Skin cytokine expression in patients with fibromyalgia syndrome is not different from controls

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Abstract
Background: Fibromyalgia syndrome (FMS) is a chronic pain syndrome of unknown etiology. There is increasing evidence for small nerve fiber impairment in a subgroup of patients with FMS. We investigated whether skin cytokine and delta opioid receptor (DOR) gene expression in FMS patients differs from controls as one potential contributor to small nerve fiber sensitization.

Methods: We investigated skin punch biopsies of 25 FMS patients, ten patients with monopolar depression but no pain, and 35 healthy controls. Biopsies were obtained from the lateral upper thigh and lower calf. Gene expression of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF), interleukin (IL)-6, and IL-8 and of the anti-inflammatory cytokine IL-10 was analyzed using quantitative real-time PCR and normalizing data to 18sRNA as housekeeping gene. Additionally, we assessed DOR gene expression.

Results: All cytokines and DOR were detectable in skin samples of FMS patients, patients with depression, and healthy controls without intergroup difference. Also, gene expression was not different in skin of the upper and lower leg within and between the groups and in FMS patient subgroups.

Conclusions: Skin cytokine and DOR gene expression does not differ between patients with FMS and controls. Our results do not support a role of the investigated cytokines in sensitization of peripheral nerve fibers as a potential mechanism of small fiber pathology in FMS.

Keywords: Fibromyalgia syndrome, Skin biopsy, Monopolar depression, Cytokines, Opioid receptor
PCR [11]. The same group also reported an increased expression of skin opioid receptors in FMS patients compared to controls [12].

Here we investigated cutaneous gene expression of pro- and anti-inflammatory cytokines and of the delta opioid receptor (DOR) in skin samples of patients with FMS and compared our results with those of patients with monopolar depression but without pain as disease controls and with healthy controls. We hypothesized that patients with FMS have a higher cutaneous gene expression of pro-inflammatory, algesic cytokines as the basis of peripheral nerve fiber sensitization and that DOR expression is reduced leading to an impaired effect of endogenous opioids with increased pain.

Methods
Patients and controls
Between 2007 and 2011 we prospectively recruited 25 patients with FMS (23 women, two men; median age: 59 years, range: 50–70). The diagnosis was made according to the 1990 criteria of the American College of Rheumatology (ACR) [13]. Patients were recruited from all over Germany mainly by contacting self-help organisations. After a telephone interview suitable patients were invited to the Department of Neurology and were examined neurologically and electrophysiologically to check the in- and exclusion criteria as described earlier [2]. Twenty-five patients could be included. Our inclusion criteria were: male and female patients >18 years of age; medically confirmed diagnosis of FMS according to the 1990 ACR criteria; other possible differential diagnoses excluded (e.g. rheumatologic, orthopaedic). Exclusion criteria were: other differential diagnoses explaining the pain (e.g. rheumatologic, orthopaedic); other and additional pain sources (e.g. pain due to arthritis); abnormalities in routine blood tests.

Depressive symptoms are frequently present in FMS patients [1]. To control for possible confounding effects of depression on study results, we additionally investigated a group of ten patients (nine women, one men; median age: 50 years, range 39–75) with monopolar major depression without pain. These patients were enrolled between 2010 and 2011 at the Department of Psychiatry at the University of Würzburg. An additional group of 35 healthy controls was recruited. The group consisted of 15 men and 20 women with a median age of 51 years (20–84 years). Our study was approved by the Würzburg Medical School Ethics Committee. Written informed consent was obtained from all study participants before enrolment. Data on clinical examination, examination with electrophysiological measurements, pain and depression questionnaires have been published elsewhere [2].

Skin punch biopsies
Five-mm diameter skin punch biopsies (Stiefel, Offenbach, Germany) were obtained for histological analysis and for quantitative real-time PCR (qRT-PCR) analysis as previously described [8]. Two biopsies were taken from each participant (lateral proximal thigh and distal calf). One patient with FMS refused a biopsy at the thigh and another patient refused skin punch biopsy at all. Skin samples were divided in two pieces. One was processed for immunohistochemical analysis to determine intraepidermal nerve fiber density. Methodology and results are presented elsewhere [2]. The second half was flash-frozen in liquid nitrogen and stored at −80°C before further processing for gene expression analyses.

