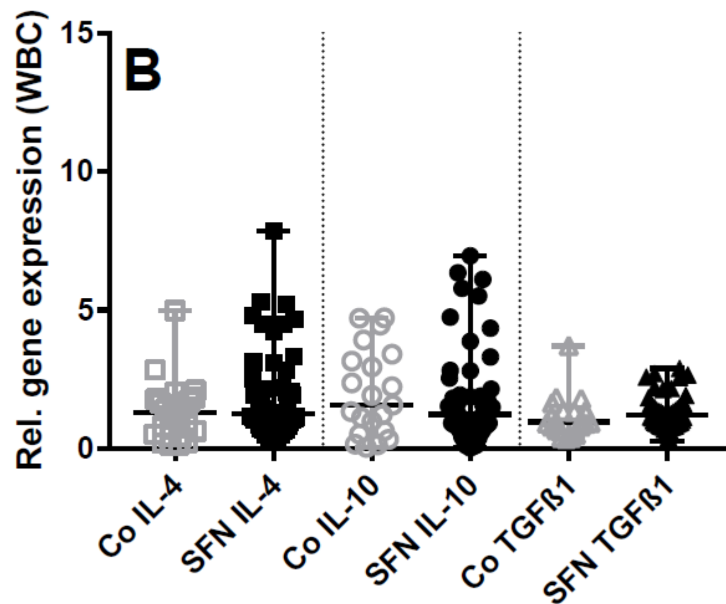


Figure 2: Gene expression of anti-inflammatory cytokines in WBC of SFN patients compared to healthy controls. Number of samples investigated: n (SFN) = 55; n (Co) = 24.
Abbreviations: Co = controls; IL- = interleukin; rel. = relative; SFN = small fiber neuropathy; TGFß1 = transforming growth factor beta; WBC = white blood cells.

3.3 Skin punch biopsy

For patients consent getting a skin punch biopsy was a precondition for taking part in the study. Thus, results base on data obtained from 55 patients. Despite, participants of the control group were asked to acquiesce in taking a biopsy. 26/31 (84%) controls agreed.

3.3.1 Reduction of the IENFD

When comparing IENFD data with the normative values (124 women, 56 men), distal IENFD was reduced in 34/55 (62%) patients and proximal IENFD in 19/55 (35%) patients. 3/19 (16%) patients had a reduction of the proximal IENFD while distal innervation was normal. Generalized nerve fiber reduction was found in 16/55 (29%) patients. In the healthy control group, the following results were given: distal IENFD was reduced in 13/26 (50%) subjects and proximal IENFD in 5/26 (19%) subjects. In 4/26 (15%) subjects distal and proximal

IENFD was reduced, while in 1/26 (4%) subject only proximal IENFD was reduced when comparing the IENFD of the control group with the normative values (Fig. 3).

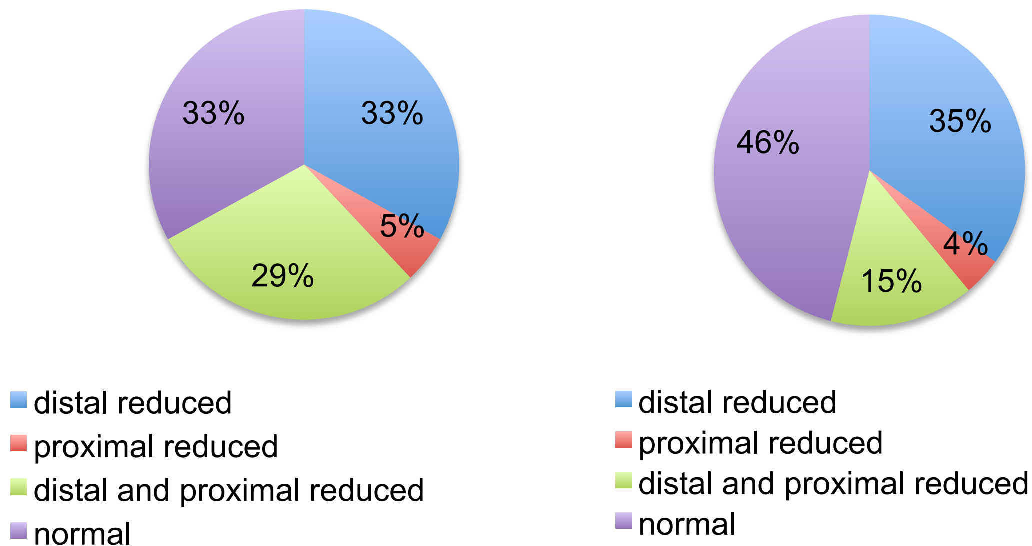


Figure 3: Skin innervation of the study cohort distinguishing patients and controls.

3.3.2 Length-dependence of skin denervation

Skin denervation was length-dependent (LD) in 8/55 (15%) patients and non-length dependent (NLD) in 3/26 (12%) patients.

3.3.3 Cell cultures of primary fibroblasts and keratinocytes

3.3.3.1 Fibroblasts

Fibroblast cultures were obtained from 54/55 (98%) of the proximal and 55/55 (100%) of the distal skin punch biopsy samples of SFN patients. In the control group, fibroblasts were collected from 26/26 (100%) of the thigh biopsies and 25/26 (96%) of the calf specimens. One sample of each group was lost due to contamination. One example of the typical spindle-shaped fibroblasts is shown

in Figure 4. Morphological differences were not detected when comparing fibroblasts of the patients and controls.

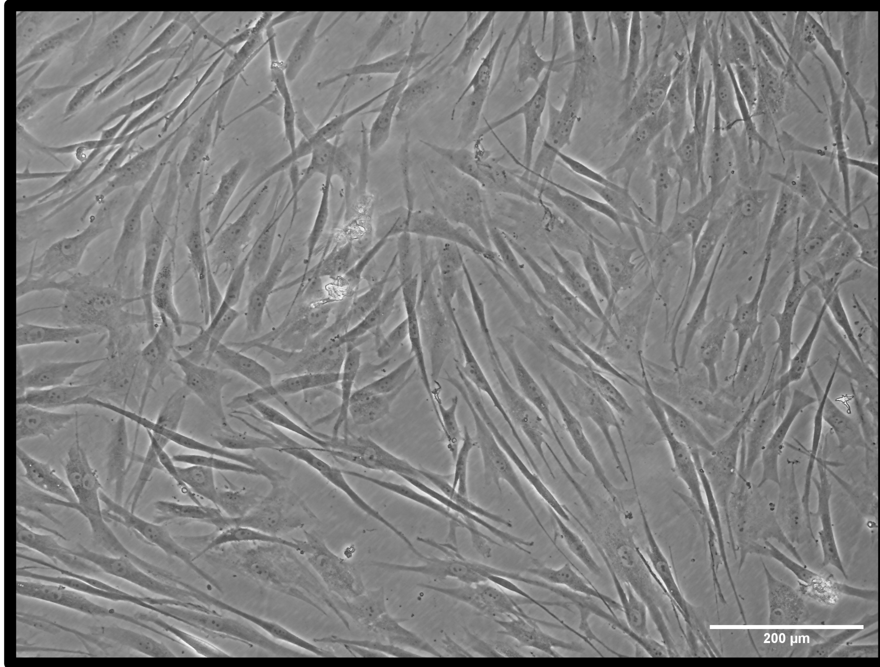


Figure 4: Cultured fibroblasts obtained from skin biopsy. Scale bar represents 200 µm.

3.3.3.2 Keratinocytes

Keratinocytes were isolated from 48/55 (87%) of patients from the skin biopsy obtained from the thigh and 47/55 (85%) of patients from the biopsy obtained from the calf. In the control group, keratinocytes were collected from 26/26 (100%) of controls from proximal biopsy and 24/26 (92%) of controls from distal skin punch biopsy. In about 50%, cultures were mixed cultures with various number of fibroblasts inside. Cells appeared in cobblestone morphology. Despite cell morphology did not differ between patients and controls, high variations of the cell morphology between samples of each group appeared.

