Untersuchung cornealer und kutaner Faktoren im Rahmen der Kleinfaserpathologie beim Fibromyalgie-Syndrom / Corneal and cutaneous factors contributing to small fiber pathology in fibromyalgia syndrome

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1. Introduction

1.1 Fibromyalgia Syndrome (FMS)

1.1.1 Definition and diagnostic criteria of FMS

Fibromyalgia syndrome (FMS) is a chronic pain condition of unknown etiology. According to current diagnostic criteria, FMS core symptoms are chronic widespread pain, sleep disturbances or non-refreshing sleep, and fatigue (Eich et al., 2017; Wolfe et al., 2010). Following the criteria established by the American College of Rheumatology in 2010 (ACR 2010 criteria) the diagnosis of FMS requires two symptom scores: the widespread pain index (WPI) and the symptom severity (SS) scale. The WPI gives the number of painful body areas out of 19 predefined areas, while the SS scale is calculated by evaluating the severity of sleep disturbances, fatigue, cognitive impairment, and general somatic symptoms (including pain as well as additional symptoms) during the past week. FMS diagnosis then requires either the combination of WPI ≥ 7 and SS scale ≥ 5 or WPI 3 – 6 and SS scale ≥ 9 (Wolfe et al., 2010).

The previous ACR 1990 diagnostic criteria used tenderness upon pressure with 4 kg at defined points in combination with chronic widespread pain for the diagnosis of FMS (Wolfe et al., 1990). This concept of tender points has been abandoned by the recent ACR 2010 criteria, whereas the German 2017 S3-guideline still allows a clinical diagnosis of FMS based on either the combination of widespread pain and tender points or the combination of widespread pain and SS scale (Eich et al., 2017). Furthermore, the FMS diagnosis can only be made when any other somatic disease which could explain patients` symptoms is excluded (Eich et al., 2017; Wolfe et al., 2010).

1.1.2 Epidemiology of FMS

In a population-based German study using the ACR 2010 criteria (Wolfe et al., 2010), a prevalence of 2.1% with no difference between male and female participants was estimated for FMS (Wolfe et al., 2013). However, FMS is diagnosed less often, and FMS diagnosis is made more frequently in women as shown in an analysis of German health insurance data: 0.3% of the analyzed population was diagnosed with FMS and >90% of the diagnosed FMS cases were females (Marschall et al., 2011). The common age of onset for FMS is 40 – 60 years (Marschall et al., 2011; Wolfe et al., 2013).

1.1.3 Etiology and pathophysiology of FMS

The etiology of FMS remains unclear although several associations have been observed: rheumatic diseases (Lee et al., 2013), gene polymorphisms (Lee et al., 2012), vitamin D deficiency (Hsiao et al., 2015), history of physical and/or sexual abuse (Afari et al., 2014), depressive disorder (Chang et al., 2015), and lifestyle factors such as smoking, obesity, and lack of physical activity (Choi et al., 2010; Mork et al., 2010). However, a causal attribution to FMS pathogenesis cannot be proven for any of these factors so far (Üçeyler et al., 2017).

Likewise, there is no generally accepted model of FMS pathophysiology. Instead diverse pathophysiological alterations have been found, each affecting subgroups of FMS patients: malfunctioning central procession of pain (Clauw, 2014; Dehghan et al., 2016), increased hippocampal neuro-transmitter concentrations (Aoki et al., 2013), dysfunction of the sympathetic

nervous system (Martinez-Martinez et al., 2014), and, most recently, pathology of small Aδ- and C- nerve fibers (Oudejans et al., 2016; Serra et al., 2014; Üçeyler et al., 2013). None of these factors are specific for FMS patients (Üçeyler et al., 2017).

By the latest version of the 2017 German S3-guideline, FMS is considered to represent a heterogenous group of patients who share a common clinical presentation caused by various biological, psychological, and social factors through diverse pathophysiological pathways (Üçeyler et al., 2017).

1.1.4 Current treatment options for FMS patients

Due to the lack of pathophysiological knowledge about FMS, treatment options are limited. The German S3-guideline recommends a multi-modal therapy concept including exercise and psychotherapeutic approaches to which a temporary pharmacological treatment can be added. Currently, no drug has an official approval for FMS treatment in Germany. However, there are positive recommendations for the use of the antidepressants amitriptyline and duloxetine and the anticonvulsant pregabalin, especially in the case of co-morbid depressive or anxiety disorder. The use of classic analgesic drugs, e.g. nonsteroidal anti-inflammatory drugs, acetaminophen, opioids, is not recommended (Sommer et al., 2017).

1.2 Small fiber pathology (SFP)

Small fiber pathology (SFP) is a term describing damage to or misfunction of thinly myelinated Aδ- and un-myelinated C-nerve fibers. It can result in symptoms matching the function of these fibers which include nociception,

cold- and warm-perception, itch, and autonomic regulation (Üçeyler, 2016). However, SFP has also been detected in diseases that present without or just with few of the typical symptoms of small fiber dysfunction, e.g. multiple sclerosis (Bitirgen et al., 2017) and Parkinson's disease (Kass-Iliyya et al., 2017).

1.2.1 Experimental assessment of SFP

Currently, several methods to study small nerve fiber function and morphology exist. Perception and pain thresholds of diverse modalities including those conducted by small fibers can be assessed by quantitative sensory testing (QST) (Rolke et al., 2006). Indirect information about conduction of small fibers are obtainable via pain-related evoked potentials (PREP), contact-heat evoked potentials, or laser evoked potentials (Lefaucheur, 2019). Electrophysiological properties of single Aδ- and C-fibers can be assessed using microneurography (Serra et al., 1999). Determination of intraepidermal nerve fiber density (IENFD) in skin punch biopsies (Lauria et al., 2005) and the determination of corneal nerve fiber density (CNFD) using corneal confocal microscopy (CCM) (Tavakoli et al., 2008) are methods to study small nerve fiber morphology.

1.2.2 Small fiber pathology and small fiber neuropathy (SFN)

Although SFP is a defining feature of small fiber neuropathy (SFN), these two terms cannot be used interchangeably. SFN is defined as a sensory peripheral neuropathy which only or at least predominantly affects small fibers (Lacomis, 2002; Lauria and Lombardi, 2007). While SFP may be found in

addition to large fiber damage in many cases of polyneuropathy, the diagnosis of SFN requires clinical and electrophysiological exclusion of large fiber pathology (Devigili et al., 2008). Typical clinical presentation of SFN patients include mostly acral and symmetric neuropathic pain, par-/dysesthesias, thermal allodynia, and autonomic dysfunction (Devigili et al., 2008; Lauria and Lombardi, 2007). Diverse causes of SFN are known including metabolic, infectious, immune-mediated, toxic, and hereditary etiologies with the most common being diabetes mellitus and impaired glucose tolerance (Cazzato and Lauria, 2017). SFN etiology remains idiopathic in a large proportion of patients.

1.2.3 SFP in FMS

Several studies have lately shown SFP in subgroups of FMS patients. Patients had reduced IENFD at their ankle and upper thigh, QST profiles showing increased cold and warm detection thresholds, decreased potential amplitudes, and increased potential latencies in PREP (Evdokimov et al., 2019a; Üçeyler et al., 2013). Other studies found reduced corneal innervation in CCM examination (Erkan Turan et al., 2018; Evdokimov et al., 2019a; Oudejans et al., 2016; Ramirez et al., 2015) and hyperexcitable C-fibers in microneurography (Evdokimov et al., 2019a; Serra et al., 2014). SFP was not detected in all FMS patients, but patients with generalized reduction of small nerve fibers may form a more severely affected population which indicates a relevant role of SFP for FMS pain and its additional symptoms (Evdokimov et al., 2019a).

The molecular and cellular background of SFP is yet incompletely understood. Electron microscopic studies have shown abnormal morphology of Schwann cells around unmyelinated dermal nerve fibers and a thinning of these fibers which stands out not only in comparison to healthy controls, but also to SFN patients (Doppler et al., 2015; Kim et al., 2008). One light-microscopic study found increased numbers of mast cells in the dermis of FMS patients (Blanco et al., 2010). Gene expression studies of skin samples have mainly focused on pro- and anti-inflammatory cytokines with conflicting results: while some found elevated levels of pro-inflammatory cytokines (Salemi et al., 2003), others did not find differences in cytokine expression levels between FMS patients and healthy controls (Blanco et al., 2010; Üçeyler et al., 2014). More recently, decreased expression of insulin like growth factor-1 receptor (IGF-1R) accompanied by increased expression of its regulator microRNA miR let-7d was detected in FMS skin (Leinders et al., 2016). Separate expression analysis of specific skin cell populations revealed overexpression of the axon guidance protein ephrin A4 (EFNA4) and its receptor EPHA4 in fibroblasts and keratinocytes. Transforming growth factor-ß1 (TGF-ß1) was overexpressed in fibroblasts and the anti-inflammatory cytokine interleukin 10 (IL-10) was overexpressed in keratinocytes (Evdokimov et al., 2019b).

1.3 Langerhans cells (LC) in FMS

Langerhans cells (LC) are a population of antigen presenting cells located within the basal epidermis. LC have also been shown to populate the cornea with their cell bodies located within the basal epithelial layers and the sub-basal nerve plexus (Zhivov et al., 2005). As antigen presenting cells, LC are

capable of initiating immune reactions or tolerance to detected antigens (Doebel et al., 2017). LC have also been shown to influence the homeostasis of cutaneous (Doss and Smith, 2014) and corneal (Hamrah et al., 2016) nerve fibers. This makes LC a promising target for research on SFP. Indeed, increased densities of corneal LC combined with reduced CNFD and CNFL were found in various conditions including diabetic neuropathy (Tavakoli et al., 2011), chronic inflammatory demyelinating polyneuropathy (Stettner et al., 2016), herpes zoster ophthalmicus (Cavalcanti et al., 2018), multiple sclerosis (Bitirgen et al., 2017), and dry eye disease (Choi et al., 2017). No increase in corneal LC density was, however, found in patients suffering from SFN (Bucher et al., 2015).

1.4 Micro RNA expression in FMS

Micro RNA (miRNA) are a group of small non-coding RNA molecules. miRNA play an important role in the regulation of gene expression by repression of mRNA transcription or even complete mRNA degradation. miRNA can also be found in exosomes and are assumed to participate in intercellular communication (Kress, 2016). Deregulated miRNA expression signatures have been linked to chronic and neuropathic pain states. It has been hypothesized that the ability of specific miRNA to regulate neuronal, immune cells, and neuro-immune crosstalk plays a key role in the pathogenesis of pain syndromes (Kress et al., 2013).

Research on miRNA expression in FMS patients has so far shown several up- or downregulated miRNA in blood sera and cerebrospinal fluid though no disease specific miRNA signature was found (D'Agnelli et al., 2019).

Research in FMS skin samples has also shown miRNA alterations and in the case of miR let-7d concomitant downregulation of one of its target mRNA (IGF-1R) was found (Leinders et al., 2016).

1.5 Study objectives

1.5.1 Analysis of corneal LC and potential correlations with small nerve fiber alterations in FMS patients

LC are a promising target for research on the background of SFP in FMS. LC can be assessed in the human cornea via CCM while simultaneously analyzing the corneal sub-basal nerve plexus for signs of SFP. We set out to determine corneal LC densities, study LC morphology, and quantify LC-nerve fiber contacts in FMS patients and compare the results to findings in healthy controls and disease controls suffering from SFN. We hypothesized that in FMS patients more LC would be found than in our control groups and increased LC density would correlate with signs of SFP.

1.5.2 Identification and evaluation of differentially expressed RNA in distinct skin cell populations in FMS patients

Dermal fibroblasts and epidermal keratinocytes are in close proximity to the cutaneous endings of small sensory nerve fibers and are likely to have a relevant influence on these fibers. Molecular mechanisms behind this assumed interplay are not yet fully understood. We separately cultured fibroblasts and keratinocytes from skin punch biopsies of FMS patients with differing degrees of small nerve fiber affection and healthy controls and screened expression levels of mRNA and miRNA via next generation

sequencing (NGS). We hypothesized that differential expression of a specific set of RNA would distinguish FMS patients from controls and FMS patients with high SFP from those with low SFP.

2 Methods

2.1 Study design and patient recruitment

For this case-control-study, participants were recruited between September 2014 and August 2018 at the Department of Neurology, University of Würzburg, Germany. We included 143 FMS patients and 65 age- and sex-matched healthy controls in our study. We first interviewed FMS patients by telephone who had actively contacted us for study participation. The telephone interview was used to inform the patients and to screen for in- and exclusion criteria, which were the following:

Inclusion criteria:

- Age ≥ 18 years
- ACR 1990 or ACR 2010 diagnostic criteria fulfilled (Wolfe et al., 1990; Wolfe et al., 2010; Wolfe et al., 2011)
- Completed diagnostic work-up to exclude alternative diagnoses according to the 2012 version of the German S3-guideline (Eich et al., 2012)

Exclusion criteria:

- Polyneuropathy
- History of diabetes mellitus, renal insufficiency, drug or alcohol misuse
- Untreated thyroid dysfunction
- Malignancy within the past 5 years
- Acute inflammatory disease at the time of examination
- Any severe psychiatric disorder currently requiring treatment
- Usage of hard contact lenses
- History of eye diseases or eye surgery

- Pain of other cause which was undistinguishable from FMS pain
- Pending compensation claims

We compared our data on corneal innervation and immune cells to those of 41 female SFN patients as disease controls. These patients were recruited between May 2015 and August 2018 as part of another ongoing study in our research team. We recruited SFN patients who had been seen in our clinic as in- or out-patients previously. All patients fulfilled the inclusion criteria of being ≥ 18 years of age and diagnosis of SFN according to current criteria (Devigili et al., 2008). Exclusion criteria were the same as for FMS patients.
Healthy controls were recruited among relatives and friends of the patients and were matched to our patient group for sex and age. Exclusion criteria for healthy controls were the same as for patients.
FMS patients were recruited by Dr. Johanna Frank and Dr. Dimitar Evdokimov, SFN patients were recruited by Dr. Nadine Egenolf and Luisa Kreß, healthy controls were recruited by Dr. Johanna Frank, Dr. Nadine Egenolf, Luisa Kreß, and Alexander Klitsch.
All patients and controls gave written and informed consent to study participation and the study received a positive vote by the Ethics Committee of the University of Würzburg Medical Faculty (121/14).

2.2 Laboratory and electrophysiological assessment

Serum samples of all patients were taken to measure concentrations of electrolytes, thyroid stimulation hormone (TSH), vitamin B12, renal and hepatic marker proteins, and HbA1c levels. All participants underwent oral

glucose tolerance testing (OGTT) to exclude diabetes or impaired glucose tolerance (IGT). To exclude polyneuropathy all FMS and SFN patients underwent electrophysiological measurements of the right sural and tibial nerve according to an established protocol (Kimura, 2001).