RNA extraction from skin samples
RNA extraction followed a protocol described earlier [8]. After thawing skin samples were immersed in 1 ml TRIzol reagent (Invitrogen, Karlsruhe, Germany), and dispersed (Polytron 1600E, Luzern, Switzerland). Samples were then incubated in 200 μl chloroform (25°C, 3 minutes) and were centrifuged (12,000 g, 15 minutes, 4°C). Supernatants were mixed with 500 μl of isopropanol and incubated again (25°C, 10 minutes). After another centrifugation step (12,000 g, 10 minutes, 4°C), the pellet was washed with 75 % ethanol and spun again (7,500 g, 5 minutes, 4°C). The samples were air-dried and the pellet was dissolved in diethylpyrocarbonate-treated water. Afterwards, samples were incubated in a water bath (55°C, 10 minutes).

Reverse transcription PCR
All PCR reagents and cyclers were purchased from Applied Biosystems (Darmstadt, Germany). Extracted mRNA (500 ng) was reverse transcribed using TaqMan Reverse Transcription Reagents. Using 10 μl 10× PCR-buffer, 6.25 μl Multiscribe reverse transcriptase, 2 μl RNase inhibitor, 22 μl MgCl₂, 20 μl dNTPs the reactions were performed in the ABI PRISM 7700 Cycler at the following conditions: 25°C, 10 min; 48°C, 60 min; 95°C, 5 min.

Gene expression analysis
TaqMan Universal Master Mix and 5 μl of cDNA were used for qRT-PCR performed in the GeneAmp 7700 sequence detection system with the following gene specific TaqMan Assays: TNF (ID: Hs00174128_m1), IL-6 (ID: Hs00174131_m1), IL-8 (ID: Hs00174103_m1), and IL-10 (ID: Hs00174086_m1). Additionally, we assessed gene expression of DOR (ID: Hs00538331_m1). 18sRNA (ID: Hs99999901_s1) served as endogenous control. The 25 μl-reaction mix contained 12.5 μl TaqMan Master Mix and 1.25 μl primer. The cycler conditions were: 50°C, 2 min; 95°C, 10 min; 45 cycles with 95°C, 15 sec;
Table 1 Basic data of patient groups

|                  | FMS   | Depression | Healthy controls |
|------------------|-------|------------|------------------|
| M, F             | 2, 23 | 1, 9       | 15, 20           |
| Median age (range) [yrs] | 59 (50–70) | 50 (39–75) | 51 (20–84) |
| Median disease duration (range) [yrs] | 21 (3–50) | 23 (3–35) | Not applicable |
discrepancy, since already small differences in Ct-values may appear large when transferred to x-fold changes.

In our previous study we showed that small fiber impairment is present in a subgroup of patients with FMS [2]; our findings were confirmed by others [3-6]. The open question is how reduced intraepidermal nerve fibers may be linked with peripheral hyperalgesia and pain. One possibility is that the remaining peripheral nerve endings are sensitized by local influences such as pro-inflammatory and algesic cytokines. However, we did not find a positive support for this assumption in our study and suggest that other local factors like chemokines need to be investigated and also the ion channel repertoire of the remaining supposedly diseased nerve fibers.

Our study has several limitations. The number of patients was small and with the relatively high variation of the individual qRT-PCR values potential group differences may have been masked. The lack of difference in gene expression patterns does not necessarily mean lack of difference in the actually functional protein levels, however, we were not able to conduct additional protein
analyses due to the limited amount of bio material. This was also the reason for the selected panel of investigated targets. Also, the assessment of whole skin samples instead of separated skin cells may be another reason why potentially present differences between groups may have been missed.

Conclusion
We conclude that cytokine gene expression is not restricted to FMS skin and that for the investigated cytokines there is no intergroup difference that might have been a plausible factor contributing to intraepidermal nerve fiber sensitization. The possibility of a ganglionic impact on fiber hyperexcitability and of other potential mechanisms of peripheral nerve sensitization need to be considered as well as other algesic mediators in FMS skin.

Abbreviations
ACR: American college of rheumatology; DOR: Delta opioid receptor; FMS: Fibromyalgia syndrome; IL: Interleukin; PCR: Polymerase chain reaction; TNF: Tumor necrosis factor-alpha.

Competing interests
The authors declare the following competing interests.

Authors’ contribution
NJ: Study design, patient recruitment, data collection, data assessment, manuscript preparation. SK: Patient recruitment, data collection, data assessment. WK: Patient recruitment, data collection, data assessment. SKS: Patient recruitment, data collection, data assessment, manuscript preparation. All authors read and approved the final manuscript.

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