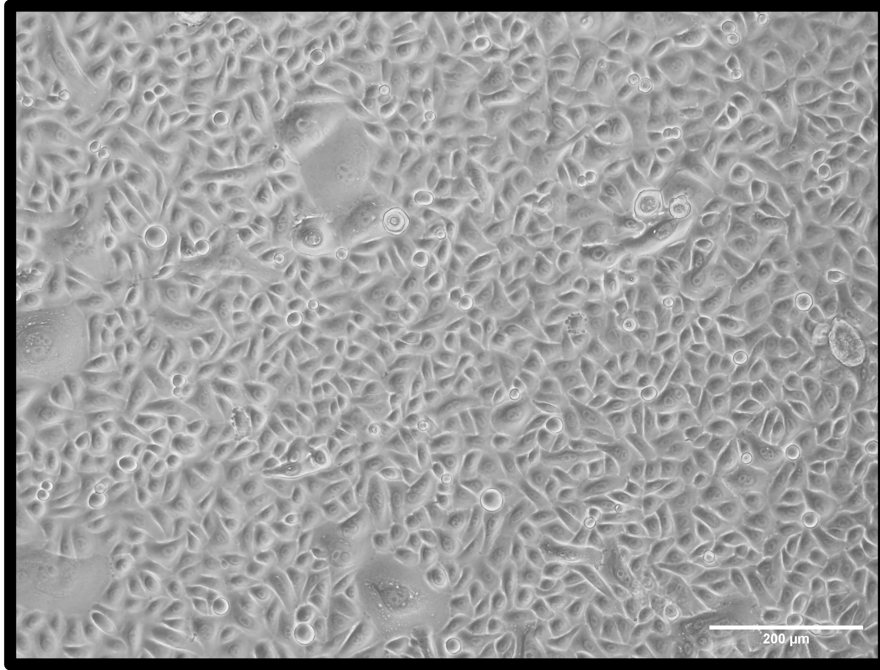
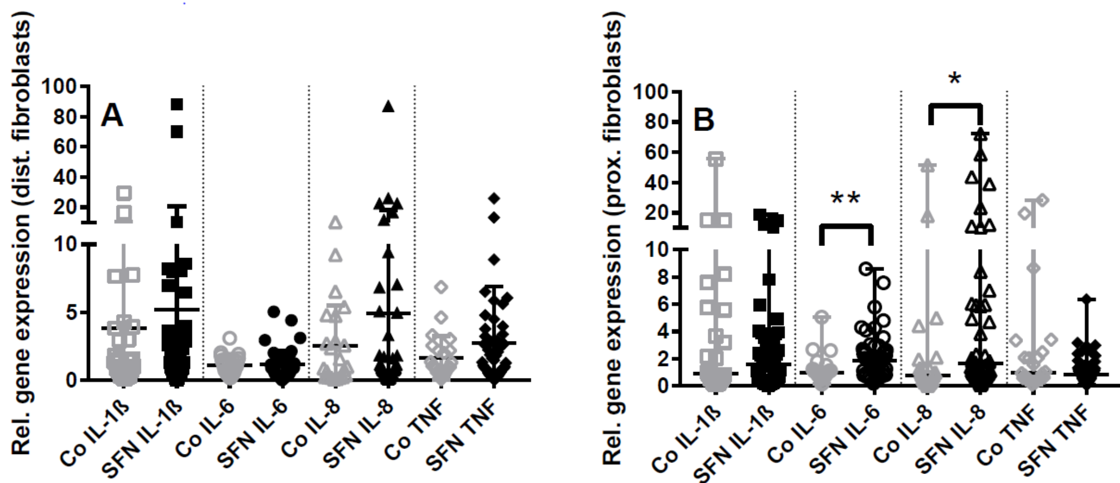


Figure 5: Cultured keratinocytes obtained from skin biopsy. Scale bar represents 200 µm.

3.3.4 Cytokine profiles of dermal fibroblasts

Comparing gene expression of the investigated pro- and anti-inflammatory cytokines, no differences were found between patients and controls in dermal fibroblasts from the calf. In fibroblasts from the thigh, gene expression of IL-6 ($p<0.01$) and IL-8 ($p<0.05$) was higher in SFN patients compared to healthy controls (Fig. 6). Measurement of IL-2 was impossible in fibroblasts, because skin cells do not express it.



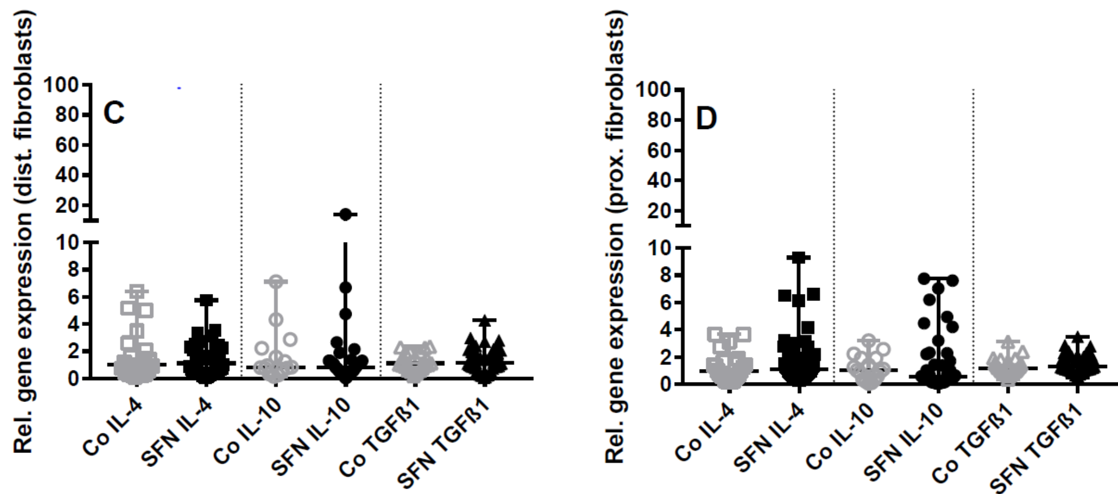


Figure 6: Gene expression of pro- and anti-inflammatory cytokines in fibroblasts of SFN patients compared to healthy controls. Number of samples investigated: n (SFN) prox. = 54; n (Co) prox. = 25; n (SFN) dist. = 55; n (Co) dist. = 26.

Abbreviations: Co = controls; dist. = distal; IL- = interleukin; prox. = proximal; rel. = relative; SFN = small fiber neuropathy; TGFß1 = transforming growth factor beta; TNF = tumor necrosis factor.

*p<0.05; **p<0.01.

3.3.5 Axon guidance molecule profiles of dermal fibroblasts

When analyzing the expression of axon guidance cues in skin fibroblasts, higher gene expression of NTN1 and Unc5b was found in fibroblasts from the calf of SFN patients in comparison to the control group (p<0.05, each). Cells from the thigh did not reveal intergroup differences for NTN1 and Unc5b gene expression (Fig. 7). Gene expression of EFNA4 and EFNA4 in fibroblasts of the patients did not differ from control group in both areas.

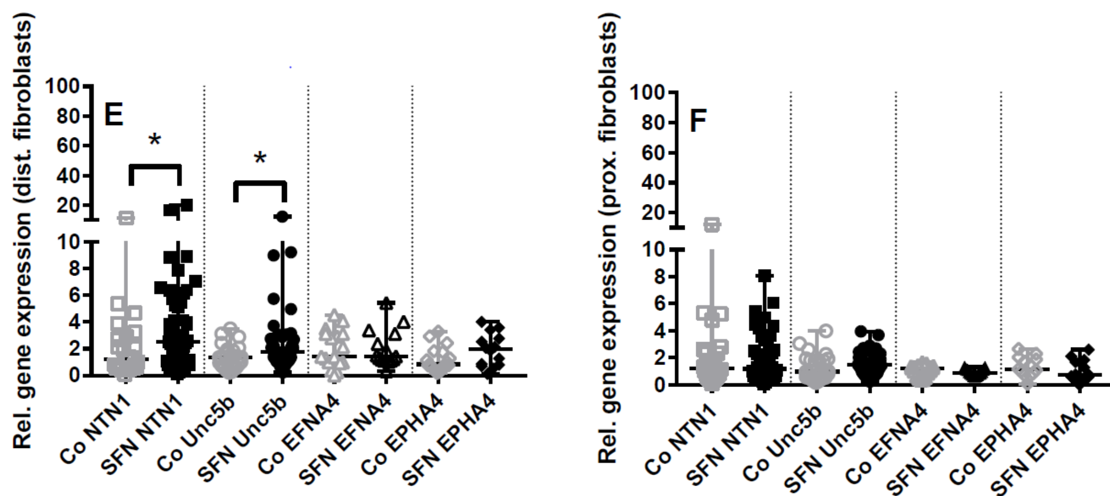


Figure 7: Gene expression of axon guidance molecules in fibroblasts of SFN patients compared to healthy controls. Number of samples investigated: n (SFN) prox. = 54; n (Co) prox. = 25; n (SFN) dist. = 55; n (Co) dist. = 26.