2.3 Questionnaires

All FMS patients were asked to fill in several questionnaires to characterize pain and disease specific symptoms, and also patients' psychological status. The questionnaires used in this study were (only German versions were used):

- Fibromyalgia Impact questionnaire (FIQ) (Offenbächer et al., 2000)
- Neuropathic Pain Symptom Inventory (NPSI) (Bouhassira et al., 2004; Sommer et al., 2011)
- Graded Chronic Pain Scale (GCPS) (Von Korff et al., 1992)
- German version of the Center for Epidemiological Studies Depression scale (CES-D) questionnaire (Allgemeine Depressionsskala; ADS) (Radloff, 1977)
- State-Trait Anxiety Inventory (STAI-S / STAI-T) (Spielberger et al., 1970)

2.4 Corneal assessment

All study participants underwent a corneal assessment including slit lamp examination, Cochet-Bonnet esthesiometry, and CCM. We compared CCM data of 134 female FMS patients to data of 60 healthy female controls and of 41 female SFN patients.

All patients and controls underwent slit lamp examination by an ophthalmologist to exclude corneal pathologies that might interfere with CCM examination. We asked every participant whether they regularly used contact lenses. Corneal sensitivity was tested with a Cochet-Bonnet esthesiometer (Luneau Ophtalmologie, Chartres Cedex, France). The monofilament of the esthesiometer was used to apply light pressure onto the central cornea just below the pupil. The resulting pressure is defined by the length of the monofilament. We assumed detection of this irritation by the patient at any length < 5 cm as pathologic sensibility (Dua et al., 2018).

2.4.1 Corneal confocal microscopy (CCM)

CCM image acquisition was performed according to a previously published protocol (Tavakoli and Malik, 2011). We used a Heidelberg Retina Tomograph (Heidelberg Engineering GmbH, Heidelberg, Germany) in combination with the Rostock Cornea Module (Heidelberg Engineering GmbH, Heidelberg, Germany) capped with a sterile TomoCap® (Heidelberg Engineering GmbH, Heidelberg, Germany). Participants' eyes were anaesthetized using Conjuncain EDO® eye drops containing 0.4% oxybuprocaine hydrochloride (Bausch & Lomb GmbH, Berlin, Germany) and lubricated using Corneagel EDO® (Bausch & Lomb GmbH, Berlin, Germany) before microscopy. The microscope was then carefully positioned to allow direct contact between the TomoCap® and the ocular surface. The fine focus of the Rostock Cornea Module was tuned to set the focal plane of the microscope into the corneal sub-basal nerve plexus. A mean of 70 images per eye of the corneal center and pericenter were taken using section mode. Image resolution was 384 x

384 pixels representing a field of view of 400 x 400 µm. To avoid visual or corneal complications CCM examination time was restricted to 5 minutes per eye.

All images of both eyes were presented to a second investigator blinded to group allocation, who chose three images per eye for further analysis. Image selection was based on image quality, contrast, and field of view within the central cornea. We excluded all images from further analysis which had the focal plane set incorrectly or were obscured by artefacts such as pressure lines. The analysis of three representative images as chosen by this method has been shown to produce results on corneal nerve fiber parameters and Langerhans cell density comparable to the analysis of wide-field mapped composite images of the whole cornea (Kheirkhah et al., 2015a).

Images were analyzed using purpose-written, proprietary software ACCMetrics and CCMetrics® (M.A. Dabbah, Imaging Science, Manchester, UK). ACCMetrics was used to determine corneal nerve fiber density (CNFD, i.e. number of main nerve fiber bundles per mm² [no./mm²]), nerve fiber length (CNFL, i.e. total length of nerve fiber bundles [mm/mm²]), nerve fiber width (CNFW, i.e. the average axial diameter of all nerve fiber bundles analyzed [mm]), and nerve fiber fractal dimension (CFracDim, i.e. measure of spatial distribution and structure complexity of corneal nerve fibers) in a fully automated way. CCMetris® was used to determine corneal nerve branch density (CNBD, i.e. number of branches arising from the main nerve fiber bundles [no./mm²]) in a semi-automated way.

LC were counted and analyzed manually using no special software. All bright reflective structures showing a cell body like shape were counted as LC. We

counted LC on the same images we used for nerve fiber quantification. In the following text the number of LC per mm^2 will be referred to as LC_{total}. We further subclassified LC based on their morphology (Mayer et al., 2012) into dendritic LC (dLC, i.e. cells showing dendrite like elongations emerging from their cell body, Figure 1A) and non-dendritic LC (ndLC, i.e. cells only consisting of a cell body, Figure 1B). It was determined for every LC whether it showed visible contact to nerve fibers (i.e. cell body or dendrite visually overlapping with a nerve fiber, abbreviated as: $LC_{fiber\ contact}$/ $LC_{no\ contact}$, Figure 1A,B) in analogy to a previously described method (Stettner et al., 2016). We calculated the ratio of $LC_{fiber\ contact}$/CNFL, $dLC_{fiber\ contact}$/CNFL, and $ndLC_{fiber\ contact}$/CNFL to exclude misinterpretation of high or low counts of $LC_{fiber\ contact}$ solely based on high or low CNFL increasing/ decreasing the probability of morphological overlapping of LC and nerve fiber bundles.

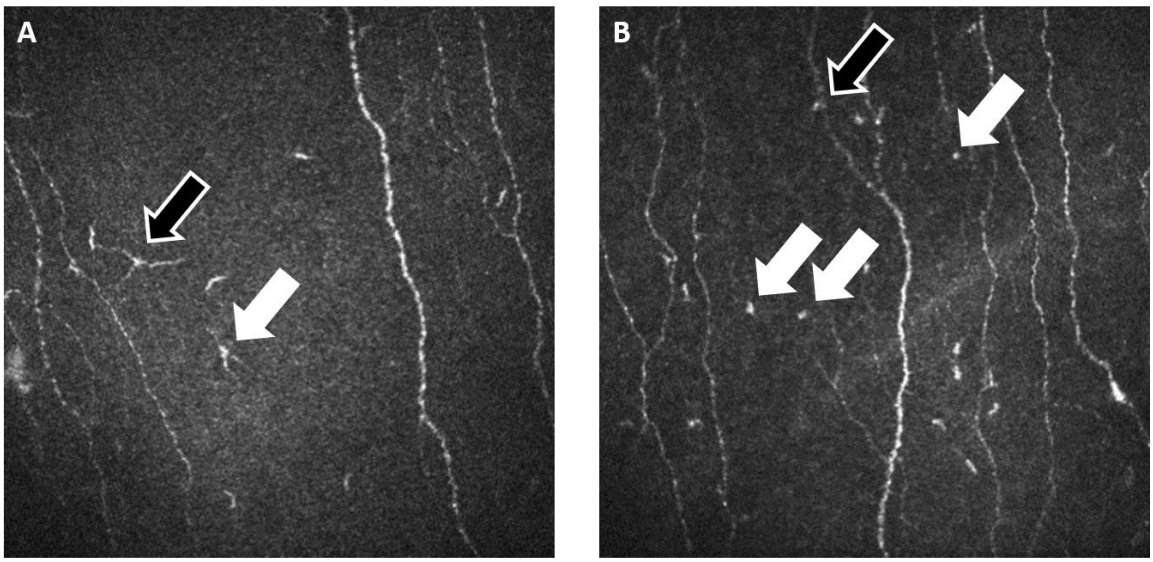

Figure 1: Representative CCM images

Photomicrographs were taken at the level of the sub-basal nerve plexus within Bowman's layer of the cornea.
A: Arrows indicate LC showing dendrites (dLC), black arrow: dLC with nerve fiber contact ($dLC_{fibercontact}$), white arrow: dLC without nerve fiber contact ($dLC_{no\ contact}$), **B:** Arrows indicate LC consisting only of a cell body without dendrites (ndLC). Black arrow: ndLC with nerve fiber contact ($ndLC_{fiber\ contact}$), white arrows: ndLC without nerve fiber contact ($ndLC_{no\ contact}$). Adapted from Klitsch et al., 2020, *J Peripher Nerv Syst*.
Abbreviations: CCM = corneal confocal microscopy, dLC = dendritic Langerhans cells, LC = Langerhans cells, ndLC = non-dendritic Langerhans cells.

2.5 Screening for xerophthalmia

All study participants underwent a screening procedure for dry eye disease (DED) / xerophthalmia. Prior to CCM examination, a Schirmer's test (Haag-Streit UK Ltd, Harlow Essex, UK) without anesthesia was applied to both eyes and participants were interviewed using the German version of the Ocular Surface Disease Index (OSDI) (Schiffman et al., 2000). The Schirmer's test result was considered pathologic when the wetting of the paper strip inserted beneath participants' lower eye lid was ≤ 5 mm/5 min (Bron et al., 2007).

OSDI results were classified as normal (score 0 – 12), mild DED (score 13 – 22), moderate DED (score 23 – 32), and severe DED (score ≥33) (Wolffsohn et al., 2017). As criteria for a positive screening for xerophthalmia we chose an OSDI score ≥13 plus a pathologic Schirmer's test result in at least one eye.

2.6 RNA expression analysis in skin cells

We analyzed the expression of messenger RNA (mRNA) and microRNA (miRNA) in dermal fibroblasts and epidermal keratinocytes of FMS patients to explore their potential role in FMS small fiber pathology. For this purpose, we took skin punch biopsies at two standardized sites in patients and controls and thereafter cultured fibroblasts and keratinocytes as separate cell lines. Cells were then lysated for RNA extraction. Expression analysis consisted of two steps: a screening using next generation sequencing (NGS) technique and a validation using quantitative real-time polymerase chain reaction (qRT-PCR).

2.6.1 Skin punch biopsy

Two 6-mm skin punch biopsies were taken from every patient, one at the lateral ankle and another at the upper thigh of the right leg. The same was done in 28/65 healthy controls, 37/65 healthy controls refused skin punch biopsy. Skin punch biopsy was performed according to a previously published protocol (Üçeyler et al., 2010). Half of the biopsy sample was used for cell culture experiments (Evdokimov et al., 2019b), while the other half was used to perform immune-fluorescence staining to determine intraepidermal nerve fiber density (IENFD).

2.6.2 Cell culture of keratinocytes and fibroblasts

After taking skin punch biopsies, we mechanically separated dermis and epidermis. Fibroblasts and keratinocytes were then incubated at 5% CO_2 and 37°C as monocellular cultures. We applied growth media and trypsinization reagents adhering to a previously published protocol (Evdokimov et al., 2019b) cultivating the cells for two passages. Next, fibroblasts and keratinocytes were lysated for RNA extraction. Dr. Dimitar Evdokimov, Daniela Urlaub, Luisa Kreß, and Tobias Malzacher performed cell culture work in our team at the Department of Neurology, University of Würzburg, Germany.

2.6.3 RNA extraction

We used QIAzol Lysis Reagent (Quiagen, Hilden, Germany) to lysate cultured cells and stored the created lysate at -80°C until further processing. We then used the MiRNeasy Mini Kit (Quiagen, Hilden, Germany) according to manufacturer's instructions to extract total RNA including small RNA from the lysated fibroblasts and keratinocytes. Isolated RNA was examined using a NanoDrop™ One spectrophotometer (Thermo Fisher, Waltham, Massachusetts, USA) to determine RNA concentration and then again stored at -80°C until further processing.

2.6.4 Selection of patients and samples for RNA expression analysis

We examined RNA samples from three different groups of study participants by next generation sequencing (NGS). These were 6 FMS patients with

reduced IENFD at both sites of skin punch biopsy (FMS $IENFD_{reduced}$), 6 FMS patients with normal IENFD at both sites of skin punch biopsy (FMS $IENFD_{normal}$), and 5 healthy controls. The differentiation into FMS $IENFD_{reduced}$ and FMS $IENFD_{normal}$ was made because of previous findings showing a positive correlation between the extent of IENFD reduction and symptom severity in FMS patients (Evdokimov et al., 2019a). We selected participants for each group aiming to create groups with comparable median age, body mass index (BMI), and disease duration. To increase homogeneity, we only included women in all three groups. The criteria for subjects to be included in the FMS $IENFD_{reduced}$ group were:

- IENFD at the upper thigh < 8.0 fibers/mm and
- IENFD at the lower leg < 6.0 fibers/mm.

Criteria for subjects to be included in the FMS $IENFD_{normal}$ group were:

- IENFD at the upper thigh ≥ 8.0 fibers/mm and
- IENFD at the lower leg ≥ 6.0 fibers/mm.

We aimed at including subjects in our FMS $IENFD_{reduced}$ group who additionally showed high current pain intensity (i.e. NRS > 5), high symptom severity as reflected by the questionnaires (ADS, GCPS, FIQ, STAI-T), and signs of corneal small fiber involvement as observed by CCM. For our FMS $IENFD_{normal}$ group, we selected patients with comparably low current pain intensity (i.e. NRS ≤ 5), low symptom severity as reflected by the questionnaires (ADS, GCPS, FIQ, STAI-T), and no signs of corneal small fiber involvement as observed by CCM.

As biomaterial for NGS screening, we selected RNA extracted from keratinocytes from the proximal biopsy site at the upper thigh. Keratinocytes

physiologically are in close contact with epidermal nerve fiber endings and may play a role in small fiber sensitization and small fiber morphology. We previously showed greater disease severity in FMS patients with reduced proximal IENFD (Evdokimov et al., 2019a), which is why we chose to investigate material from the proximal biopsy site.

2.6.5 miRNA and whole exome screening by next generation sequencing (NGS)

2.6.5.1 miRNA library preparation and sequencing

For miRNA screening, we created cDNA libraries according to the NEBNext Multiplex Small RNA Library Preparation Guide for Illumina using the extracted total RNA from all three groups. We diluted adapters and primers 1:4. RNA was then amplified through 13 cycles of PCR. RNA libraries were pooled without previous size selection and sequencing was performed on a NextSeq 500 with a read length of 75 bp. 3'- Adapters were trimmed using Cutadapt (ver. 1.12) (Martin, 2011). Read quality was assessed by calculating Phred score and all reads with a Phred score below Q20 were discarded. All reads shorter than 18 bp were also discarded. We then aimed to filter small non-coding RNAs (rRNAs, tRNAs, snRNAs, and sRNAs) by mapping the reads to Rfam database (Griffiths-Jones et al., 2003). We used the miRDeep2 pipeline (Friedlander et al., 2012) to map remaining reads against the human genome (GRCh38, Release 10) and, in the following step, against miRNA precursor sequences from miRbase release v-21 (Kozomara and Griffiths-Jones, 2014). Resulting miRNA expression counts were then analyzed using DESeq2 1.16.1 (Love et al., 2014) to compare expression levels between our

three groups. Significantly differential expression of genes was assumed at a p-value <0.05 after Benjamini-Hochberg correction.