Abbreviations: Co = controls; dist. = distal; EFNA4 = ephrin-A4; EPHA4 = ephrin-receptor-A4; NTN1 = netrin1; prox. = proximal; rel. = relative; SFN = small fiber neuropathy.

*: $p<0.05$.

3.3.6 Cytokine profiles of epidermal keratinocytes

Pro-inflammatory cytokine assessment of keratinocytes did not show an intergroup difference of gene expression. Among the investigated anti-inflammatory cytokines, gene expression of TGF-ß1 was higher in keratinocytes from the calf ($p<0.05$) and the thigh ($p<0.01$) of SFN patients than in controls (Fig. 8). Measurement of IL-2 was impossible in keratinocytes, because skin cells do not express it.

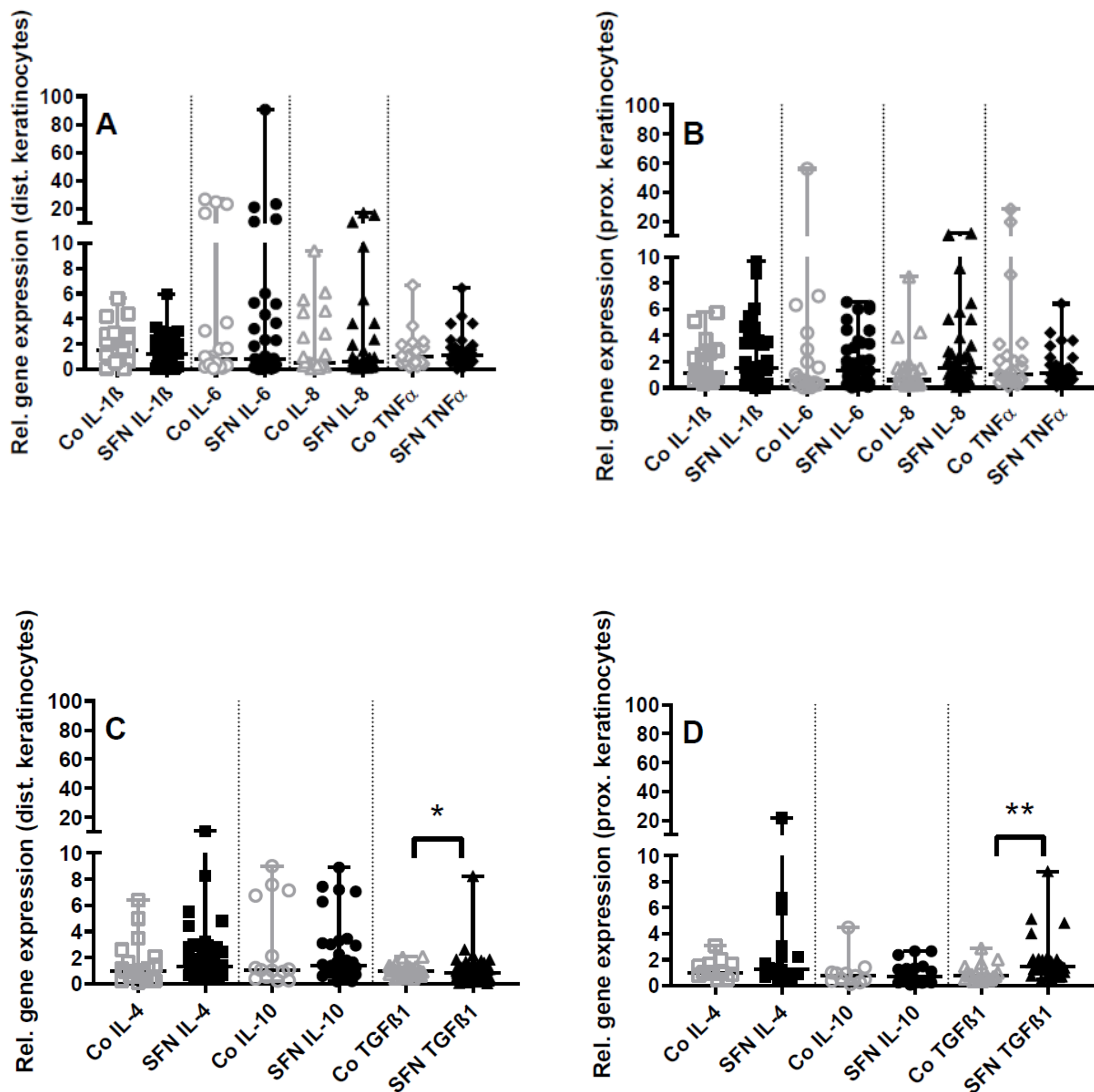


Figure 8: Gene expression of pro- and anti-inflammatory cytokines in keratinocytes of SFN patients compared to healthy controls. Number of samples investigated: n (SFN) prox. = 48; n (Co) prox. = 26; n (SFN) dist. 47; n (Co) dist. = 24.

Abbreviations: Co = controls; dist. = distal; IL- = interleukin; prox. = proximal; rel. = relative; SFN = small fiber neuropathy; TGFß1 = transforming growth factor beta; TNF = tumor necrosis factor.

*p<0.05; **p<0.01.

3.3.7 Axon guidance molecule profiles of epidermal keratinocytes

Patients showed a higher gene expression for NTN1 ($p<0.05$) in keratinocytes from the thigh than controls. When comparing gene expression of patients and controls for Unc5b in keratinocytes of the thigh and the calf, no intergroup differences were found. Results are presented in Fig. 9. EFNA4 and its receptor did not show intergroup difference in both areas.

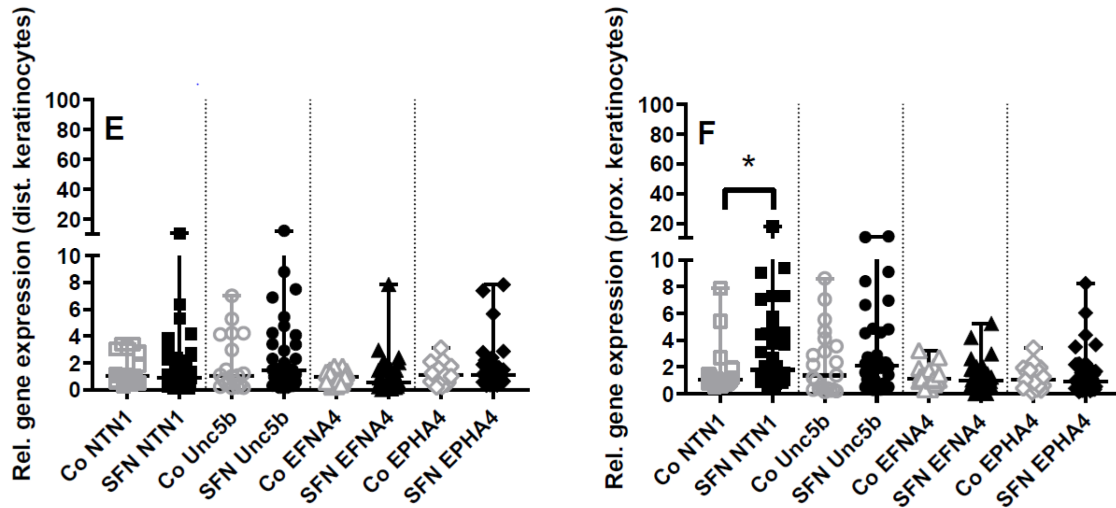


Figure 9: Gene expression of axon guidance molecules in keratinocytes of SFN patients compared to healthy controls. Number of samples investigated: n (SFN) prox. = 48; n (Co) prox. = 26; n (SFN) dist. = 47; n (Co) dist. = 24.

Abbreviations: Co = controls; dist. = distal; EFNA4 = ephrin-A4; EPHA4 = ephrin-receptor-A4; NTN1 = netrin1; prox. = proximal; rel. = relative; SFN = small fiber neuropathy.

*: $p<0.05$.

3.3.8 Subgroup analysis for IL-6 and IL-8 in fibroblasts of the thigh and for NTN1 and Unc5b in fibroblasts of the calf

The patient group was stratified for pain distribution, etiology of SFN, and length-dependence of skin innervation to analyze potential intergroup differences in gene expression. Described subgroups were chosen because pain is a major symptom of the patients, etiology as further classification option of disease, and length-dependence of skin innervation based on the results of the previous study (Üçeyler et al. 2010). Analysis was done for those target genes, which showed intergroup differences in gene expression when

comparing the patient group with healthy controls. Table 6 illustrates gene expression data in patient subgroups compared to healthy controls and lists the respective p-values.

Table 6: Gene expression of IL-6, IL-8, NTN1, and Unc5b in fibroblasts of different SFN subgroups compared to the control group.

	IL-6 thigh	**IL-8 thigh**	**NTN1-calf**	**Unc5b-calf**
Assumed etiology (n=22)	p<0.01	p<0.05	-	p<0.05
Idiopathic SFN (n=33)	p<0.05	p<0.05	p<0.05	-
Acral pain (n=17)	p<0.05	p<0.05	p<0.05	-
Generalized pain (n=22)	p<0.01	p<0.05	-	p<0.05
LD-SFN (n=8)	-	-	-	-
NLD-SFN (n=47)	p<0.01	p<0.05	p<0.05	p<0.05

Abbreviations: IL- = interleukin; LD-SFN = Length-dependend SFN; NLD-SFN = non length-dependend SFN; NTN1 = netrin1.
-: no significant difference when comparing the subgroup of SFN patients and the healthy controls.

The patient and control groups were additionally separated into subgroups by focusing on results of IENFD assessment. Analysis was done for IL-6 and IL-8 in proximal and for NTN1 and Unc5b in the distal skin sample. Table 7 summarizes the different subgroups and their p-values by comparison of the target levels of different SFN groups and the control group.

Table 7: Subgroups and p-values considering the differences of gene expression in IL-6, IL-8, NTN1, and Unc5b.

Compared subgroups	**p-values IL-6 prox.**	**p-values IL-8 prox.**	**p-values NTN1 dist.**	**p-values Unc5b dist.**
SFN red. vs. SFN normal	-	-	-	-
SFN red. vs. all controls	p<0.001	-	p<0.05	-

SFN normal vs. all controls	-	-	-	-
SFN normal vs. controls normal	-	-	-	-
SFN red. vs. controls red.	p<0.001	p<0.05	p<0.05	-
SFN red. vs. controls normal	-	-	-	-

Abbreviations: dist. = distal; IL- = interleukin; NTN1 = netrin1; prox. = proximal, red. = reduced, vs. = versus.
N-values: n SFN red. = 18 proximal, 34 distal; n SFN normal = 18; n controls = 26; n controls red. = 5 proximal, 13 distal; n controls normal = 12.-: no significant difference when comparing the subgroup of SFN patients and the healthy controls.
Reduced means a reduction of the IENFD when comparing the participants' results of skin punch biopsy with the normative values of the laboratory, based on 199 healthy controls. Normal means an IENFD between the normative ranges based on laboratory evaluated data.

3.3.9 Subgroup analysis for TGF-ß1 and NTN1 in keratinocytes of the thigh and for TGF-ß1 in keratinocytes of the calf

Subgroup analysis of keratinocytes was done following the same stratification as described for fibroblasts in 3.2.7 considering proximal biopsy samples for TGF-ß1 and NTN1 as well as distal skin punch biopsies for TGFß1. Table 8 gives an overview of the results and provides the particular p-values.

Table 8: Gene expression of TGF-ß1 and NTN1 in keratinocytes of different SFN subgroups compared to the control group.

	TGF-ß1 thigh	**TGF-ß1 calf**	**NTN1-thigh**
Assumed etiology (n=22)	p<0.01	-	-
Idiopathic SFN (n=33)	p<0.01	-	-
Acral pain (n=17)	p<0.001	p<0.001	-
Generalized pain (n=22)	p<0.05	-	-
LD-SFN (n=8)	p<0.05	-	-

NLD-SFN (n=47)	p<0.001	p<0.05	-

Abbreviations: LD-SFN = Length-dependent SFN; NLD-SFN = non length-dependent SFN; NTN1 = netrin1; TGF-ß1 = transforming growth factor-ß1.
-: no significant when comparing the subgroup of SFN patients and the healthy controls.

Subgroup analysis of keratinocytes was done by following the same stratifications as described for fibroblasts considering IENFD results. Variations in gene expression, observing different subgroups in patients and controls, are presented in Table 9 together with the respective p-values.

Table 9: Subgroups and p-values considering the differences of gene expression in TGF-ß and NTN1.

Compared subgroups	**p-values TGF-ß1 prox.**	**p-values TGF-ß1 dis.**	**p-values NTN1 prox.**
SFN red. vs. SFN normal	-	-	-
SFN red. vs. all controls	p<0.01	p<0.05	p<0.05
SFN normal vs. all controls	p<0.01	-	-
SFN normal vs. controls normal	-	-	-
SFN red. vs. controls red.	p<0.01	-	p<0.05
SFN red. vs. controls normal	-	p<0.05	-

Abbreviations: dist. = distal; NTN1 = netrin1; prox. = proximal; red. = reduced; TGF-ß1 = transforming growth factor beta 1; vs. = versus.
N-values: n SFN red. = 18 proximal, 34 distal; n SFN normal = 18; n controls = 26; n controls red. = 5 proximal, 13 distal; n controls normal = 12.-: no significant difference when comparing the subgroup of SFN patients and the healthy controls.
Reduced means a reduction of the IENFD when comparing the participants' results of skin punch biopsy with the normative values of the laboratory, based on 199 healthy controls. Normal means an IENFD in-between the normative range based on laboratory evaluated data.

4 Discussion

4.1 Overview

In this study a large group of SFN patients was phenotyped and their systemic (WBC) and cutaneous (fibroblasts, keratinocytes) cytokine expression profiles as well as the expression of skin axon guidance molecule was investigated. A method was further established to obtain primary fibroblast and keratinocyte cell cultures from 3-mm diagnostic skin punch biopsies for cell-specific in vitro assessment. Higher levels of IL2, IL8, and TNF in WBC, IL6 and IL8 in fibroblasts, and TGF-ß1 in keratinocytes of patients with SFN compared to healthy controls. It was demonstrated that NTN1 and Unc5b in fibroblasts and NTN1 in keratinocytes is higher expressed in patients compared to control cells. Data contribute to the concept of an immune involvement in SFN pain and a potential role of skin cells in cutaneous innervation.

4.2 Role of skin cells in neuropathic pain

Skin cells and pain perception:

There is growing evidence that skin cells are actively involved in pain perception (Talagas et al. 2018), e.g. keratinocytes take part in sensory transduction (Huet and Misery 2019) and regulation of noxious sensory neuron activity (Talagas et al. 2019). Keratinocytes and fibroblasts carry pain-associated receptors such as voltage-gated sodium channels and TRP channels (Gouin et al. 2017) and secrete algesic mediators, e.g. cytokines and chemokines (Feldmeyer et al. 2010, Niebuhr et al. 2010). Recent data show that defined stimuli including temperature, touch, and pain may induce nociceptor expression in skin and may be transformed from sensory stimuli to action potentials and lead to nociceptor sensitization (Lumpkin and Caterina 2007).

While the exact mechanism remains to be determined, primary cell cultures of keratinocytes and fibroblasts obtained from patients and healthy controls are promising concepts to investigate the interplay between skin cells and

nociceptors in pain development e.g. using co-culture systems as has been successfully done with keratinocytes and dorsal root ganglion (DRG) neurons (Kumamoto et al. 2014).

To characterize axonal excitability and baseline axonal responses in the presence of keratinocytes, another group used microfluidic chambers and seeded rat DRG neurons in the cell body compartment and rat keratinocytes in the axonal compartment of the chambers (Tsantoulas et al. 2013). Under co-cultivation conditions, neurites spread out, but axonal crossing and viability of neurons did not differ with or without keratinocytes (Tsantoulas et al. 2013).