2.6.5.2 mRNA library preparation and sequencing

For mRNA screening we created cDNA libraries according to the standard Illumina TruSeq protocol using the extracted total RNA from all three groups. Library sequencing was performed on a NextSeq 500 using a High Output 1 x 75 cycles kit to generate reads with a length of 75 bp. 3'- Adapters were trimmed using Cutadapt (ver. 1.12) (Martin, 2011). Quality of raw reads was assessed using FastQC 0.11.3 (Andrews, 2010) and all reads with a Phred score below Q20 were discarded. We used the short read aligner STAR-2.5.2b (Dobin et al., 2013) with genome and annotation files retrieved from GENCODE to map remaining reads against the human genome (GRCh38, Release 10). We also checked the proportion of reads mapping to the mouse reference genome. In our samples it ranged between 86% and 89%. Quantification of sequences aligning to specific human genes was calculated using BED Tools' sub-command intersect (ver. 2.15.0) (Quinlan and Hall, 2010). Resulting mRNA expression counts were then analyzed using DESeq2 1.16.1 (Love et al., 2014) to compare expression levels between our three groups. Significantly differential expression of genes was assumed at a p-value <0.05 after Benjamini-Hochberg correction.

NGS and associated data processing and analysis for miRNA and mRNA screening was performed by Dr. Richa Bharti, and Dr. Konrad U. Förstner at the Core Unit Systemmedizin / Institute for Molecular Infection Biology, University of Würzburg, Germany.

2.6.6 Selection of target genes for further analysis

We used quantitative real-time PCR (qRT-PCR) to validate the screening results retrieved from NGS screening. All genes showing differential expression between FMS patients and controls (i.e. $p<0.05$ after Benjamini-Hochberg correction for multiple testing) were analyzed in samples from 27 FMS patients and 9 healthy controls which included the 12 patients and 5 controls examined in our NGS screening.

Additionally, we used qRT-PCR to analyze the expression of miR let-7d in 73 FMS patients compared to 13 healthy controls. This target was chosen based on previous findings in our group (Leinders et al., 2016) and not on NGS screening results. We did not restrict our miR let-7d analysis on RNA from keratinocytes from the upper thigh but used and separately measured biomaterial from keratinocytes and fibroblasts from both biopsy sites.

2.6.7 Quantitative real-time PCR (qRT-PCR) RNA expression analysis

We applied two differing protocols to quantify miRNA and mRNA expression via qRT-PCR.

To determine miRNA expression levels, samples of total isolated RNA were diluted to a concentration of 5 ng/µl using RNase-Free water. cDNA synthesis was then performed using 2 µl of diluted RNA and the Universal cDNA synthesis kit II (Exiqon, Vedbaek, Denmark). Sample preparation and reverse-transcriptase PCR were performed according to manufacturer's instructions. For expression quantification via real-time PCR the ExiLENT SYBR® Green Master Mix Kit (Exiqon, Vedbaek, Denmark) was used. Specimens were

diluted with nuclease-free water 1:80 and mixed with kit reagents and primers according to manufacturer's instructions. For each sample we prepared a triplet of qRT-PCR wells. To determine a suitable endogenous control primer for miRNA qRT-PCR we tested four commercially available small RNA household gene primers (5S, U6, SNORD44, SNORD48), of which SNORD44 (5' -3' CCTGGATGATGATAAGCAAATGCTGACTGAACATGAAGGTCTTAATTAGC TCTAACTGACT) showed the most stable qRT-PCR results. It was therefore chosen as endogenous control primer for our miRNA experiments.

To determine mRNA expression levels, a defined amount of 250 ng of total isolated RNA from each sample was used for reverse-transcriptase PCR to create cDNA. For this we used TaqMan Reverse Transcription Reagents® (Life Technologies, Carlsbad, CA, USA). PCR cycler conditions were:

1) 10 minutes at 25°C: annealing
2) 60 minutes at 48°C: reverse transcription
3) 5 minutes at 95°C: enzyme inactivation

For expression quantification via real-time PCR we used the TaqMan Universal Master Mix (Life Technologies, Carlsbad, CA, USA). Triplets of cDNA from each sample were prepared for qRT-PCR (5 µl cDNA, 1.75 µl aqua dest., 2 µl Master Mix, 0.25 µl primer). As a suitable endogenous control primer for mRNA qRT-PCR we chose 18S RNA (Hs99999901_s1).

qRT-PCR amplification for both protocols was performed on a StepOnePlus-Cycler (Applied Biosystems, Darmstadt, Germany). Expression levels of examined miRNA and mRNA were then calculated using the $2^{(-\Delta\Delta CT)}$ method (Livak and Schmittgen, 2001).

2.7 Statistical analysis

CCM and qRT-PCR data were analyzed using SPSS 25 (IBM, Ehningen, Germany). We applied the non-parametric Kruskal Wallis test with post-hoc testing by the non-parametric Man-Whitney U test for intergroup comparison, because our data did not show a normal distribution. Categorical data were analyzed using the $\chi 2$ test. For our correlation analysis the bivariate Spearman correlation coefficient was used, and we further applied linear univariate regression models to our data. P-values of correlation analysis and linear regression models were adjusted using the Bonferroni-Holm method. We analyzed NGS results as described in section “miRNA and whole exome screening by next generation sequencing (NGS)”. P-values < 0.05 were considered statistically significant.

3 Results

3.1 Characterization of the study population

We recruited 143 FMS patients, 41 SFN patients, and 65 healthy controls. Table 1 (see appendix) gives an overview on demographic and baseline laboratory data of this complete cohort. For the included SFN patients we also assessed which etiology may be causative of their disease: In 17 (42%) patients SFN etiology remained idiopathic. In nine cases (22%) diabetes mellitus or impaired glucose tolerance may have been causative, in six cases (15%) each, thyroid dysfunction and autoimmune factors, and in three cases (7%) parainfectious factors were likely contributors to SFN. In eight cases (20%) a genetic mutation associated to SFN was known.

As male FMS patients were highly underrepresented in our study population (9 male versus 134 female), we chose to include only female participants into our CCM analysis. Baseline data for FMS patients and controls, therefore, differ from the complete cohort: For FMS patients (n=134) median age was 51 (21 – 74) years, median BMI was 24 (16 – 42) kg/m², and median disease duration was 12 (0.75 – 56) years. For controls (n=60) median age was 50 (22 – 64) years and median BMI was 24 (17 – 42) kg/m². All 41 SFN patients included in our study were female.

Subjects included in our NGS screening experiments were carefully selected as described above (see section “Selection of patients and samples for RNA expression analysis”). Table 2 (see appendix) gives an overview on the demographic and baseline laboratory parameters of these 17 FMS patients and controls.

Similar data are shown for all subjects included in qRT-PCR experiments for NGS screening validation in Table 3 (see appendix) and for miR let-7d analysis in Table 4 (see appendix).

3.2 Reduction of IENFD in a subgroup of FMS patients

We obtained biomaterial of skin punch biopsies from the upper thigh and the lower calf from 142/143 FMS patients, in one FMS patient only from the upper thigh, and in 28/65 (43%) healthy controls from both biopsy sites. 37/65 (57%) of healthy controls refused skin punch biopsy. IENFD was determined in every biopsy taken. Table 5 gives an overview of the amount of successful cell cultures for keratinocytes and fibroblasts (i.e. monocellular cultures with sufficient numbers of cells for RNA extraction).

Table 5: Amount of successful cell cultures per biopsy site and cell type:

	FMS patients		Healthy controls	
	keratinocytes	fibroblasts	keratinocytes	fibroblasts
upper thigh (proximal)	73/143 (51%)	140/143 (98%)	16/28 (57%)	26/28 (93%)
lower calf (distal)	68/142 (48%)	141/142 (99%)	12/28 (43%)	25/28 (89%)

Cell culture was considered successful when it was monocellular, had no visible contamination with other cell types, and the number of cells was sufficient for RNA isolation for further experiments.

Abbreviations: FMS = fibromyalgia syndrome

Of the FMS patients, 45/143 (31%) had normal IENFD at both biopsy sites (i.e. proximal IENFD ≥ 8 fibers/mm and distal IENFD ≥ 6 fibers/mm, “FMS

IENFD$_{normal}$"), 67/143 (47%) had reduced IENFD at the upper thigh, 66/142 (46%) had reduced IENFD at the lower leg, and 36/143 (25%) showed a reduction of IENFD at both biopsy sites ("FMS IENFD$_{reduced}$"). In contrast, healthy controls had normal IENFD at both biopsy sites in 17/28 (61%) cases, 5/28 (18%) had reduced IENFD at the proximal biopsy site, 10/28 (36%) had reduced IENFD at the distal biopsy site, and in 4/28 (14%) cases IENFD was reduced at both biopsy sites.

3.3 Results of eye and corneal assessment in FMS, SFN, and healthy controls

3.3.1 FMS and SFN patients show lower density of LC contacting corneal nerve fibers

We did not find an intergroup difference for LC_{total} density between FMS patients *(median 19.8 cells/mm² [0 – 255.2])*, SFN patients *(13.5 cells/mm² [0 – 143.8])*, and healthy controls *(22.9 cells/mm² [0 – 152.1]; Figure 2A)*. $LC_{fiber\ contact}$ was not different between FMS patients *(5.2 cells/mm² [0 – 53.1])* and controls *(7.3 cells/mm² [0 – 61.5])* but was lower in SFN patients *(3.1 cells/mm² [0 – 37.5])* than in controls *($p<0.01$; Figure 2B)*. No intergroup difference was found in $LC_{no\ contact}$ *(FMS: 12.5 cells/mm² [0 – 214.6], SFN: 10.4 cells/mm² [0 – 106.3], controls: 15.6 cells/mm² [0 – 100.0]; Figure 2C)*. Analyzing $dLC_{total,}$ we did not find an intergroup difference between our three study groups *(FMS: 11.5 cells/mm² [0 – 160.4], SFN: 7.3 cells/mm² [0 – 99.0], controls: 14.1 cells/mm² [0 – 147.9]; Figure 2D)*. However, FMS patients and SFN patients both had lower $dLC_{fiber\ contact}$ values than controls *(FMS: 2.1 cells/mm² [0 – 45.8], SFN: 1.6 cells/mm² [0 – 32.3], controls: 4.2 cells/mm² [0*

– 58.3], FMS versus controls: p<0.05, SFN versus controls: p<0.01; Figure 2E). There was no intergroup difference for $dLC_{no\ contact}$ *(FMS: 8.3 cells/mm² [0 – 129.2], SFN: 5.2 cells/mm² [0 – 66.7], controls: 9.9 cells/mm² [0 – 97.9])*. Non-dendritic LC densities did not differentiate between FMS patients, SFN patients, and healthy controls:

$ndLC_{total}$ *(FMS: 5.2 cells/mm² [0 – 105.2], SFN: 7.3 cells/mm² [0 – 67.7], controls: 5.7 cells/mm² [0 - 20.8])*,

$ndLC_{fiber\ contact}$ *(FMS: 1.0 cells/mm² [0 – 37.5], SFN: 1.0 cells/mm² [0 – 8.3], controls: 1.0 cells/mm² [0 – 9.4])*,

$ndLC_{no\ contact}$ *(FMS: 4.2 cells/mm² [0 – 85.4], SFN: 4.7 cells/mm² [0 – 65.6], controls: 3.6 cells/mm² [0 – 17.7])*.

The $LC_{fiber\ contact}$/CNFL ratio in SFN patients was lower than in controls ($p<0.01$), while in FMS patients it was as high as in controls (data not shown). The $dLC_{fiber\ contact}$/CNFL ratio in both, FMS and SFN patients, was lower than in healthy controls ($p<0.05$ each, Figure 2F). There was no difference in the $ndLC_{fiber\ contact}$/CNFL ratio between the three study groups (data not shown).

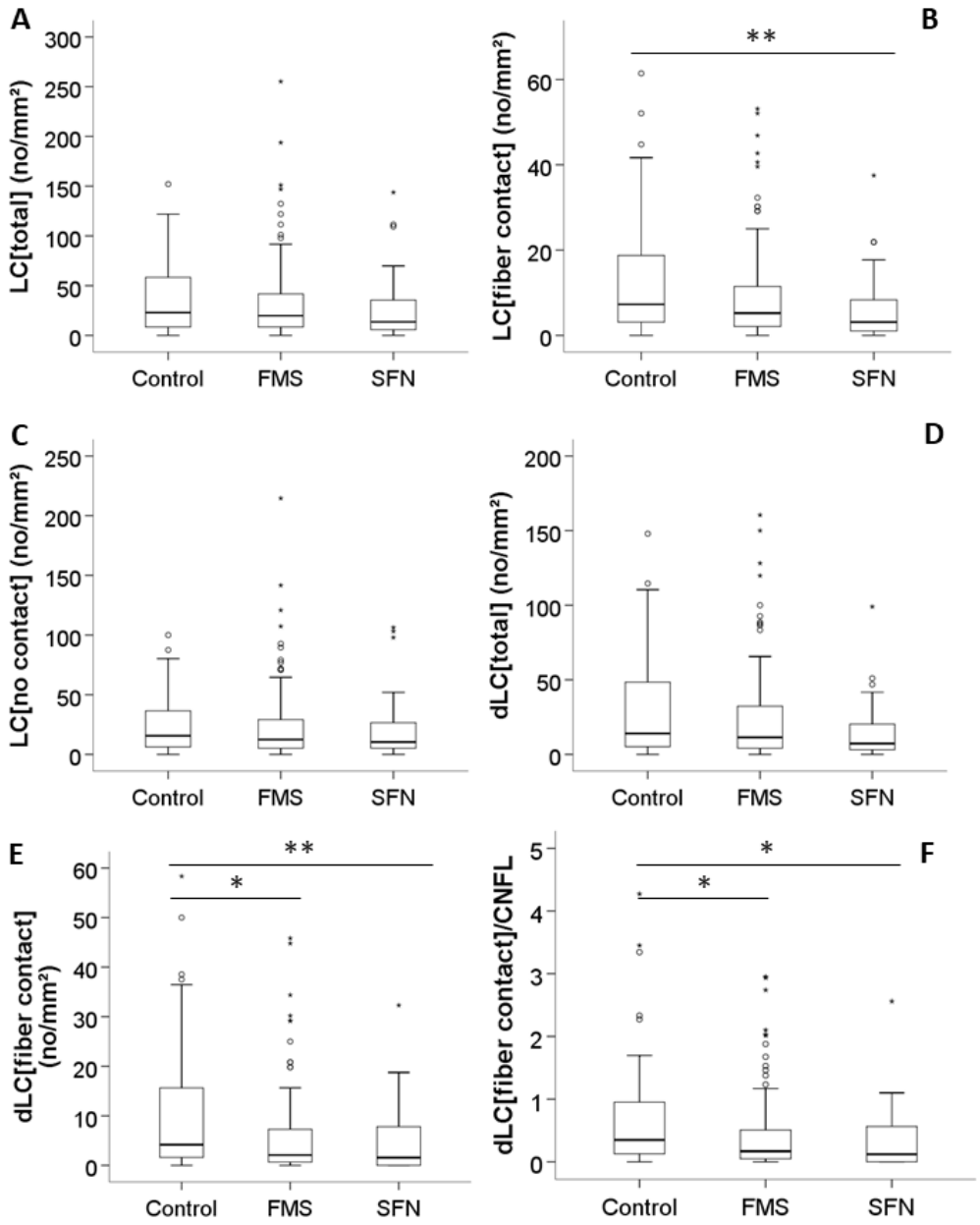

Figure 2: Comparison of LC between FMS patients, SFN patients, and healthy controls.