Skin cells and pain development

Close vicinity between nociceptors and fibroblasts and keratinocytes in the skin suggests communication and functional interactions as shown using live cell imaging, super-resolution imaging, and 3D-structured illumination super-resolution microscopy (Keppel Hesselink et al. 2017, Krishnan-Kutty et al. 2017, Talagas et al. 2018). Physical interaction was investigated by phalloidin as an actin stain and applying 3D-structured illumination super-resolution microscopy, which showed nerve fiber ensheathment by keratinocytes in co-cultures (Krishnan-Kutty et al. 2017). Functional interaction of human keratinocyte-neuron cell cultures were suggested due to elevated Ca^{2+} levels under co-cultivation conditions (Krishnan-Kutty et al. 2017). Current work in zebrafish and drosophila melanogaster shows, somatosensory neurons to influence epidermal sheaths, which influences dendrite branch stabilization and nociceptive sensitivity (Jiang et al. 2019). Thus, blocking the link between epidermal cells and branches of somatosensory neurons resulted in reduced nociceptive response, which underscores the notion that neurite ensheathment influences nociceptor morphology and functionality (Jiang et al. 2019).

Skin cells and skin innervation:

As shown in a co-culture model of human skin cells of patients with atopic eczema and porcine DRG, keratinocytes and fibroblasts may influence skin innervation, neurite outgrowth, and morphology by secretion of neurotrophic

factors such as NGF and glial cell-line-derived neurotrophic factor (GDNF) (Roggenkamp et al. 2012, Kumamoto et al. 2014). Recent studies in patients with painful peripheral neuropathy reported a reduction of the IENFD assessed with PGP9.5 immunostaining, a promotion of regenerating nerve sprouts assessed with growth-associated protein-43 (GAP43) immunostaining, and thus a higher GAP43/PGP9.5 ratio, which may result in dysregulation of skin homeostasis (Galosi et al. 2018). The authors suggest that neuropathic pain is associated with the density of regenerating nerve sprouts, which are sensitized e.g. by voltage gated sodium channels or spontaneous C-fiber activity (Galosi et al. 2018). Axon guidance cues, which regulate axonal pathfinding and may repel or attract nerve fibers, direct tissue innervation (Furukawa et al. 2014). In a rat model of diabetic SFN, a correlation between Sema3A as an axon guide, mTOR signalling, and fiber density was found (Wu et al. 2018). This protocol of isolating primary fibroblast and keratinocyte cell cultures serves as a basis for obtaining a human, SFN specific co-culture model of skin cells from affected skin and patient-derived sensory neurons for in-depth analysis.

4.3 Fibroblast and keratinocyte skin cell cultures

Generating patient-derived 2D cell cultures of fibroblasts and keratinocytes from 3-mm diagnostic skin punch biopsies was succeeded. So far, neuropathic pain pathophysiology was mainly studied in animal models, preferentially rat, mouse or pig (Rice et al. 2019). To obtain human biomaterial, previous studies used large skin samples of several cm^2 to generate 2D in vitro cell culture models of human keratinocytes for further investigation of molecular mechanisms (Johansen 2017). Another group utilized human keratinocyte progenitors and stem cells from adult oral mucosa with a high cellular proliferation rate to produce ex vivo tissue for treatment of oral mucosa defects (Izumi et al. 2013). Using juvenile foreskin as a highly proliferative tissue, enabled to create an in vitro 3D human cell culture model which allowed human individualized analysis of cell-cell and cell-matrix functionality (Reuter et al. 2017).

The previous study was the first to analyze potential contributors to neuropathic pain in SFN investigating whole skin punch biopsies of patients and healthy

controls (Üçeyler et al. 2010). The current protocol allows the individual investigation of fibroblasts and keratinocytes from few mm skin biopsy samples taken from a low proliferative area, i.e. calf or thigh. Biopsies are obtained from patients and healthy controls and enable disease specific analysis. While the previous study reported differences in cytokine expression patterns, the impact of the single cell types remained unclear. The importance of axon guidance molecules on skin cells were not investigated so far. Using primary cell cultures may detect additional subtle differences and allow to attribute alterations in gene expression profiles to the particular cell type.

4.4 Cytokine expression profiles in WBC

Many studies investigated the systemic gene expression of pro- and anti-inflammatory cytokines in animal models and in patients with neuropathic pain (Allison et al. 2016, Wang et al. 2016, Hung et al. 2017). Expression of the pro-inflammatory cytokines TNF, IL-1ß, IL-6, and IL-17 and of the anti-inflammatory cytokines IL-4, IL-10, and TGFß-1 were mostly altered when comparing patients and healthy controls (Allison et al. 2016, Wang et al. 2016, Hung et al. 2017). In patients with polyneuropathies, higher levels of IL-1, IL-2, IL-8, and TNF were detected in patients with painful neuropathy compared to healthy controls, whereas the group of painless polyneuropathy patients only showed differences for IL-8 and TNF in intergroup comparison of peripheral blood mononuclear cells (PBMC) (Langjahr et al. 2018). In the previous study, using whole blood samples obtained from 24 SFN patients and matched healthy controls, higher expression of IL-2, IL-10, and TGFß1 was reported in SFN patients compared to controls (Üçeyler et al. 2010). After enlarging the group to 55 patients and 31 healthy controls, higher levels of IL-2, IL-8, and TNF were found in WBC of SFN patients compared to healthy controls.

Potential reasons for the discrepancy in peripheral blood cytokine levels may be the lack of direct comparability of the studies: the number of study participants varies between the different studies, one study analyzed cytokine expression in PBMC, whereas the others used whole blood, which contains additional cytokine-producing cells. Various underlying reasons of the disease may also

have influenced the results. The group of patients with polyneuropathy includes many subgroups e.g. immune mediated, diabetes associated, which additionally exert influence on cytokine expression apart from the two subgroups “painless” and “painful” neuropathies. In comparison with the previous study, the number of SFN patients was lower (n=24) and whole blood was used in contrast to WBC in the current study. Individual cell analysis bears the potential to differentiate between WBC and other cells producing cytokines, but also has the risk to miss cytokine alterations, which result from the interaction of different cells in dynamic systems. In the current study, TNF provides encouraging data. This may lead to further investigations on its influence on fiber stimulation and sensitization via induction of spontaneous A- and C-fiber activity, which then might be linked to contributing to SFN pain. Alternatively, IL-2 as a relatively constant result in different studies, remains a promising candidate. To further elucidate the underlying mechanism linking these cytokines with neuropathic pain in SFN, research on protein level will be performed in future studies.

4.5 Cytokine expression profiles in isolated fibroblasts and keratinocytes

Recent studies focused on the analysis of cytokines in affected nerve and skin samples obtained from human patients (Üçeyler and Sommer 2008, Hänel et al. 2013, Johnston et al. 2017, Noske 2018, Sommer et al. 2018). One study using skin biopsies obtained from patients with psoriasis detected an increase of IL-1 and IL-36 expression in keratinocytes (Johnston et al. 2017). Studies focusing on neuropathy and neuropathic pain showed elevated levels of IL-6 and IL-10 in sural nerve biopsy specimens in patients with painful neuropathies compared to patients with painless neuropathies (Üçeyler and Sommer 2008, Sommer et al. 2018). Additionally, higher gene expression of IL-6 and IL-10 were reported in skin of patients with neuropathy compared to healthy controls (Deprez et al. 2001, Koike et al. 2008, Üçeyler and Sommer 2008, Sommer et al. 2018). In the previous study, local cytokine expression profiles of whole skin biopsy samples showed higher levels of IL-6 and IL-8 in the affected distal skin sample of patients with SFN compared to healthy controls (Üçeyler et al. 2010). Variability

of local cytokine expression in various diseases and tissues suggests that changes in cytokine expression are tissue, symptom or disease specific. Therefore, the current study distinguished between fibroblasts and keratinocytes to approach the cytokine producing cell type in SFN and enlarged the study groups. Higher levels of IL-6 and IL-8 were detected in fibroblasts, and previously subtle or undetected differences of TGF-ß1 expression was shown in keratinocytes when comparing patients with controls. Similarly, higher gene expression profiles of TGF-ß1 in fibroblasts and higher gene expression profiles of IL-10 in keratinocytes of patients compared to controls were found when investigating other conditions with small fiber pathology such as fibromyalgia syndrome (FMS) (Evdokimov et al. 2019). In this study TGF-ß1 levels were elevated in fibroblasts and IL-10 expression was higher in keratinocytes when comparing the FMS patients to the control group (Evdokimov et al. 2019). Prior studies investigated whole skin biopsies in FMS patients and detected IL-1ß, IL-6, and TNF expression in patients compared to controls (Salemi et al. 2003, Üçeyler et al. 2014).

Potential reasons for the diversity in cytokine expression of skin cells in different diseases may be caused by the underlying individual pathophysiological mechanisms. The comparison of the previous (Üçeyler et al. 2010) and the current study within the SFN group, using whole skin samples or isolated fibroblast and keratinocyte cell cultures may have influenced the results. Individual cell analysis bears the potential to perform profound analysis of cytokine profiles in particular cell types, but also has the risk of missing cytokine alterations caused by co-interactions between skin cells. In the current study, IL-6 was higher expressed in fibroblasts of patients compared to healthy controls. This may indicate that dermal cells may contribute to SFN pain via secretion of algesic mediators and thus affecting dermally localized nerve fibers. Alternatively, higher dermal IL-6 expression may influence neurites along their way through the skin, which may result in local symptoms. To further elucidate the underlying mechanisms of cytokine and neuron interaction, co-cultures of skin cells and neurons will be performed in future studies.

4.6 IL-8 as a generally increased mediator in SFN

Several studies investigated IL-8 in animal models and reported this chemokine to be elevated after nerve injury and thus to be of potential importance in the development and maintenance of neuropathic pain e.g. by inflammatory hypersensitivity (Brandolini et al. 2017). Another group, which measured the IL-8 levels in serum, DRG, and sciatic nerve after sciatic nerve injury in rats, reported increased IL-8 levels in DRG and injured nerve, whereas the serum levels remained unchanged (Khan et al. 2017). Following these results, IL-8 was suggested to be specific for nerve injury (Khan et al. 2017). IL-8 was used as a target gene in a rat model of lumbar disc herniation with radicular neuropathic pain (Kim et al. 2011). Intrathecal injection of IL-8 receptor inhibitor reduced mechanical allodynia in rats and promoted IL-8 as a potential therapeutic target of neuropathic pain (Kim et al. 2011). In human studies IL-8 was measured in plasma of peripheral blood and saliva samples obtained from patients with burning mouth syndrome as a neuropathic orofacial pain syndrome (Barry et al. 2018). Significant elevated levels of IL-8 were found in peripheral blood when comparing the patient group with the control group (Barry et al. 2018). In saliva on the contrary, IL-8 expression only followed a trend of higher levels in patients group compared to controls (Barry et al. 2018). Thus, systemic inflammation is suggested more than local to be associated with orofacial neuropathic pain symptoms (Barry et al. 2018). In the current study, higher IL-8 expression in WBC and skin cells compared to healthy controls were identified. Possible explanations for the discrepancy in local chemokine expression are explained e.g. by differences between humans and animal models. Variability in duration of pain symptoms and potentially applied therapeutics may also have influenced the results. IL-8 as systemically elevated cytokine, makes it a promising target as a potential biomarker for neuropathic pain. Furthermore, the local elevated gene expression of IL-8 in patients with SFN, makes it a point of application for local drugs. However, it remains unclear, whether increased IL-8 levels are linked to neuropathic pain or enhanced IL-8 expression is a result of experiencing pain. To further investigate whether IL-8 is cause or result of neuropathic pain or even a combination of

both, further studies e.g. prospective monitoring of SFN patients should be performed.

4.7 Axon Guidance Molecule expression profiles in isolated fibroblasts and keratinocytes

In the central nervous system, morphological changes and functionality of axon guidance molecules were investigated in several studies assessing animal models of neuropathic pain (Masuda et al. 2009, Wu et al. 2016, Khangura et al. 2019, Li et al. 2019, Niftullayev and Lamarche-Vane 2019). Another group developed an in vitro co-culture system of epidermal keratinocytes and DRG neurons to investigate interactions between these two cell types and to quantify keratinocyte dependent nerve fiber growth (Kumamoto et al. 2014). The authors showed a reduction of nerve fiber outgrowth by adding Sema3A to keratinocytes as a known inhibitor of neurite outgrowth (Kumamoto et al. 2014). Local alterations in axon guidance molecule expression were analyzed in recent studies e.g. by showing elevated levels of NTN1 in Schwann cells and macrophages after sciatic nerve injury in rats (Webber et al. 2011). Apart from neuropathic pain, the role of keratinocytes as cells discharging axon guidance molecules was reported in an animal model of atopic dermatitis (Yamaguchi et al. 2008) and in human patients (Botchkarev et al. 2006, Tominaga et al. 2008). Sema3A was reported to act through nerve fiber repulsion and decreased epidermal nerve fiber density (Yamaguchi et al. 2008). The expression of the NTN1 and Unc5b as well as EFNA4 and EPHA4 was recently investigated in skin fibroblasts and keratinocytes of FMS patients (Evdokimov et al. 2019). Higher expression of EFNA4 and EPHA4 was found in fibroblasts and keratinocytes when comparing patients with controls (Evdokimov et al. 2019). Applying the same methodology in the current study, elevated levels of NTN1 and Unc5b in fibroblasts were found when comparing SFN patients with healthy controls. Keratinocytes presented a higher gene expression of NTN1 in the SFN group compared to healthy controls.

Potential aspects for the discrepancy in axon guidance expression of the FMS cohort and the SFN cohort may be various underlying mechanisms of fiber

reduction and sensitization in different diseases. In SFN, NTN1 - higher expressed in patients' fibroblasts and keratinocytes - is a promising target to influence axonal outgrowth and being involved in epidermal nerve fiber reduction. In light of reports on literature NTN1 to act in two different ways attractive e.g. via Deleted in Colorectal Cancer (DCC) receptor or repulsive via receptors of the Unc5 family, the focus on interactions between NTN1 and its receptor will be performed in future studies. The current study collected gene expression data of NTN1 and Unc5b in skin cells but did not measure DCC levels, interactions between DCC and Unc5b among themselves or with NTN1, with the result of missing information in interpreting the influence of NTN1 on axonal growth and functionality.

4.8 Conclusion and Outlook

The importance of cytokines in the development and maintenance of neuropathic pain as well as in the pathophysiology of SFN is incompletely understood. Homeostasis and interaction of cytokine expression may play a role (Martini and Willison 2016). To investigate their relevance for SFN pathophysiology, the interactions between cytokines among themselves, between surrounding cells, and functional analysis e.g. on protein level should be determined. Even though IL-8 is supposed to function as a potential biomarker, some factors remain to be elucidated: the potential dynamics in cytokine expression, the duration and the kind of tissue in which cytokines are elevated, as well as endogenous and exogenous factors e.g. drugs, hormones, or temperature, which potentially influence cytokine expression. MicroRNA, small non-coding RNAs, are known to be important in numerous physiological and pathophysiological processes (Niederberger et al. 2011) and are promising candidates which are involved in cytokine production and regulation (Jiangpan et al. 2016, Tian et al. 2019). One study reported a downregulation of miR-129-5p after CCI in a rat model of neuropathic pain, which correlated with increased levels of pro-inflammatory cytokines and promoted inflammatory processes through increased high mobility group protein B1 (HMGB1) expression (Tian et al. 2019). Alterations of miR-129-5p expression may act as one of the causal

factors in neuropathic pain. miR-21 is reported to be involved in inflammatory processes and leads to enhanced production of IL-10 and decreased synthesis of TNF (Sheedy 2015). Applying a rat model of neuropathic pain, miR-21 synthesis was induced by intrathecal application of IL-1. It remains to be elucidated, if this knowledge leads to additional understanding of neuropathic pain in SFN. Therefore, and for assignment of one target as a potential diagnostic or prognostic biomarker, further studies on human biomaterial are necessary.

Apart from the importance of cytokines in SFN, the results of the axon guidance analysis provide evidence for neuropathic pain development by influencing nerve fiber outgrowth and functionality of cutaneous free nerve endings. To further elucidate the underlying mechanism, in depth analysis of cell-nerve fiber interactions in skin will be performed in future studies. The already established patient-derived in vitro 3D skin model is one opportunity to determine the cross-talk between sensory neurons and skin cells individualized in SFN patients (Karl et al. 2019).