A: No intergroup difference in LC_{total} density.

B: Lower $LC_{fiber\ contact}$ density in SFN patients than in controls ($p<0.01$), but no difference between FMS patients and controls.

C: No intergroup difference in $LC_{no\ contact}$ density.

D: No intergroup difference in dLC_{total} density.

E: Lower $dLC_{fiber\ contact}$ density in FMS patients ($p<0.05$) and in SFN patients ($p<0.01$) compared to controls.

F: Lower $dLC_{fiber\ contact}$/CNFL ratio in FMS and SFN patients than in controls ($p<0.05$ each). Adapted from Klitsch et al., 2020, *J Peripher Nerv Syst*. Abbreviations: dLC_{total} = all dendritic cells, $dLC_{fiber\ contact}$ = dendritic cells with nerve fiber contact, $dLC_{no\ contact}$ = dendritic cells without nerve fiber contact, FMS = fibromyalgia syndrome, LC = Langerhans cells, LC_{total} = total cell number, $LC_{fiber\ contact}$ = total number of cells with nerve fiber contact, $LC_{no\ contact}$ = total number of cells without fiber contact, SFN = small fiber neuropathy. *p<0.05, **p<0.01.

3.3.2 Comparison of changes in corneal innervation between FMS and SFN patients and healthy controls

Reduced corneal sensitivity, i.e. Cochet-Bonnet esthesiometry result <5cm, was found in 17/132 (13%) FMS patients, 4/40 (10%) SFN patients, and 6/59 (10%) controls, revealing no difference between the groups (χ^2 *[2] = 0.42, p>0.05*)

Both patient groups showed lower CNFL than healthy controls ($p<0.05$ each, Figure 3A). Only FMS patients also showed lower CNFD than controls ($p<0.05$), there was no difference in CNFD between SFN patients and controls or between SFN patients and FMS patients (Figure 3B). CNBD did not differ between FMS patients and controls, but lower CNBD distinguished SFN patients from controls and from FMS patients ($p<0.001$ each, Figure 3C). We did not detect any intergroup differences in CNFW and CNFracDim (Figure 3D, E).

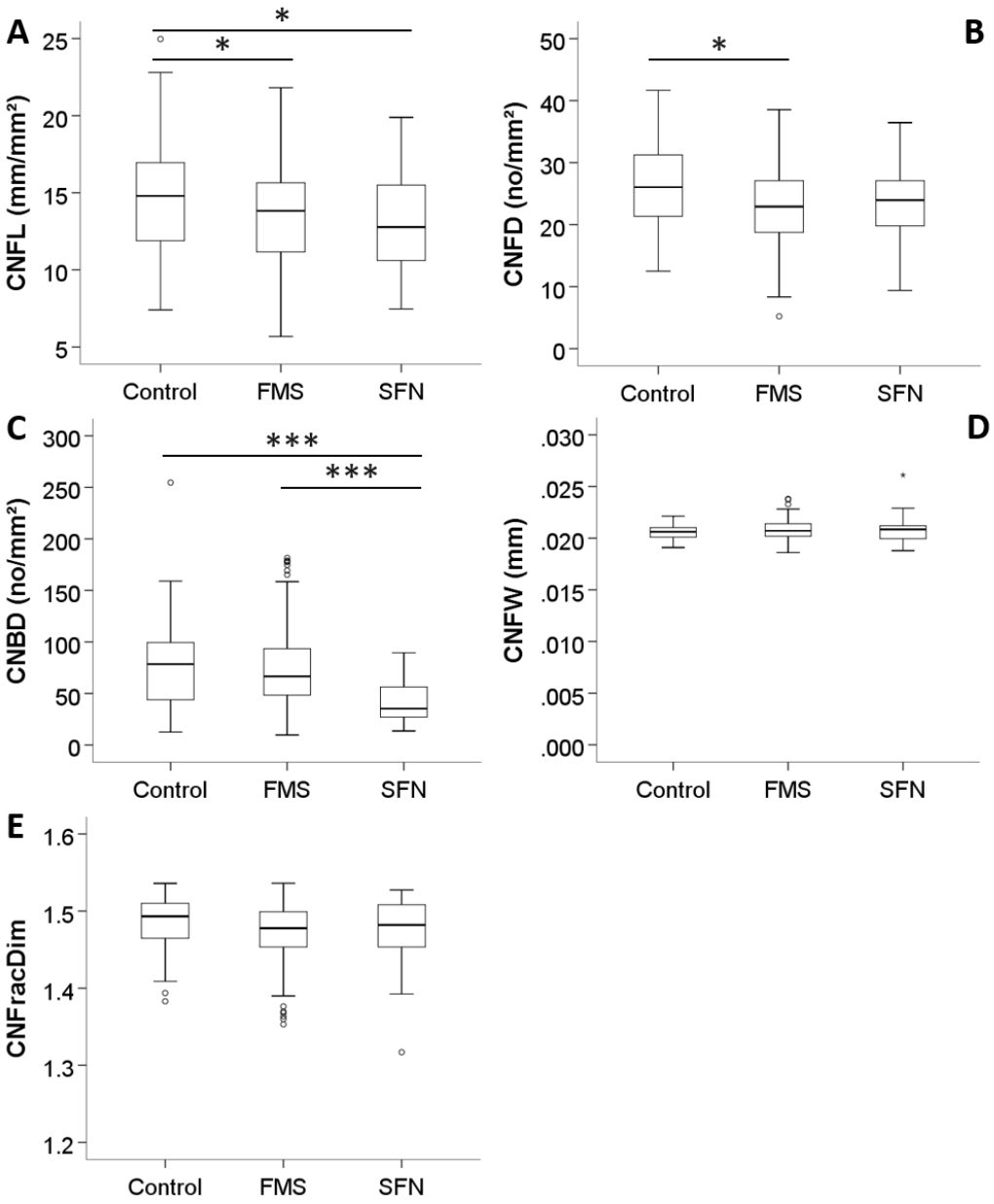

Figure 3: Comparison of corneal nerve fiber measurements between FMS patients, SFN patients, and healthy controls.

A: Lower CNFL in FMS and SFN patients than in controls ($p<0.05$ each).

B: Lower CNFD in FMS patients than in controls ($p<0.05$).

C: Lower CNBD in SFN patients than in controls and FMS patients ($p<0.001$ each).

D: No intergroup difference in CNFW.

E: No intergroup difference in CNFracDim. Adapted from Klitsch et al., 2020, *J Peripher Nerv Syst*.

Abbreviations: CNBD = corneal nerve branch density, CNFD = corneal nerve fiber density, CNFL = corneal nerve fiber length, CNFracDim = corneal nerve fractal dimension, CNFW = corneal nerve fiber width, FMS = fibromyalgia syndrome, SFN = small fiber neuropathy. *p<0.05, ***p<0.001.

3.3.3 Low LC density does not predict low nerve fiber density

Our correlation analysis with consecutive application of linear regression models did not reveal any correlation between LC_{total} or $dLC_{fiber\ contact}$ and corneal nerve fiber parameters (CNFD, CNFL, CNBD) in FMS patients, SFN patients, and controls. Neither were LC_{total} or $dLC_{fiber\ contact}$ predictive of nerve fiber parameters in any of the three groups. Age and disease duration (defined as reported duration of pain due to disease) did not correlate with the density of corneal LC dLC and ndLC or with corneal nerve fiber parameters (CNFD, CNFL, CNBD, CNFW, CFracDim) in any of the three groups. Regression analysis, however, revealed linear models predicting LC_{total} *($F[1,38]=8.605$, $p<0.05$, $R^2=0.185$)*, $LC_{fiber\ contact}$ *($F[1,38]=11.287$, $p<0.05$, $R^2=0.229$)*, dLC_{total} *($F[1,38]=11.315$, $p<0.05$, $R^2=0.229$)*, $dLC_{fiber\ contact}$ *($F[1,38]=10.798$, $p<0.05$, $R^2=0.221$)*, and $dLC_{no\ contact}$ *($F[1,38]=9.138$, $p<0.05$, $R^2=0.194$)* by age in SFN patients. We found no such models in FMS patients and healthy controls. Neither did we find disease duration to be predictive of any LC or nerve fiber parameter in any of the three groups.

3.3.4 Prevalence of xerophthalmia in FMS and SFN patients is higher than in controls, but does not influence LC density or nerve fiber density

Schirmer's test was positive (i.e. <5 mm/5 minutes) in at least one eye in 33/134 (25%) FMS patients, in 17/40 (43%) SFN patients, and in 12/60 (20%) healthy controls. A positive Schirmer's test in both eyes was found in 20/134

(15%) FMS patients, in 9/40 (23%) SFN patients, and in 2/60 (3%) healthy controls. A comprehensive overview of OSDI scores of FMS patients, SFN patients, and healthy controls is depicted in Figure 4. The proportion of patients with a positive screening result for xerophthalmia, i.e. the combination of a pathological Schirmer's test and a pathological OSDI score, was highest in SFN patients (11/40, 28%), followed by FMS patients (22/131, 17%), and lowest in healthy controls (3/59, 5%). χ^2-Test revealed a higher xerophthalmia frequency in FMS patients (χ^2 *[1] =4.881, p<0.05*) and SFN patients compared to controls (χ^2 *[1] =9.865, p<0.01*), but no difference between FMS and SFN patients (χ^2 *[1] =2.255, p>0.05*).

To control whether this difference in xerophthalmia frequency had an influence on our above described findings of CCM examination, we compared participants with and without a positive screening within each group (FMS, SFN, controls). We did not find any difference in CNFL, CNFD, CNBD, CNFW, CFracDim, dLC_{total}, $dLC_{fiber\ contact}$, or $LC_{fiber\ contact}$ between participants with and without dry eyes (Figure 5, see appendix).

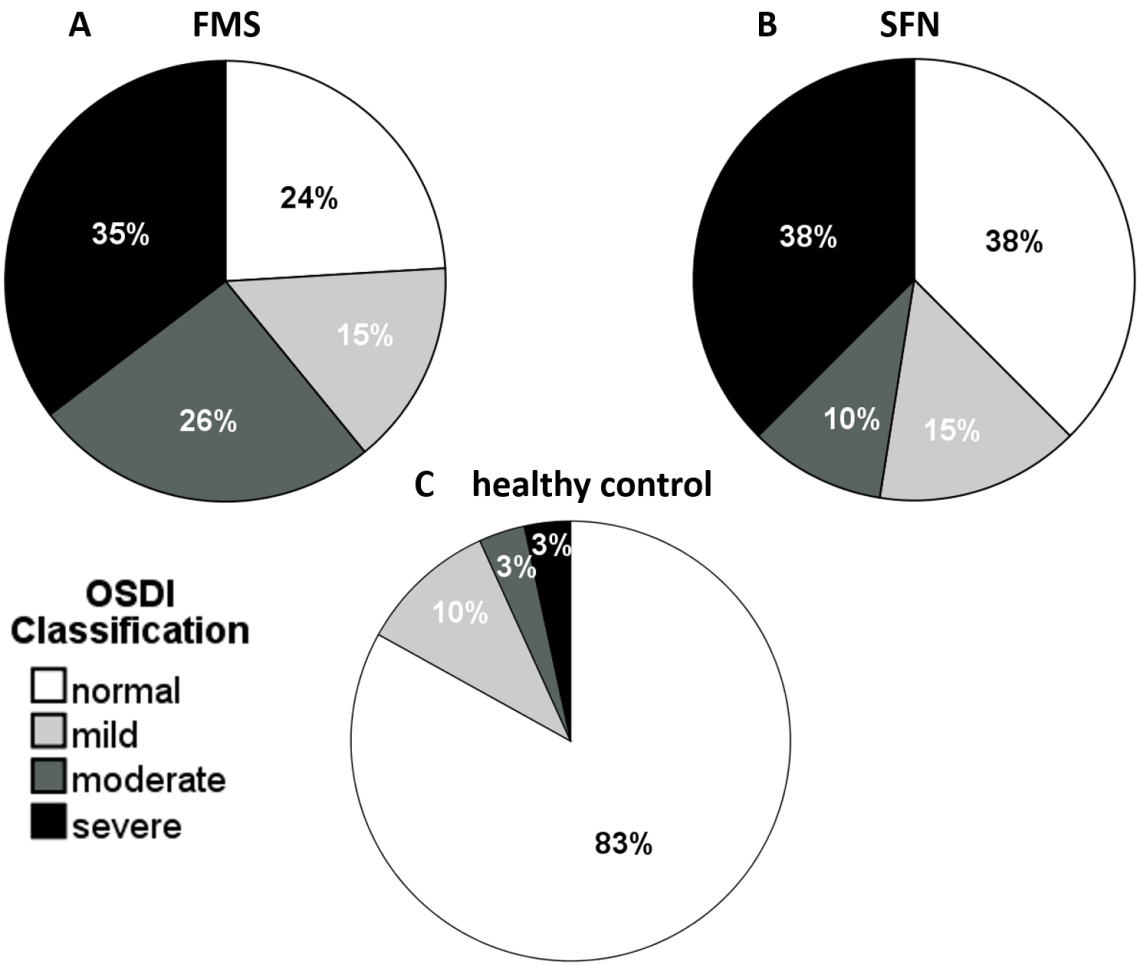

Figure 4: DED severity in FMS patients, SFN patients, and healthy controls as assessed by the OSDI score. Adapted from Klitsch et al., 2020, *J Peripher Nerv Syst*.