4.9 Limitations and strengths of the study

One limitation of the study is the control cohort, which also included participants with physiologically reduced IENFD in skin punch biopsy, but without any symptoms or signs of neurological disease. Cell culture experiments do not represent the current state of skin at the time of biopsy. Furthermore, the culturing process influences production and release of gene products. Further confounders are: The very small size of skin biopsy, and the area of biopsy as a low proliferative tissue. These conditions make it difficult to preserve enough cells for further processing and comparison of results between study participants. Cell quality, especially of the keratinocytes, varies highly between individuals and quantity is often low. Because of differences in growth rate and the applied separation method between keratinocytes and fibroblasts, fibroblasts often overgrow the keratinocytes and mixed cultures with variation in keratinocyte density are obtained. Also, the cultivation time differed because of the inconstant growth rate.

The focus of the study was on fibroblasts and keratinocytes as major cell types of human skin, thus conclusions about the role of other cell types such as mast cells or macrophages are not allowed. A larger biopsy is necessary to analyze protein levels additionally to gene expression as aftereffect parameter.
The strength of the study is the large panel of potentially relevant cytokines and axon cues investigated in individually cultured keratinocytes and fibroblasts. This approach allows stratification of the results for the respective cell type and subgroup analysis, which guides the way for future studies.

5 Summary / Zusammenfassung

Der Pathomechanismus von Schmerz bei SFN ist unklar. Auf Grundlage tierexperimenteller und klinischer Studien wird die Einwirkung kutaner und systemischer Schmerzmediatoren auf sensibilisierte Nozizeptoren in der Haut als mögliche Ursache diskutiert. In diesem Zusammenhang gab es Hinweise auf die Bedeutung von pro- und anti-inflammatorischen Zytokinen in der Pathophysiologie neuropathischer Schmerzen. Ziel der Studie war es, die systemische und lokale Genexpression pro- und anti-inflammatorischer Zytokine in Leukozyten sowie kutanen Fibroblasten und Keratinozyten von Patienten mit SFN zu messen. Ferner wurde untersucht, inwieweit die Expression repellierender Axon Guidance Moleküle und deren Rezeptoren in Hautzellen die IENFD regulieren könnten. Insgesamt konnten 55 SFN PatientInnen und 31 gesunde KontrollprobandInnen prospektiv rekrutiert werden. Nach ausführlicher klinischer Phänotypisierung und Blutentnahme wurden bei allen StudienteilnehmerInnen 6-mm Hautstanzbiopsien am lateralen Unter- und Oberschenkel entnommen. Die Messung der systemisch relativen Genexpression (ΔG) der Zytokine Interleukin IL-1β, IL-2, IL-6, IL-8 und des TNF-α erfolgte aus Leukozyten. Aus den Hautstanzbiopsien, die u.a. zur Bestimmung der IENFD verwendet wurden, wurden außerdem Primärzellkulturen von Keratinozyten und Fibroblasten angelegt, aus denen die lokale ΔG von Axon Guidance Molekülen NTN1, Unc5b, EPHA4 und EFNA4 sowie der Zytokine IL-1β, IL-4, IL-6, IL-8, IL-10, TNF-α und TGF-β1 mittels qRT-PCR erfolgte. Systemisch zeigte sich eine höhere Genexpression für IL-2, IL-8 und TNF bei SFN Patienten im Vergleich zu gesunden Kontrollen. In Keratinozyten konnten höhere Expressionen von NTN1 und TGF-β1 bei Vergleich der Patientengruppe mit der Kontrollgruppe nachgewiesen werden. In Fibroblasten zeigte sich im Gruppenvergleich eine höhere Genexpression für NTN1, Unc5b sowie für IL-6 und IL-8. Die systemisch und lokal bei SFN Patienten nachgewiesene höhere Expression algetischer, pro-inflammatorischer Zytokine verglichen mit Kontrollen unterstützt eine mögliche pathophysiologische Rolle bei der Entstehung von neuropathischen Schmerzen. Ferner weisen die Daten darauf hin, dass Fibroblasten und

Keratinozyten durch die Expression von NTN1 und Unc5b Einfluss auf das subepidermale und intraepidermale Nervenfaserwachstum nehmen und durch lokale Denervierung bei der Entstehung neuropathischer Schmerzen mitwirken könnten.

6 Appendices

6.1 Overview Tables of the selected cytokines

Overview 1: Pro-inflammatory cytokines and their function.

cytokine	selected functions
IL-1ß	- Stimulation, sensitization and induction of spontaneous activity of A- and C-fibers (Wang et al. 2012) - Induction of e.g. IL-6, cyclooxygenase 2 (COX2), and phospholipase A2 expression (Vallejo et al. 2010)
IL-2	- Produced by cluster of differentiation (CD) 4^+ and $CD8^+$ T-cells, B-cells and dendritic cells (Gaffen and Liu 2004) - Proliferation of $CD4^+$ and $CD8^+$ T-cells (Gaffen and Liu 2004)
IL-6	- Regulation and secretion of pro-inflammatory cytokines by influencing cytokine cascade (Austin and Moalem-Taylor 2010, Vallejo et al. 2010) - Hypersensitivity of pain (Austin and Moalem-Taylor 2010)
IL-8	- Chemotaxis (Bote et al. 2013) - Elevated gene expression levels in patients with neuropathic pain (Sommer et al. 2018)
IGF	- Prevention of neuronal apoptosis (Rauskolb et al. 2017) - Promotion of axonal growth and myelinization (Rauskolb et al. 2017)
TNF	- Stimulation, sensitization and induction of spontaneous activity of A- and C-fibers (Wang et al. 2012) - Allodynia, hyperalgesia (Zhao et al. 2017)

Abbreviations: CD = cluster of differentiation, IGF = insulin-like growth factor; IL- = interleukin; TNF = tumor necrosis factor.

Overview 2: Anti-inflammatory cytokines and their function.

cytokine	selected functions
IL-4	- Activation of microglial cells (Okutani et al. 2018)

	- Decrease of pain (Hung et al. 2017)
IL-10	- Ambiguous effects on pain development depending on concentration and target structure (Hung et al. 2017) - Alleviation of hyperalgesia and allodynia (Hung et al. 2017)
TGF-ß1	- Prevention and reversion of neuropathic pain (Chen et al. 2013) - Influence varies between different anatomical location (Hung et al. 2017) - Modulation of immune response (Chen et al. 2013)

Abbreviations: IL- = interleukin; TGF- = transforming growth factor.

6.2 Abbreviations

ADS	“Allgemeine Depressionsskala”
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BMI	body mass index
°C	degree-celsius
CCM	corneal confocal microscopy
CD	cluster of differentiation
cDNA	copy-DNA
cm	centimeter
CMAP	compound muscle action potential
Co.	controls
CO_2	Carbon dioxide
DCC	Deleted in Colorectal Cancer
Dist.	distal
dl	deciliter
DMEM/F-12	Dulbecco`s Modified Eagle Medium F-12 Nutrient Mixture (Ham), (+) L-Glutamine
dmL	distal motor latency
DMSO	Dimetyl Sulfoxide
DNA	deoxyribonucleic acid
DRG	dorsal root ganglions
EDTA	ethylenediaminetetraacetic acid
EFNA4	ephrin-A4
EL	erythrocyte lysis
EPH	ephrin receptors
EPHA4	ephrin receptor A4
F	female
FCS	fetal calf serum
Fig.	figure
FMS	fibromyalgia syndrome
FTSM	full thickness skin models
g	gram

GAP43	growth-associated protein 43
GDNF	glial cell-line-derived neurotrophic factor
GCPS	Graded Chronic Pain Scale
HbA1c	Haemoglobin A1c
hdf	human dermal fibroblasts
hek	human epidermal keratinocytes
HIV	human immunodeficiency virus
HMGB1	high mobility group protein B1
h	hour
HSAN	hereditary sensory and autonomic neuropathy
IENFD	intraepidermal nerve fiber density
IGF	insulin-like growth factor
IL	interleukin
DA	dalton
k	kilo-
LD	length-dependent
M	male
mm	millimeter
m^2	square meter
MAP	mitogen-activated protein
min	minutes
miRNA	microRNA
ml	milliliter
mRNA	messenger-RNA
mV	millivolt
n	number
N	Number
N.	nervus
NA	not applicable
NCV	Nerve conduction velocity
NGF	nerve growth factor
NLD	non-length-depenent