Abbreviations: DED = dry eye disease, FMS = fibromyalgia syndrome, OSDI = ocular surface disease index, SFN = small fiber neuropathy

3.4 RNA expression analysis in skin cells

3.4.1 NGS screening reveals differential expression of two mRNA, but no miRNA in FMS patients' keratinocytes

Figure 5 shows the MA-plots generated during NGS experiments. Our NGS screening experiments detected differential expression of two mRNA (*PRSS21 [serine protease 21]: Log2FoldChange=2.527, adjusted p-value=0.0005, CD86 [cluster of differentiation 86]: Log2FoldChange=-2.265,*

adjusted p-value=0.0035) between FMS patients (n=12) and healthy controls (n=5). After post-hoc testing PRSS21 revealed to be expressed at a higher level in FMS $IENFD_{normal}$ (n=6) compared to controls (n=5, $p<0.001$ after correction, Figure 6A) but not in FMS $IENFD_{reduced}$ (n=6) compared to controls (n=5) ($p>0.05$ after correction, Figure 6B). CD86 revealed to be expressed at a lower level in FMS $IENFD_{normal}$ (n=6) compared to controls (n=5) ($p<0.01$ after correction, Figure 6A), but not in FMS $IENFD_{reduced}$ (n=6) compared to controls (n=5) ($p>0.05$ after correction, Figure 6B). Post-hoc testing showed no differential expression of any mRNA between FMS $IENFD_{normal}$ (n=6) and FMS $IENFD_{reduced}$ (n=6) ($p>0.05$ after correction, Figure 6C).

We found no difference in expression of any miRNA between FMS patients (n=12) and healthy controls (n=5) ($p>0.05$ after correction, Figure 6D).

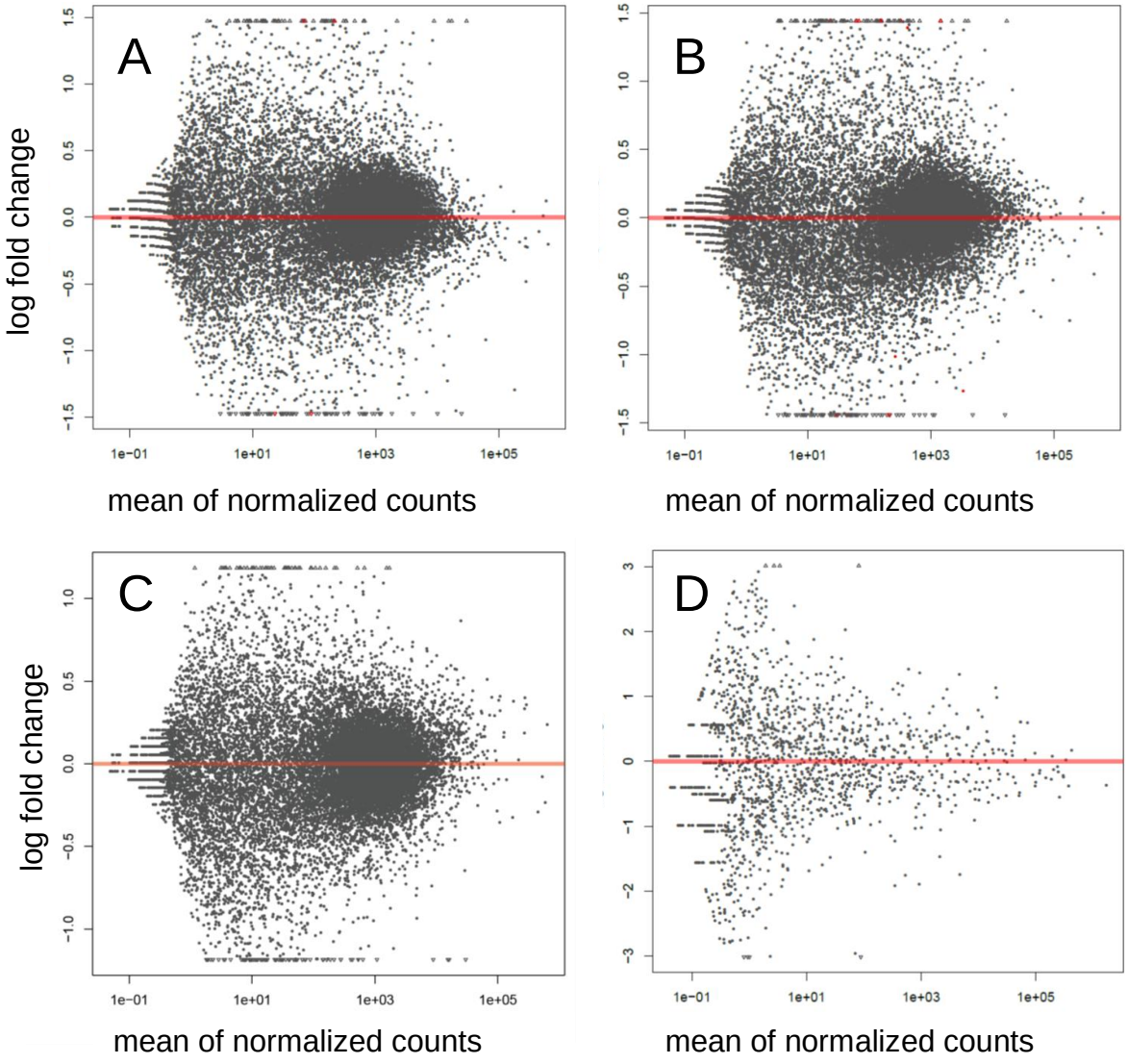

Figure 6: MA-plots generated by our NGS screening experiments.
Every dot represents one specific gene. The x-axis ("M") shows the mean expression level across all samples included in depicted comparison, while the y-axis ("A") shows the mean difference in expression between two compared groups. Examined biomaterial was mRNA/miRNA extracted from keratinocytes out of skin punch biopsies of the upper thigh after two passages of cell culture. A: mRNA, FMS $IENFD_{normal}$ versus controls. B: mRNA, FMS $IENFD_{reduced}$ versus controls. C: mRNA, FMS $IENFD_{normal}$ versus FMS $IENFD_{reduced}$. D: miRNA, FMS patients versus controls.
Abbreviations: FMS = fibromyalgia syndrome, IENFD = intraepidermal nerve fiber density, miRNA = micro-ribonucleic acid, mRNA = messenger-ribonucleic acid, NGS = next generation sequencing.

3.4.2 Validation of screening results via qRT-PCR

A larger cohort of patients (FMS $IENFD_{normal}$: n=17, FMS $IENFD_{reduced}$: n=10) and healthy controls (n=9) was tested to validate NGS results on PRSS21 and CD86 expression using qRT-PCR. In this cohort expression of PRSS21 was higher in FMS patients than in controls ($p<0.001$; Figure 7A) and both patient subgroups, FMS $IENFD_{normal}$ ($p<0.001$) and in FMS $IENFD_{reduced}$ ($p<0.05$), showed higher PRSS21 expression in post-hoc testing than controls (Figure 7B). There was no difference in CD86 expression found when comparing all FMS patients and controls of this cohort, neither was there an expression difference detectable in one of the two patient subgroups ($p>0.05$ each, Figures 7C, D).

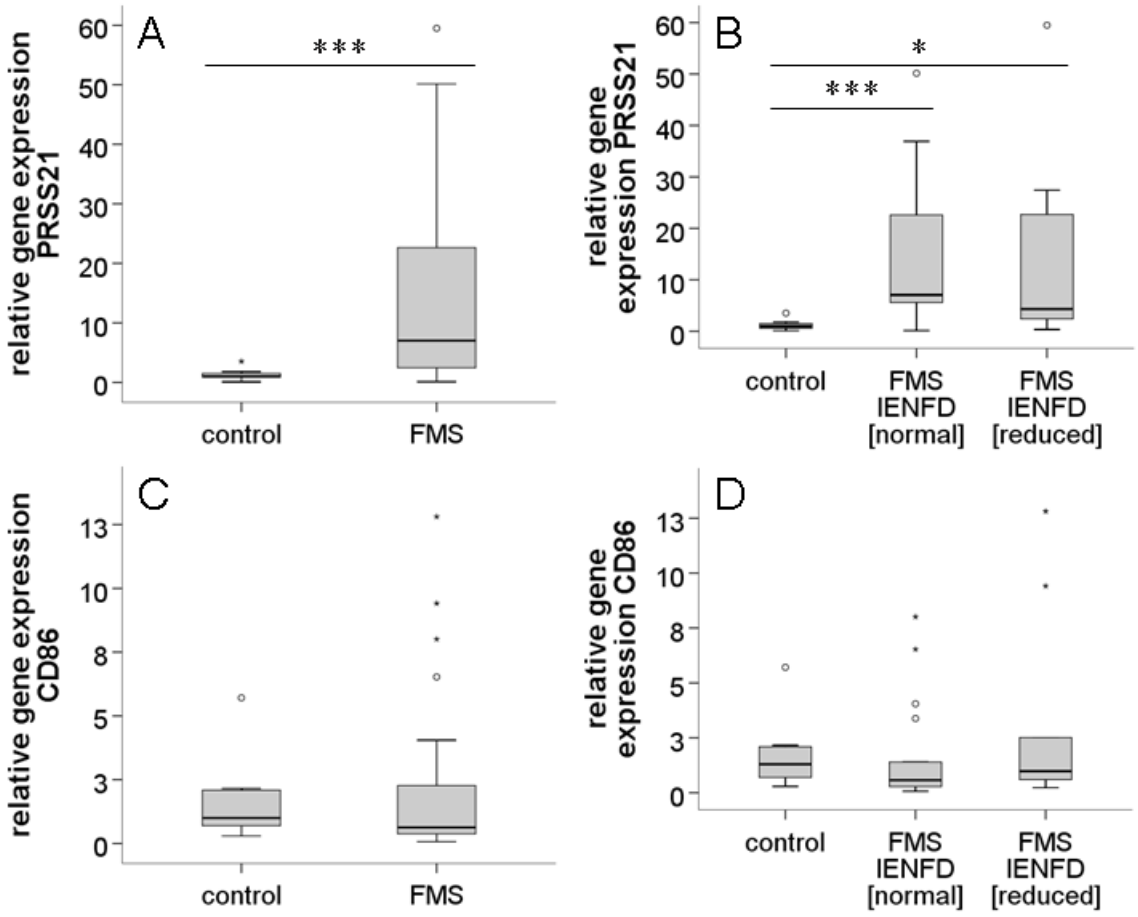

Figure 7: NGS screening validation by qRT-PCR.

A: PRSS21 expression was higher in FMS patients (n=27) than in controls (n=9). B: In both FMS patient subgroups PRSS21 expression was higher than in controls (FMS $IENFD_{normal}$: n=17, $p<0.001$; FMS $IENFD_{reduced}$: n=10, $p<0.05$). C & D: No differential expression of CD86 between FMS patients (n=27) and controls (n=9) was detectable by qRT-PCR.

Abbreviations: CD86 = cluster of differentiation 86, FMS = Fibromyalgia Syndrome, IENFD = intraepidermal nerve fiber density, NGS = next generation sequencing, PRSS21 = serine protease 21, qRT-PCR = quantitative real-time polymerase chain reaction.

3.4.3 miR let-7d is expressed equally in cultured skin cells of FMS patients and healthy controls

We compared expression levels of miR let-7d in fibroblasts (Figures 8A, B) and keratinocytes (Figures 8C, D) each taken from the upper thigh (Figures 8A, C) and the ankle (Figures 8B, D) between FMS patients and healthy controls. In none of the four comparisons did we find a differential miR let-7d expression ($p>0.05$ each).

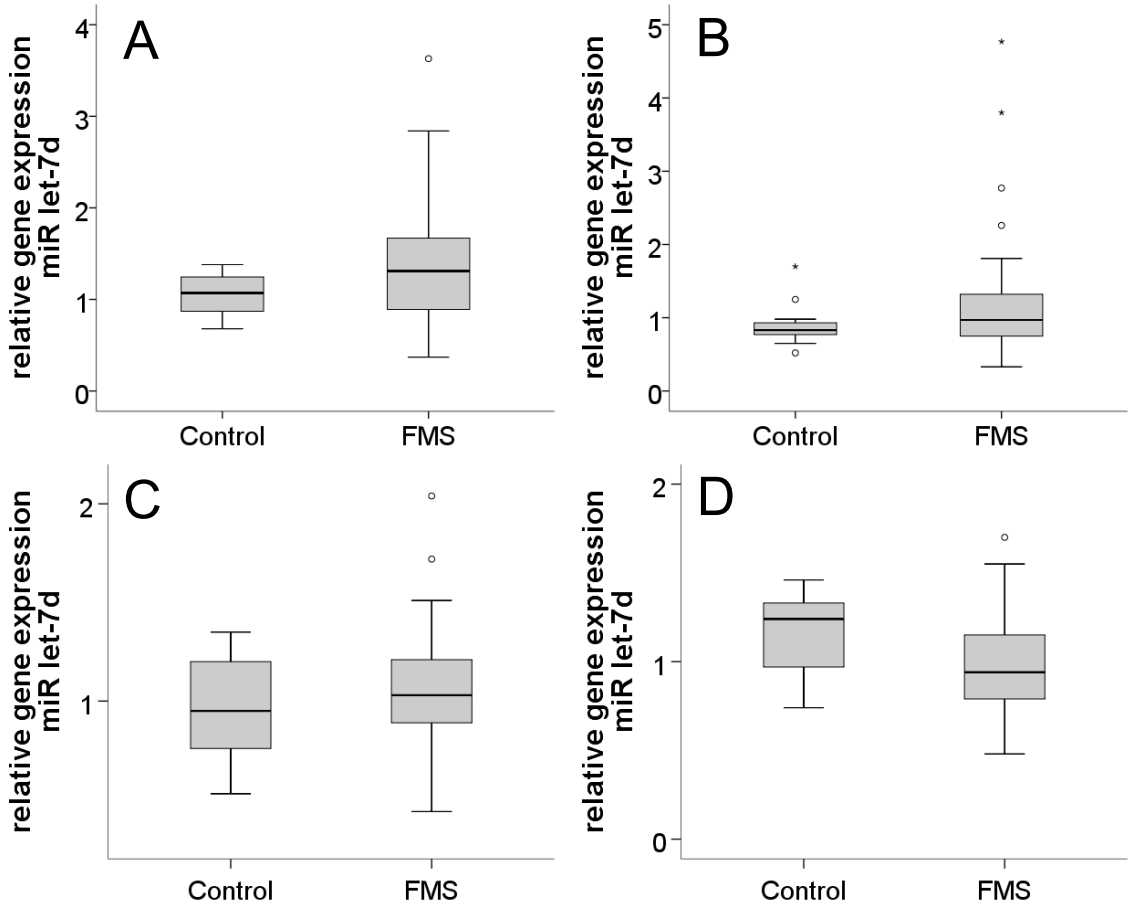

Figure 8: miR let-7d expression analysis by qRT-PCR
A: No difference between FMS patients (n=62) and controls (n=12) in fibroblasts from the upper leg. B: No difference between FMS patients (n=73) and controls (n=11) in fibroblasts from the lower leg. C: No difference between FMS patients (n=37) and controls (n=10) in keratinocytes from the upper leg.

D: No difference between FMS patients (n=46) and controls (n=9) in keratinocytes from the lower leg.

Abbreviations: FMS = Fibromyalgia Syndrome, qRT-PCR = quantitative real-time polymerase chain reaction.

4 Discussion

4.1 Main results

We examined corneal LC in association with sub-basal nerve fiber bundles and RNA expression levels in skin cells in a large cohort of FMS patients and found a lower density of corneal $dLC_{\text{fiber contact}}$ and higher expression of PRSS21 in keratinocytes of FMS patients than in healthy controls.

Our hypothesis on corneal LC density was that a higher LC density is found in FMS patients than in our control groups and that LC density correlates with the degree of SFP. We did not find a higher density of LC in FMS patients compared to healthy controls, neither did we find a correlation between LC density and signs of SFP.

Our hypotheses on RNA expression in FMS skin cells was that differential expression of a specific set of RNA distinguishes FMS patients from controls and FMS patients with high SFP from those with low SFP. We did not find any miRNA that were differentially expressed between FMS patients and healthy controls, but we found one mRNA with differential expression between FMS patients and healthy controls (PRSS21).

Additionally, we confirmed previous findings of reduced IENFD and CNFL in FMS and SFN patients, but detected a new difference in corneal innervation between both patient groups: CNBD was normal in FMS patients but reduced in SFN patients. We also reproduced the finding of increased rates of dry eye disease (DED) in FMS patients and found a similar frequency in SFN patients. We could not reproduce the previous finding of increased miR let-7d expression in FMS skin.

4.2 Corneal LC and SFP

We assessed morphological signs of SFP in FMS patients as others have done before. The new aspects of our study were the determination of corneal LC density and the comparison of FMS patients to patients with isolated SFN. Previous studies on small fiber morphology reported lower IENFD, CNFD, CNFL, CNBD, and thinner dermal and corneal stromal nerve fibers in subgroups of FMS patients than in healthy controls (Caro and Winter, 2014; Doppler et al., 2015; Erkan Turan et al., 2018; Evdokimov et al., 2019a; Giannoccaro et al., 2014; Harte et al., 2017; Oudejans et al., 2016; Ramirez et al., 2015; Üçeyler et al., 2013). We confirmed these findings on IENFD, CNFD, and CNFL but not on CNBD level. We did not assess dermal or corneal stromal nerve fiber thickness. One reason for our result on CNBD in FMS patients differing from previous findings may be that we exclusively used semi-automated nerve branch counting by CCMetrics® on patients and controls while Oudejans et al. (2016) used fully-automated ACCMetrics on FMS patients and compared the results to published reference values assessed using CCMetrics® (Tavakoli et al., 2015). We did not analyze corneal stromal nerve thickness but instead determined the width of nerve fiber bundles in the sub-basal nerve plexus (CNFW) which did not differ between FMS patients and controls.

We found no increase in corneal LC density as originally hypothesized. Instead a difference between FMS patients and healthy controls was only visible in terms of lower $dLC_{fiber\ contact}$ density in FMS patients. To further investigate this finding, we calculated the ratio of $dLC_{fiber\ contact}$/CNFL, which was reduced in FMS patients, too. We conclude from this that lower dLC_{fiber}

$_{contact}$ density in FMS patients represents an actual loss of this sub-population of LC instead of merely a reduced chance for dLC to contact nerve fibers because of low CNFL. Our findings on corneal LC in FMS patients can be interpreted as a sign of distorted LC-nerve communication, possibly in terms of a lack of neurotrophic signaling by LC. It has been shown that corneal LC play a key role in maintaining corneal and epidermal innervation and influence the local levels of neurotrophic factors (Choi et al., 2017; Doss and Smith, 2014). In various other diseases, all with a known autoimmune component, an increase in corneal LC density was found and interpreted as a sign of autoimmune activity damaging small nerve fibers, e.g. diabetic neuropathy (Leppin et al., 2014), CIDP (Stettner et al., 2016), multiple sclerosis (Bitirgen et al., 2017), and immune mediated DED (Kheirkhah et al., 2015b). Our findings on corneal LC in FMS patients are an argument against the hypothesis of autoimmune damage to small nerve fibers in FMS.

Previous CCM studies in SFN patients found lower CNFD, CNFL, and CNBD than in healthy controls, but no difference in LC density (Brines et al., 2018; Bucher et al., 2015). In our study, we confirmed these findings except for CNFD, which did not differ between SFN patients and healthy controls in our cohort. Compared to FMS patients, SFN patients showed many similar changes: CNFL, $dLC_{fiber\ contact}$ density, and $dLC_{fiber\ contact}$/CNFL ratio did not differ between FMS and SFN patients and were lower than in healthy controls. In CNFD no statistical difference to FMS patients or controls was found. $LC_{fiber\ contact}$ density in SFN patients was lower than in healthy controls but not than in FMS patients. The most notable difference between FMS and SFN was lower CNBD in SFN patients than in FMS patients and healthy controls. We

conclude from these findings that corneal SFP in FMS is morphologically different from isolated SFN. While in FMS less main nerve fiber bundles can be found in the corneal sub-basal nerve plexus (CNFD) which show a normal branching (CNBD), in SFN we see a normal CNFD with reduced branching from main nerve fiber bundles. This may indicate nerve fiber loss at a more proximal site in FMS patients than in SFN patients. A reduction of LC- and dLC-nerve fiber contact may be involved in the loss of small fibers in SFN, as described for FMS patients above.

4.3 DED in FMS and SFN patients

Higher prevalence of DED in FMS patients compared to healthy controls was reported in multiple studies (Aykut et al., 2018; Chen et al., 2016; Erkan Turan et al., 2018; Gallar et al., 2009; Turkyilmaz et al., 2013) and DED is associated with changes in corneal innervation and LC density on its own (Choi et al., 2017; Kheirkhah et al., 2015b; Machetta et al., 2014). We, therefore, applied a DED screening consisting of the OSDI questionnaire and a Schirmer's test without anesthesia to all study participants. DED frequency was higher in FMS and SFN patients than in healthy controls, but patients with and without DED did not differ in any CCM parameter. We concluded that increased DED frequency did not confound our findings on corneal nerves and LC.

4.4 RNA in FMS skin cells

We created monocellular cultures of epidermal keratinocytes from skin punch biopsies of the upper thigh of FMS patients and controls and analyzed miRNA

and mRNA expression profiles using NGS and qRT-PCR. We compared the expression levels not only between FMS patients and controls but also between patients with generalized (FMS $IENFD_{reduced}$) and no (FMS $IENFD_{normal}$) small fiber affection.

Previous studies examining blood sera or cerebrospinal fluid (CSF) found numerous miRNA (D'Agnelli et al., 2019) and mRNA to be differentially expressed in FMS patients compared to controls. Microarray analysis of blood samples revealed as much as 482 differentially expressed mRNA in FMS patients compared to controls, the genes considered most interesting by the authors were involved in allergic/hypersensitivity reactions (e.g. CPA3, MS4A2, FCER1A and IL-3RA) or immune cell signaling (e.g. IL-10 and IL-25) (Jones et al., 2016). Among the reported miRNA expression differences between FMS patients and controls, some (miR 145-5p, miR223-3p, miR23a-3p) were even found to be up- /downregulated in the same direction in blood and CSF samples throughout different studies (Bjersing et al., 2013; Cerda-Olmedo et al., 2015; Masotti et al., 2017). For miR 145-5p a correlation between CSF expression level and FMS pain and fatigue was found (Bjersing et al., 2013).

One study identified higher expression of miRNA let-7d in RNA samples from white blood cells and whole skin sections in combination with downregulation of its target mRNA IGF-1R in FMS patients compared to healthy controls (Leinders et al., 2016). In another study, using monocellular cultures of skin cells, higher expression of IL-10, EFNA4, and EPHA4 was found in keratinocytes, and higher expression of TGF- β1, HCN2 (hyperpolarization-

activated cyclic nucleotide-gated ion channel 2), EFNA4, and EPHA4 was found in fibroblasts (Evdokimov et al., 2019b).

Through NGS screening and following qRT-PCR validation, we identified no differentially expressed miRNA in FMS patients and only one differentially expressed mRNA compared to controls: PRSS21 was overexpressed in FMS $IENFD_{normal}$ patients, but not in FMS $IENFD_{reduced}$ patients compared to healthy controls. Its expression was, however, not different between FMS $IENFD_{normal}$ patients and FMS $IENFD_{reduced}$ patients. PRSS21 protein expression has previously been described in testicular germinal cells and various tumor cell lines, its appearance seems to be restricted to premeiotic stem cells physiologically (Hooper et al., 1999; Hooper et al., 2000), but mRNA expression of PRSS21 is found in various tissues (Inoue et al., 1998). There is no data on PRSS21 function in the contexts of pain or neuropathy. This information makes PRSS21 an unlikely player in the pathophysiology of SFP in FMS. Also, its expression being increased only in the FMS $IENFD_{normal}$ group indicates a role for PRSS21 in FMS without SFP. Its expression at mRNA and protein level needs to be analyzed in skin samples of FMS patients before further conclusions on its role in FMS.

NGS screening showed one further differentially expressed gene: CD86 was expressed at a lower level in the FMS $IENFD_{normal}$ group compared to controls, but this difference could not be confirmed in our qRT-PCR validation. CD86, also known as B7-2, is a cell surface protein usually expressed by antigen presenting cells (APC) such as LC, it is upregulated upon APC activation and acts as a co-stimulatory signal in T-cell activation (Chen and Flies, 2013). It has already been reported to be expressed at low levels in keratinocytes of

different epithelial tissues (Marshall et al., 2017; Romero-Tlalolini et al., 2013). Although reduced CD86 expression fits well to our finding of lower corneal $dLC_{fiber\ contact}$ in FMS patients compared to controls, with both resembling a lower status of immune system activation in FMS, we cannot confirm differential CD86 expression between FMS patients and controls. Perhaps our cohort used for validation was too small to detect the relatively small difference in mRNA expression levels of CD86 between FMS and controls and the examination of a larger cohort might bring different results. In that case CD86 protein expression should be assessed.

Our NGS screening did not detect differential expression of any of the RNA previously reported to be differentially expressed in FMS skin or keratinocytes (miR let-7d, IGF-1R, IL-10, EFNA4, and EPHA4). This may reflect the assumed pathophysiological heterogeneity in FMS patients which in combination with our relatively small sample size of 12 FMS patients versus 5 controls might have hindered these differences to reach statistical significance. We tried to overcome this problem by creating groups homogenous in baseline, clinical, and small fiber characteristics, but these characteristics do not necessarily translate into homogenous molecular SFP mechanisms in our groups.

We determined miR let-7d expression levels in larger cohorts of FMS patients and controls via qRT-PCR in fibroblasts and keratinocytes each from a proximal and a distal biopsy site, independent of its unremarkable NGS screening result. In contrast to previous findings (Leinders et al., 2016) miR let-7d expression in FMS skin cells was not different from healthy control skin cells. This may mean that differential expression levels of miR let-7d in skin

originate not from keratinocytes or fibroblasts but another cell type. Alternatively, differential expression may be lost during the two passages of cell culture we performed.

4.5 Strengths and limitations

We examined a large cohort of well characterized FMS patients using multiple approaches to elucidate the background of SFP in FMS. This allowed us to detect even subtle differences between patients and healthy controls. We compared our CCM results not only to healthy controls but also to disease controls suffering from isolated SFN which allowed a better characterization of the details in FMS small fiber changes in the cornea. We also assessed DED through an objective (Schirmer's test) and a subjective test (OSDI) and by doing this were able to control for one major possible confounder on our CCM results. In our RNA expression analysis, we did not only compare FMS patients and controls but also searched for differences between subgroups differing in their degree of clinical and small fiber affection giving us the opportunity to determine a possible role for our findings in SFP. NGS and qRT-PCR were performed in biomaterial from monocellular cell cultures of the two most abundant cell types in the skin - keratinocytes and fibroblasts - giving the possibility of assigning findings in RNA expression to one cell type. This approach, however, may also have hindered us from reproducing previous findings from whole skin RNA examination as there is a chance that these changes were caused by less common cells in the skin, like Langerhans cells or mast cells. Due to the method's high cost we had to restrict our NGS screening to keratinocytes and could only examine relatively small groups (12

FMS patients and 5 controls). Even though we considered keratinocytes the most promising targets for our research, NGS mRNA and miRNA expression analysis of fibroblasts might yield further information. Another limitation of our study lies in the resolution limitation of CCM. We can only assume that the observed contact between corneal LC and nerves is a cell-cell contact with biochemical interaction, but to prove this electron microscopy would be needed.

4.6 Conclusion and outlook

Our findings of corneal LC changes and RNA expression differences in keratinocytes between FMS patients and controls narrow in the range of possible mechanisms behind SFP in FMS and open new possibilities for further research. Low corneal $dLC_{\text{fiber contact}}$ density can be suspected to contribute to loss of small nerve fibers through lack of local neurotrophic signaling. In which way reduced small nerve fiber density leads to FMS pain remains elusive, but patients with extensive SFP also report higher pain than those with no SFP (Evdokimov et al., 2019a). It can also be suspected that LC have a direct impact on FMS pain by secreting pro-nociceptive local mediators or by secreting less analgesic local mediators than they would physiologically. These capabilities have been described for other immune cells, e.g. monocytes/ macrophages and T-cells (Ji et al., 2016). Alternatively, the recruitment of monocytes/macrophages and T-cells by LC into the proximity of nerve fiber endings may be reduced and a lack of their analgesic signaling contribute to FMS pain. It should also be considered that reduced $dLC_{\text{fiber contact}}$ density can also result from SFP instead of causing it.

From a clinical perspective $dLC_{fiber\ contact}$ density adds diagnostic value to CCM examination in FMS patients. If reduced, this parameter can be considered typical but not specific for FMS. For routine clinical use, $dLC_{fiber\ contact}$ determination should be automated as our manually counting approach turned out to be very time consuming. Future studies will need to examine LC in different tissues, e.g. skin punch biopsies, with a dedicated look at LC nerve contacts. A promising source of information about LC-nerve interactions may be tear fluid in which levels of neurotrophic factors can be measured and correlated with corneal $dLC_{fiber\ contact}$ density.