NLG	“Nervenleitgeschwindigkeit”
NMDA	N-methyl-D-aspartate
NRS	numeric rating scale
NTN1	netrin 1
OGTT	oral glucose tolerance test
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PCS	Pain Catastrophizing Scale
pg	picogram
PGP9.5	protein-gene product 9.5
PREP	pain related evoked potentials
Prox.	proximal
QSART	quantitative sudomotor axon reflex test
QST	quantitative sensory testing
Rel.	relative
RNA	ribonucleic acid
RT	room temperature
RTK	receptor protein-tyrosine kinase
s	second
SCN9A	sodium voltage-gated channel α subunit 9
SFN	Small fiber neuropathy
SNAP	sensory nerve action potential
SNRI	serotonin norepinephrine reuptake inhibitors
TCA	tricyclic antidepressants
TGF	transforming growth factor
TNF	tumor necrosis factor
TRP	transient receptor potential
TRPM3	transient receptor potential subfamily M member 3
TRPV3	transient receptor potential vanilloid 3
TrypLE Express	TrypLE™ Express Enzyme (-) phenol red TrypLE Express
TSH	thyroid stimulating horme
TSL	thermal sensory limen

U	units
μl	microliter
μV	microvolt
WBC	white blood cells
WDT	warm detection threshold

6.3 Materials

6.3.1 Technical equipment

- Advanced Primus 96-PCR cycler (Peqlab Biotechnology, Erlangen, Germany)
- Analog vortex mixer (VWR, Radnor, USA)
- Centrifuge 5417R (Eppendorf, Hamburg, Germany)
- Centrifuge rotina 420R (Hettich, Tuttlingen, Germany)
- Centrifuge rotofix 32 (Hettich, Tuttlingen, Germany)
- Fluorescence microscope Axiophot 2 (Zeiss, Oberkochen, Germany)
- Freezer TSX Series -80°C (Thermo Scientific, Waltham, USA)
- Incubator Heraeus Thermo Scientific Cell 150 (Thermo Scientific, Waltham, USA)
- Microscope Olympus CKX41 (Olympus, Tokyo, Japane)
- Microscope Stemi 2000 (Zeiss, Oberkochen, Germany)
- Mr Frosty (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- Nanodrop® spectrophotometer (Peqlab Biotechnology, Erlangen, Germany)
- StepOnePlus™ Cycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- Vacuum pump ECOM-P 4153 (Eppendorf, Hamburg, Germany)
- Wather bath WNB 7-45 (Memmert, Schwabach, Germany)

6.3.2 Consumable Supplies

- Biopsy Punch 6 mm (Stiefel, Offenbach am Main, Germany)
- Caps PCR soft tubes 0.2 ml (Biozym Scientific, Oldendorf, Germany)
- Caps safe lock tubes 1.5 ml (Eppendorf, Hamburg, Germany)
- Caps: 0.2 ml; 1.5 ml; 2 ml (Eschendorf, Hamburg, Germany)
- Cell culture dishes Nunclon ™ Surface (NUNC A/S, Roskilde, Denmark)
- Cell Culture Flask, CELLSTAR ®, 25 ml (greiner bio-one, Kremsmünster, Austria)

- Cell Culture Flask, CELLSTAR ®, 25 ml (greiner bio-one, Kremsmünster, Austria)
- Cryovials (Fa. Biozym Scientific, Hessisch Oldendorf, Germany)
- Cutasept (Paul Hartmann, Heidenheim, Germany)
- Disoposable pipette 5 ml; 10 ml; 25 ml (greiner bio-one, Kremsmünster, Austria)
- Eclipse Needle with SmartSlip (Becton Dickinson, Franklin Lakes, New Jersey, USA)
- Falcon tubes 15 ml (greiner bio-one, Kremsmünster, Austria)
- Gazin Mullkompressen (Lohmann&Rauscher, Wien, Austria)
- Glas-pasteur-pipettes (Hartenstein, Würzburg, Germany)
- Leukostrips S (Smith&Nephew, Medical Limited, England)
- Micro Amp Optical 96-Well Reaction Plate (Applied Biosystems, Darmstadt, Germany)
- Multipipette ® Stream (Eppendorf, Hamburg, Germany)
- Optical Adhesive Covers (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- Pipette 10 µl; 100 µl; 1000 µl (Eppendorf, Hamburg, Germany)
- Pipette tips Biosphere filter tips 10 µl; 100 µl; 1000 µl (Sarstedt, Nüümbrecht, Germany)
- Pipette tips Combitips ® plus 0.1 ml; 5 ml (Eppendorf, Hamburg, Germany)
- Scalpel, (Braun, Melsungen, Germany)
- Sterile adhesive plasters, Leukomed (BSN medical, Hamburg, Germany)
- Sterile forceps (Braun, Melsungen, Germany)
- Sterile gloves (Semperit, Wien, Österreich)
- Syringe 5 ml (Braun, Melsungen, Germany)

6.3.3 Drugs

- Accu-Chek Dextro (O.G.-T., Roche Diabetes Care, Germany)
- Local anaesthesia (Scandicain 1%, Aspen Pharma, München, Germany)

6.3.4 Buffers and solutions

- Aqua ad iniectabilia (Braun, Melsungen, Germany)
- Bovine serum albumine (Sigma-Aldrich, München, Germany)
- chloroform (Carl Roth, Karlsruhe, Germany)
- Dimetyl Sulfoxide (Carl Roth, Karlsruhe, Deutschland)
- Dulbecco`s Modified Eagle Medium F-12 Nutrient Mixture (Ham), (+) L-Glutamine (DMEM/F-12) culture medium (Life Technologies, Carlsbad, CA, USA)
- EpiLife Defined Growth Supplement (Life Technologies, Carlsbad, CA, USA)
- EpiLife medium (Thermo Fisher Scientific, Waltham, Massachusetts USA)
- Erythrocyte lysis (EL)-buffer (Quiagen, Hilden, Germany)
- Ethanol 100% (Sigma-Aldrich, München, Germany)
- miRNeasy Mini Kit #217004 (Quiagen, Hilden, Germany) including:
    - QIAzol Lysis reagent (containing guanidine thiocyanate and phenol) (Quiagen, Hilden, Germany)
    - RNAse-free-water (Quiagen, Hilden, Germany)
    - RPE buffer (Quiagen, Hilden, Germany)
    - RWT buffers (Quiagen, Hilden, Germany)
    - Silica-membrane RNeasy spin column (Quiagen, Hilden, Germany)
- Paraformaldehyde 4% (Sigma-Aldrich, München, Germany)
- Penicillin/Streptomycin antibiotics (Life Technologies, Carlsbad, CA, USA)
- RNA cell protect (Quiagen, Hilden, Germany)
- StemPro Accutase (Life Technologies, Carlsbad, CA, USA)
- Sterile distilled ultra pure water (Cayman Chemical Company, Ann Arbor, USA)
- TaqMan Reverse Transcription Reagents (Applied Biosystems Darmstadt, Germany) including:
    - Random Hexamer

- Oligo-DT
- 10x PCR buffer
- Multiscribe reverse transcriptase
- Rnase inhibitor
- $MgCl_2$
- dNTPs

- TaqMan Universal Master Mix (Applied Biosystems, Darmstadt, Germany)
- Terralin liquid (Schülke, Norderstedt, Germany)
- TrypLE™ Express Enzyme (-) phenol red TrypLE Express (Life Technologies, Carlsbad, CA, USA)

6.3.5 Antibodies used in immunohisto-/cytochemistry

- Protein-gene product 9.5 (PGP9.5 1:1000 (Zytomed, Berlin, Germany)

6.3.6 Primers used for qRT-PCR

- 18sRNA (Hs99999901_s1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- IL-1ß (Hs00174097_m1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- IL-2 (Hs00174114_m1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- IL-6 (Hs00174131_m1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- IL-8 (Hs00174103_m1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- TNF-alpha (Hs00174128_m1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- IGF-1 (Hs01547656_m1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

- IL-4 (Hs00174122_m1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- IL-10 (Hs00174086_m1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- TGF-beta (Hs99999918_m1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- EFNA4 (Hs00193299_m1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- EPHA4 (Hs00953178_m1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- NTN1 (Hs00924151_m1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- Unc5b (Hs00900710_m1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

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Publikationen

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