Epidermal keratinocytes are another cell type suspected to contribute to SFP in FMS. Like immune cells, they can also induce and reduce nociception based on secreted paracrine mediators acting at nociceptors (Ji et al., 2016). In our study we find no differential expression of any known mediator between FMS patients and healthy controls which speaks against keratinocytes playing a role in FMS SFP. Different findings may, however, result from conducting NGS screening experiments on keratinocytes of a larger cohort of FMS patients. Our sole finding of higher PRSS21 expression in FMS patients than in controls is difficult to put into the context of FMS or SFP as none of its known functions are connected to pain or neuropathy. Due to its huge difference between FMS patients and controls, PRSS21 expression in keratinocytes may hold high diagnostic value should it be overexpressed in native keratinocytes, which did not undergo cell culture, too. To clarify this possible use, future experiments need to assess PRSS21 expression in native skin and should also include samples from disease controls as we do not yet know whether PRSS21 overexpression is specific for FMS. A similar approach

to ours, using NGS analysis of RNA expression levels in monocellular cultures of skin cells of FMS patients, may still be promising in other cell types than keratinocytes, e.g. fibroblasts or LC. Another promising approach is the creation of co cultures of two or more cell types, e.g. keratinocytes and fibroblasts, as these systems can imitate local cellular interactions which may be crucial for the pathophysiological processes in FMS.

5 Summary

We examined 143 patients suffering from FMS, a syndrome characterized by chronic widespread pain, sleep disturbances, and fatigue. Etiology and pathophysiology of FMS are scarcely understood. In recent years abnormalities of small Aδ- and C-nerve fibers have been found in subgroups of FMS patients. It is yet unclear how such SFP is caused in FMS patients and how it contributes to FMS symptoms.

We used CCM to analyze corneal small nerve fibers and associated LC, comparing FMS patients' results to those from 65 healthy controls and 41 disease controls suffering from SFN. We, further, assessed expression levels of mRNA and miRNA in keratinocytes taken from skin punch biopsies of FMS patients and healthy controls kept as monocellular cell cultures. A screening was performed using NGS in a small cohort of 12 FMS patients and 5 healthy controls. Results were validated in larger cohorts by qRT-PCR.

As in previous studies IENFD and CNFD were reduced in a subgroup of FMS patients. We found identical LC densities in FMS patients, healthy controls, and SFN patients. The subpopulation of $dLC_{fiber\ contact}$ in FMS and SFN patients was lower than in healthy controls. Our RNA expression analysis revealed one mRNA that was expressed higher in FMS patients than in controls: PRSS21.

We conclude that reduced neurotrophic signaling of LC may contribute to SFP in the cornea. Epidermal PRSS21 expression and $dLC_{fiber\ contact}$ density are promising biomarker candidates for FMS diagnosis.

6 Zusammenfassung

Wir untersuchten 143 PatientInnen mit FMS, einem chronischen Schmerzsyndrom mit bislang kaum verstandener Ätiologie und Pathophysiologie. In den letzten Jahren wurden bei Subgruppen von FMS-PatientInnen Pathologien der sogenannten small fibers nachgewiesen. Wie diese entstehen oder zu den Symptomen des FMS beitragen ist noch unklar. Wir untersuchten corneale Nerven und assoziierte LC mittels CCM und verglichen die Ergebnisse der FMS PatientInnen mit denen von 65 gesunden Kontrollen und 41 SFN Patientinnen. Weiterhin untersuchten wir die mRNA und miRNA Expression in Keratinozyten aus Hautstanzbiopsien von FMS PatientInnen und gesunden Kontrollen, die isoliert in Zellkultur genommen wurden. Mittels NGS wurde ein mRNA/miRNA-Screening in einer kleinen Kohorte von 12 PatientInnen und 5 Kontrollen durchgeführt. Die Ergebnisse wurden mittels qRT-PCR in einer größeren Gruppe validiert.

Wie in vorausgegangenen Studien waren IENFD und CNFD bei FMS PatientInnen-Subgruppen reduziert. Die Dichte an LC war bei FMS und SFN PatientInnen sowie gesunden Kontrollen identisch. Die Subpopulation der $dLC_{fiber\ contact}$ war bei FMS und SFN PatientInnen niedriger als bei gesunden Kontrollen. Eine mRNA, PRSS21, wurde bei FMS PatientInnen stärker als bei Kontrollen exprimiert. Wir schlussfolgern, dass eine Reduktion neurotropher Signale durch LC zur Kleinfaserpathologie bei FMS beitragen könnte. Epidermale PRSS21-Expression und $dLC_{fiber\ contact}$ Dichte stellen vielversprechende Kandidaten für Biomarker zur FMS-Diagnose dar.

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8 Curriculum Vitae

9 Publications

9.1 Journal articles

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9.2 Poster presentations

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Meyer zu Altenschildesche C, Saffer N, Kreß L, Malzacher T, **Klitsch A**, Karl F, Urlaub D, Sommer C, Kurth I, Üçeyler N. Genetische Variationen bei Small Fiber Neuropathie. Posterabstract, Deutscher Schmerzkongress, Mannheim, 2019.

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11 Appendix:

11.1 Figures:

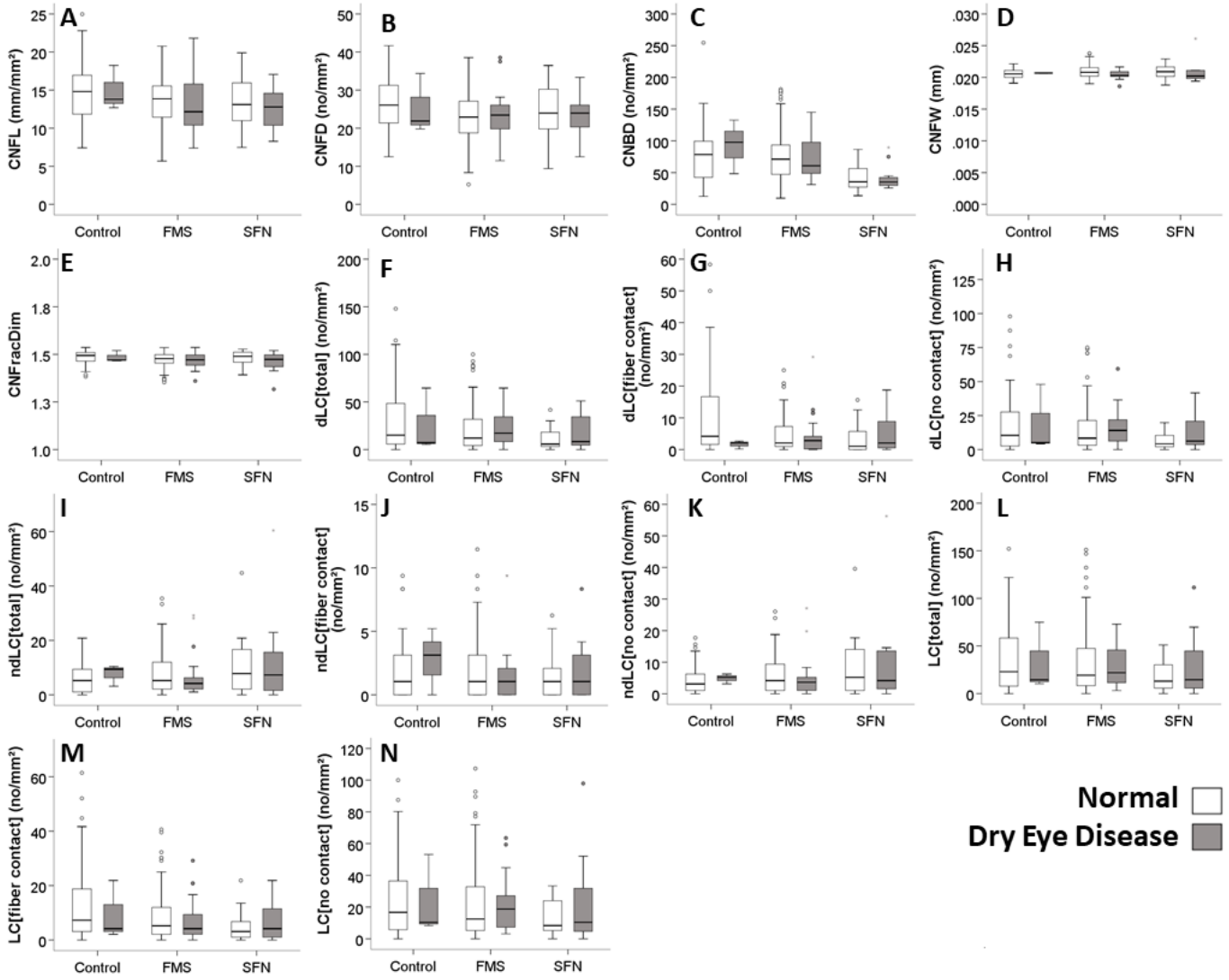

Figure 5: **Comparison of corneal parameters between study participants with and without dry eyes.**

We compared results of every parameter assessed by CCM within all three groups between participants with and without a positive dry eye screening. There was no difference in any of the analyzed parameters: A: CNFL, B: CNFD, C: CNBD, D: CNFW, E: CNFracDim, F: dLC_{total}, G: $dLC_{fiber\ contact}$, H: $dLC_{no\ contact}$, I: $ndLC_{total}$, J: $ndLC_{fiber\ contact}$, K: $ndLC_{no\ contact}$, L: LC_{total}, M: $LC_{fiber\ contact}$, N: $LC_{no\ contact}$. Adapted from Klitsch et al., 2020, *J Peripher Nerv Syst*.

Abbreviations: CNBD = corneal nerve branch density, CNFD = corneal nerve fiber density, CNFL = corneal nerve fiber length, CNFracDim = corneal nerve fractal dimension, CNFW = corneal nerve fiber width, $dLC_{fiber\ contact}$ = dendritic cells with nerve fiber contact, $dLC_{no\ contact}$ = dendritic cells without nerve fiber contact, dLC_{total} = all dendritic cells, FMS = fibromyalgia syndrome, $ndLC_{fiber\ contact}$ = non-dendritic cells with nerve fiber contact, $ndLC_{no\ contact}$ = non-dendritic cells without nerve fiber contact, $ndLC_{total}$ = all non-dendritic cells, $LC_{fiber\ contact}$ = total number of cells with nerve fiber contact, $LC_{no\ contact}$ = total number of cells without nerve fiber contact, LC_{total} = total cell number, SFN = small fiber neuropathy.

11.2 Tables:

Table 1: Baseline characteristics, IENFD, and questionnaire results of all study participants

	FMS patients (n = 143)	SFN patients (n=41)	Healthy controls (n = 65)
Proportion female/ male	134 (94%) / 9 (6%)	41 (100%) / 0 (0%)	60 (92%) / 5 (8%)
Age (years)	50 (21 – 74)	55 (22 – 73)	49 (22 – 65)
BMI (kg/m²)	24.3 (16.1 – 41.9)	25 (19 - 42)	23.9 (16.7 – 41.5)
Time since diagnosis (years)	3 (0 – 35)	0.5 (0 – 8)	N/A
duration of pain due to disease (years)	12 (0.75 – 56)	4 (0 – 20)	N/A
Laboratory findings: - HbA1c (ref.: ≤6.1%) - OGTT(2h) (ref.: ≤140 mg/dl) - TSH (ref.: 0.3 – 4.0 mIU/l) - Vitamin B12 (ref.: 197 – 866 pg/ml)	 5.4 (4.7 – 6.4; 2 pathologic) 120 (65 – 217; 23 pathologic) 1.8 (0.0 – 22.0; 19 pathologic) 446 (183 – 2000; 12 pathologic)	 5.6 (3.6 – 7.7; 5 pathologic) 127 (79 – 284; 12 pathologic) 1.6 (0.2 – 9.2; 6 pathologic) 450 (215 - 2000; 5 pathologic)	 N/A 110 (55 –159; 2 pathologic) N/A N/A

IENFD: - lower leg (fibers/mm) - upper thigh (fibers/mm)	 6.3 (0.0 – 14.4) 8.3 (1.3 – 20.0)	 5.4 (0 – 11.8) 9.7 (2.3 – 16.5)	 6.7 (1.1 – 15.2) 10.2 (4.6 – 18.5)
GCPS: - current pain intensity (NRS) - disability due to pain	 6 (0 – 9) 56.7 (6.7 – 86.7)	 4 (0 – 8) 43.3 (0 – 90)	 N/A N/A
FIQ sum score	48.1 (17.5 – 70.7)	N/A	N/A
STAI-T sum score	45 (0 – 76)	N/A	N/A
ADS sum score	22 (3 – 51)	16 (2 – 38)	N/A

Data are given as median and range in brackets.

Abbreviations: ADS = Allgemeine Depressionsskala (German version of the Center for Epidemiological Studies Depression scale (CES-D) questionnaire), BMI = body mass index, CNFD = corneal nerve fiber density, CNFL = corneal nerve fiber length, FIQ = FibromyaIgia Impact Questionnaire, FMS = fibromyalgia syndrome, GCPS = Graded Chronic Pain Scale, IENFD = intraepidermal nerve fiber density, IGT = impaired glucose tolerance, N/A = not applicable, NRS = numeric rating scale, OGTT = oral glucose tolerance test, SFN = small fiber neuropathy, STAI-T = State-Trait Anxiety Inventory – Trait, TSH = thyroid-stimulating hormone.

Table 2: Baseline characteristics, IENFD, and questionnaire results of subjects analyzed via NGS

	FMS $IENFD_{normal}$ (n = 6)	FMS $IENFD_{reduced}$ (n = 6)	Healthy controls (n = 5)
Age (years)	57 (40 – 63)	54 (45 – 72)	47 (31 – 55)
BMI (kg/m²)	21.5 (19.6 – 32.8)	26.7 (23.2 – 30.4)	24.7 (21.2 – 30.5)
Time since diagnosis (years)	4 (3 – 12)	7 (0.5 – 9)	N/A
duration of pain due to FMS (years)	18 (10 – 30)	15 (1 – 40)	N/A
Laboratory findings:			
- HbA1c (ref.: ≤6.1%)	5.4 (5.0 – 6.0)	5.5 (5.1 – 5.8)	N/A
- OGTT(2h) (ref.: ≤140 mg/dl)	113 (88 – 129)	126 (84 – 143; 1 pathologic)	94 (11 – 105)
- TSH (ref.: 0.3 – 4.0 mIU/l)	2.2 (0.7 – 22.0; 1 pathologic)	2.1 (0.1 – 10.8; 2 pathologic)	N/A
- Vitamin B12 (ref.: 197 – 866 pg/ml)	416 (349 – 1128; 1 pathologic)	406 (221 – 592)	N/A

IENFD: - lower leg (fibers/mm) - upper thigh (fibers/mm)	 8.5 (7.3 – 11.4) 11.2 (8.0 – 13.5)	 5.1 (4.1 – 5.7) 5.8 (3.5 – 7.4)	 7.0 (6.4 – 15.1) 10.0 (8.9 – 16.1)
CNFD (fibers/mm²)	24.0 (16.7 – 31.2)	14.1 (10.4 – 22.9)	31.3 (18.8 – 32.3)
CNFL (mm/mm²)	14.2 (10.0 – 17.8)	8.6 (6.2 – 14.3)	16.3 (8.7 – 17.8)
GCPS: - current pain intensity (NRS) - disability due to pain	 4 (3 – 6) 41.7 (16.7 – 60.0)	 5.5 (4 – 7) 68.3 (46.7 – 76.7)	 N/A N/A
FIQ sum score	38.5 (35.0 – 50.0)	60.9 (35.7 – 70.3)	N/A
STAI-T sum score	38.5 (28 – 39)	46 (39 -67)	N/A
ADS sum score	13.5 (8 – 36)	25.5 (17 – 48)	N/A

Data are given as median and range in brackets.

Abbreviations: ADS = Allgemeine Depressionsskala (German version of the Center for Epidemiological Studies Depression scale (CES-D) questionnaire), BMI = body mass index, CNFD = corneal nerve fiber density, CNFL = corneal nerve fiber length, FIQ = FibromyaIgia Impact Questionnaire, FMS = fibromyalgia syndrome, GCPS = Graded Chronic Pain Scale, IENFD = intraepidermal nerve fiber density, N/A = not

applicable, NRS = numeric rating scale, OGTT = oral glucose tolerance test, STAI-T = State-Trait Anxiety Inventory – Trait, TSH = thyroid-stimulating hormone.

Table 3: Baseline characteristics, IENFD, and questionnaire results of subjects analyzed via qRT-PCR to validate NGS screening

	FMS $IENFD_{normal}$ (n = 17)	FMS $IENFD_{reduced}$ (n= 10)	Healthy controls (n = 9)
Age (years)	55 (40 – 69)	55 (45 – 72)	49 (24– 65)
BMI (kg/m^2)	21.3 (16.6 – 33.8)	26.7 (23.3 – 32.1)	23.5 (21.2 – 31.6)
Time since diagnosis (years)	5 (1 – 12)	4 (0.5 – 9)	N/A
duration of pain due to FMS (years)	15 (1 – 30)	16.5 (1 – 40)	N/A
Laboratory findings			
- HbA1c (ref.: ≤6.1%)	5.3 (4.7 – 6.0)	5.5 (5.1 – 6.4; 1 pathologic)	N/A
- OGTT(2h) (ref.: ≤140 mg/dl)	114 (73 – 189; 1 pathologic)	136 (84 – 217;4 pathologic)	94 (11 – 134)
- TSH (ref.: 0.3 – 4.0 mIU/l)	1.2 (0.0 – 22.0; 2 pathologic)	2.1 (0.1 – 10.8; 3 pathologic)	N/A

- Vitamin B12 (ref.: 197 – 866 pg/ml)	381 (238.0 – 1128; 1 pathologic)	501 (221 – 780)	N/A
IENFD: - lower leg (fibers/mm) - upper thigh (fibers/mm)	 9.3 (6.8 – 13.8) 10.7 (6.9 – 14.9)	 4.7 (0.9 – 5.7) 5.2 (3.5 – 7.4)	 7.14 (6.42 – 15.17) 10.0 (8.0 – 16.1)
CNFD (fibers/mm²)	22.9 (15.6 – 31.2)	19.3 (10.4 – 34.4)	22.92 (17.71 – 32.29)
CNFL (mm/mm²)	12.9 (10.0 – 17.8)	12.7 (6.2 – 18.1)	14.5 (8.7 – 17.8)
GCPS: - current pain intensity (NRS) - disability due to pain	 5 (3 – 9) 50.0 (16.7 – 77.0)	 6 (4 – 7) 71.7 (10.0 – 83.0)	 N/A N/A
FIQ sum score	40.0 (25.0 – 68.0)	56.0 (35.7 – 70.3)	N/A
STAI-T sum score	39 (28 – 63)	50 (39 -67)	N/A
ADS sum score	16 (3 – 44)	25.5 (17 – 48)	N/A

Data are given as median and range in brackets.

Abbreviations: ADS = Allgemeine Depressionsskala (German version of the Center for Epidemiological Studies Depression scale (CES-D) questionnaire), BMI = body mass index, CNFD = corneal nerve fiber density, CNFL = corneal nerve fiber length, FIQ = Fibromyalgia Impact Questionnaire, FMS = fibromyalgia syndrome, GCPS = Graded Chronic Pain Scale, IENFD = intraepidermal nerve fiber density, N/A = not applicable, NRS = numeric rating scale, OGTT = oral glucose tolerance test, qRT-PCR = quantitative real-time polymerase chain reaction, STAI-T = State-Trait Anxiety Inventory – Trait, TSH = thyroid-stimulating hormone.

Table 4: Baseline characteristics, IENFD, and questionnaire results of subjects included in miR let-7d analysis

	FMS patients (n = 81)	Healthy controls (n = 17)
Proportion female/ male	77 (95%) / 4 (5%)	14 (82%) / 3 (18%)
Age (years)	50 (23 – 74)	48 (22 – 65)
BMI (kg/m²)	24.3 (16.1 – 36.2)	24.6 (16.7 – 30.5)
Time since diagnosis (years)	3 (0 – 35)	N/A
duration of pain due to FMS (years)	11 (0.8 – 56)	N/A
Laboratory findings - HbA1c (ref.: ≤6.1%) - OGTT(2h) (ref.: ≤140 mg/dl)	 5.5 (4.7 – 6.4; 1 pathologic) 120 (65 – 201; 1 pathologic)	 N/A 104 (59 – 159; 1 pathologic)

- TSH (ref.: 0.3 – 4.0 mIU/l) - Vitamin B12 (ref.: 197 – 866 pg/ml)	1.6 (0.1 – 22.0; 9 pathologic) 449 (221 – 1660; 7 pathologic)	N/A N/A
IENFD: - lower leg (fibers/mm) - upper thigh (fibers/mm)	 6.5 (0 – 14.4) 8.0 (1.3 – 20.0)	 6.5 (1.1 – 13.1) 10.0 (4.9 – 18.5)
Samples available*: - proximal, fibroblasts - distal, fibroblasts - proximal, keratinocytes - distal, keratinocytes	 62 73 40 47	 12 11 10 9

Data are given as median and range in brackets.

*In few cases samples had to be excluded after qRT-PCR, numbers for our statistical analysis of miR let-7d expression as given in figure 8 may therefore differ from this table.

Abbreviations: BMI = body mass index, FMS = fibromyalgia syndrome, IENFD = intraepidermal nerve fiber density, N/A = not applicable, OGTT = oral glucose tolerance test, qRT-PCR = quantitative real-time polymerase chain reaction, TSH = thyroid-stimulating hormone.

11.3 Abbreviations

ACR	American College of Rheumatology
ADS	Allgemeine Depressionsskala (German version of the Center for Epidemiological Studies Depression scale (CES-D) questionnaire)
APC	Antigen presenting cells
BMI	Body Mass Index
CCM	Corneal confocal microscopy
CD 86	Cluster of differentiation 86
cDNA	Complementary deoxyribonucleic acid
CIDP	Chronic inflammatory demyelinating polyneuropathy
CNBD	Corneal nerve branch density
CNFD	Corneal nerve fiber density
CNFL	Corneal nerve fiber length
CNFracDim	Corneal nerve fractal dimension
CNFW	Corneal nerve fiber width
CPA3	Carboxypeptidase A3
CSF	Cerebrospinal fluid
DED	Dry eye disease
$dLC_{fiber\ contact}$	Dendritic Langerhans cells with nerve fiber contact
$dLC_{no\ contact}$	Dendritic Langerhans cells without nerve fiber contact
dLC_{total}	Dendritic Langerhans cells
EFNA4	Ephrin A4
EPHA4	Ephrin type-A receptor 4

FCER1A	Fc fragment of Immunoglobulin E receptor Ia
FIQ	Fibromyalgia Impact Questionnaire
FMS	Fibromyalgia syndrome
GCPS	Graded Chronic Pain Scale
GRCh 38	Genome Reference Consortium Human build 38
HbA1c	Glycated hemoglobin
HCN 2	Hyperpolarization-activated cyclic nucleotide-gated cation channel 2
IENFD	Intraepidermal nerve fiber density
IGF-1R	Insulin-like growth factor 1 receptor
IGT	Impaired glucose tolerance
IL-10	Interleukin 10
IL-25	Interleukin 25
IL-3RA	Interleukin 3 receptor, alpha
LC	Langerhans cell
$LC_{fiber\ contact}$	Langerhans cells with nerve fiber contact
$LC_{no\ contact}$	Langerhans cells without nerve fiber contact
LC_{total}	Langerhans cells
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
MS4A2	High affinity immunoglobulin epsilon receptor subunit beta
$ndLC_{fiber\ contact}$	Non-dendritic Langerhans cells with nerve fiber contact

$ndLC_{no\ contact}$	Non-dendritic Langerhans cells without nerve fiber contact
$ndLC_{total}$	Non-dendritic Langerhans cells
NGS	Next generation sequencing
NPSI	Neuropathic Pain Symptom Inventory
NRS	Numeric rating scale
OGTT	Oral glucose tolerance test
OSDI	Ocular surface disease index
PCR	Polymerase chain reaction
PREP	Pain related evoked potentials
PRSS21	Serine protease 21
qRT-PCR	Quantitative real-time polymerase chain reaction
QST	Quantitative sensory testing
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SFN	Small fiber neuropathy
SFP	Small fiber pathology
SNORD 44	Small nucleolar ribonucleic acid 44
SNORD 48	Small nucleolar ribonucleic acid 48
snRNA	Small nuclear ribonucleic acid
sRNA	Small ribonucleic acid
SS scale	Symptom severity scale
STAI-S	State-Trait Anxiety Inventory – State
STAI-T	State-Trait Anxiety Inventory – Trait
TGF- β1	Transforming growth factor- β1

tRNA	Transfer ribonucleic acid
TSH	Thyroid-stimulating hormone
WPI	Widespread pain index

11.5 Materials

Chemicals

Chloroform		
Distilled water	Aqua ad iniectabilia Braun	B.Braun Melsungen AG, Melsungen, Germany
Ethanol 100%		
RNA isolation	miRNeasy Mini Kit QIAzol Lysis Reagent	Quiagen GmbH, Hilden, Germany

Drugs

Anesthesia, CCM	Conjuncain EDO® eye drops	Bausch & Lomb GmbH, Berlin, Germany
Anesthesia, skin punch biopsy	Scandicain 1%	AstraZeneca GmbH, Wedel, Germany
Lubrication, CCM	Corneagel EDO®	Bausch & Lomb GmbH, Berlin, Germany

Consumables

Biopsy punch 6mm		FA Stiefel, Offenbach am Main, Germany

Caps	0.2ml PCR Soft tubes	Biozym Scientific GmbH, Hess. Oldendorf, Germany
	1.5ml Safe lock tubes	Eppendorf AG, Hamburg, Germany
	2ml Mikroschraubröhre PP	Sarstedt AG&Co., Nümbrecht, Germany
CCM caps	TomoCap®	Heidelberg Engineering GmbH, Heidelberg, Germany
PCR plates	MicroAmp® Fast 96-Well reaction plate 0.1ml	Thermo Fisher Scientific Inc., Waltham, USA
PCR plate seals	Optical Adhesive Covers	
Pipette tips	10µl, 100µl, 1000µl Biosphere® Filter tips	Sarstedt AG&Co., Nümbrecht, Germany
	Combitips® Plus 1.0ml	Eppendorf AG, Hamburg, Deutschland
Scalpel		Braun, Tuttlingen, Germany
Schirmer tear test		Haag-Streit UK Ltd, Harlow Essex, UK

Sterile plasters	Leukostrip	Smith&Nephew, Medical Limited, England
Syringe, 1ml		Braun, Tuttlingen, Germany

PCR materials and primers

Nuclease-free water	Nuclease-free water Ambion™	Thermo Fisher Scientific Inc., Waltham, USA
Buffers	10x RT-Buffer	Taq Man® Reverse Transcription reagents, Life Technologies, Carlsbad, CA, USA
	25mM MgCl2	
Nucleotides	dNTP	
Primers	Oligo-DT	
	Random-Hexamers	
Reverse transkriptase	MultiScribe Reverse Transcriptase	
RNAse inhibitor		
cDNA synthesis kit for miRNA	Universal cDNA synthesis kit II	Exiqon, Vedbaek, Denmark
qRT-PCR kit for miRNA	ExiLENT SYBR® Green Master Mix Kit	
5S-RNA primer	YP00203906	

U6-RNA primer	YP00203907	
SNORD44 primer	YP00203902	
SNORD48 primer	YP00203903	
miR let-7d primer	YP00204124	
ROX Reference Dye	Invitrogen™ ROX Reference Dye	Thermo Fisher Scientific Inc., Waltham, USA
Buffers, nukleotides, DNA-polymerase	Taq Man® Universal-PCR-Master Mix	Taq Man® Gene expression assay, Life Technologies, Carlsbad, CA, USA
CD86 primer	Hs01567026_m1	
PRSS21 primer	Hs00199035_m1	
18S primer	Hs99999901_s1	

Devices

CCM image acquisition	Heidelberg Retina Tomograph III	Heidelberg Engineering GmbH, Heidelberg, Germany
	Rostock Cornea Module	
Esthesiometry	Cochet-Bonnet esthesiometer	Luneau Ophtalmologie, Chartres Cedex, France
PCR-cycler for qRT-PCR	Step One Plus RealTime PCR System	Thermo Fisher Scientific Inc., Waltham, USA
PCR-cycler for reverse transcription-PCR	Advanced primus 96	peqlab Biotechnologie GmbH,

Photometer	Nanodrop® Spectrophotometer	Erlangen, Germany
Pipettes	2.5µl, 10µl, 100µl, 1000µl Eppendorf Research	Eppendorf AG, Hamburg, Germany
	Multipipette® Stream	
Vortex mixer	GLW- L46	Gesellschaft für Laborbedarf, Würzburg, Germany

Software

CCM image analysis	CCMetrics®	M.A. Dabbah, Imaging Science, Manchester, UK
	ACCMetrics® V.2	
Literature management	EndNote X7	Thomson Reuters, New York City, USA
PCR-Cycler software	Step One Plus	Thermo Fisher Scientific Inc., Waltham, USA
Spreadsheet and text processing	Office 2016 package	Microsoft Inc., Redmond, USA
Statistical analysis	SPSS 25	IBM, Ehningen, Germany