Neural fractalkine expression is closely linked to pain and pancreatic neuritis in human chronic pancreatitis

Güralp O Ceyhan[1,2], Stefanie Deucker[2], Ihsan Ekin Demir[1], Mert Erkan[1], Martin Schmelz[3], Frank Bergmann[4], Michael W Müller[1], Thomas Giese[5], Markus W Büchler[2], Nathalia A Giese[2] and Helmut Friess[1]

The chemokine *fractalkine* induces migration of inflammatory cells into inflamed tissues, thereby aggravating inflammatory tissue damage and fibrosis. Furthermore, *fractalkine* increases neuropathic pain through glial activation, which can be diminished by blocking of its receptor, *CX3CR1*, through neutralizing antibodies. As chronic pancreatitis (CP) is characterized by tissue infiltration of inflammatory cells, fibrosis, pancreatic neuritis and severe pain, the roles of *fractalkine* and *CX3CR1* were investigated in CP ($n = 61$) and normal pancreas (NP, $n = 21$) by QRT-PCR, western blot and immunohistochemistry analyses. Their expression correlated with the severity of pancreatic neuritis, fibrosis, intrapancreatic nerve fiber density and hypertrophy, pain, CP duration and with the amount of inflammatory cell infiltrate immuno-positive for *CD45* and *CD68*. To investigate the influence of *fractalkine* on pancreatic fibrogenesis, human pancreatic stellate cells (hPSCs) were isolated from patients with CP, incubated with *fractalkine* and then *Collagen-1* and *α-smooth muscle actin* (α-SMA) expressions were measured. *CX3CR1*, but not *fractalkine*, mRNA was overexpressed in CP. In contrast, the protein levels of both *CX3CR1* and *fractalkine* were upregulated. Neuro-immunoreactivity for *fractalkine* and *CX3CR1* was strongest in patients suffering from severe pain and pancreatic neuritis. Long-term suffering from CP was noticeably related to increased neural immunoreactivity of *fractalkine*. Furthermore, *fractalkine* and *CX3CR1* mRNA overexpressions were associated with enhanced lymphocyte and macrophage infiltration. Advanced fibrosis was associated with increased *fractalkine* expression, whereas *in vitro fractalkine* had no significant impact on *collagen-1* and α-SMA expressions in hPSCs. Therefore, pancreatic *fractalkine* expression appears to be linked to visceral pain and to the recruitment of inflammatory cells into the pancreatic tissue and nerve fibers, with subsequent pancreatic neuritis. However, pancreatic fibrogenesis is probably indirectly influenced by *fractalkine*. Taken together, these novel findings suggest that *CX3CR1* represents a potential novel therapeutic target to reduce inflammation and modulate pain in CP.
Laboratory Investigation (2009) **89**, 347–361; doi:10.1038/labinvest.2008.170; published online 19 January 2009

KEYWORDS: *fractalkine*; *CX3CR1*; chronic pancreatitis; pain; pancreatic neuritis; fibrosis

Chronic pancreatitis (CP) is an inflammatory disease characterized by irreversible and progressive destruction of the pancreas, resulting in severe exocrine and endocrine insufficiency.[1,2] The destruction of the pancreatic parenchyma is accompanied by severe fibrosis with distinct accumulation of extracellular matrix (ECM) and impressive infiltration of various subsets of inflammatory cells.[1,3] There is a growing body of evidence that mononuclear cells modulate fibrogenesis by supporting the activation of human pancreatic stellate cells (hPSCs), which are the major source of intrapancreatic ECM production.[4–7] Once activated,

hPSCs not only release ECM proteins but also produce proinflammatory cytokines and chemokines.[4–7]

Another characteristic inflammatory event in CP is represented by perineural mononuclear cell infiltration. The layers of the perineurium are altered and damaged, thus they no longer provide a protective barrier. At the sites of inflammation, mononuclear cells can easily infiltrate the damaged perineurium and cause the characteristic pancreatic neuritis, which is strongly associated with abdominal pain in CP.[8–10] However, the precise pathophysiology of the neuro-immune crosstalk, and of pain initiation and maintenance in CP remains unclear.

[1]Department of Surgery, Technische Universität München, Munich, Germany; [2]Department of General Surgery, University of Heidelberg, Heidelberg, Germany; [3]Institute for Anaesthesiology Mannheim, University of Heidelberg, Mannheim, Germany; [4]Institute of Pathology, University of Heidelberg, Heidelberg, Germany and [5]Institute for Immunology, University of Heidelberg, Heidelberg, Germany
Correspondence: Dr GO Ceyhan, MD, Department of Surgery, Klinikum Rechts der Isar, Technische Universität München, Ismaninger Str. 22, D-81675 Munich, Germany.
E-mail: ceyhan@chir.med.tu-muenchen.de

At the sites of injury and inflammation, chemokines and adhesion molecules provide signals for leukocyte trafficking, including leukocyte adhesion and migration. *Fractalkine (CX3CL1)*, the unique member of the fourth class of chemokines (*CX3C*), is expressed on endothelial cells and lymphocytes, and is the only other known chemokine to be expressed on spinal neurons and sensory afferents, other than *monocyte chemoattractant protein-1* (*MCP-1*).[11–15] The highly selective fractalkine receptor *CX3CR1* is expressed on cytotoxic effector lymphocytes, including natural killer cells, cytotoxic T cells and macrophages.[16] In the nervous system, *CX3CR1* is expressed in microglia in the spinal cord, by satellite cells in the dorsal root ganglia (DRG) and in human embryonic neurons.[14,17] A distinctive feature of *fractalkine* is that it exhibits properties of both traditional chemokines and adhesion molecules. As a chemoattractant, *fractalkine* induces the migration and extravasation of natural killer cells, cytotoxic lymphocytes and macrophages into inflamed tissues and thereby aggravating tissue damage.[11,18] Moreover, by acting as an adhesion molecule, *fractalkine* can capture and enhance the migration of *CX3CR1*-expressing mononuclear cells through direct cell-to-cell interactions.[19,20] Recent data show that *fractalkine* and *CX3CR1* are involved in fibrogenesis, and are associated with renal and liver fibrosis.[21,22]

Independently from its peripheral pro-inflammatory effects, peripheral nerve damage leads to up-regulation of *fractalkine* in DRG and consequently to neuropathic pain, with activation of *CX3CR1* on spinal microglia being a crucial mechanism for neuronal sensitization.[12,14,23,24] However, also peripherally present are inflammatory mediators such as *TNF-α* and *IL-1*, which might contribute to neuropathic pain, if given access to the peripheral nerve through intraneural injection[25] or peri-sciatic-zymosan-induced inflammation.[26] In contrast, intraneural injection of *fractalkine* into the sciatic nerve was recently shown to attenuate neuropathic pain-related behavior in a spared nerve injury model of neuropathic pain in mice.[27]

In summary, *fractalkine* has pro-inflammatory, pain-modulating and fibrogenic effects. As all these three factors, that is inflammation, pain and fibrosis, determine the prognosis of CP patients and often necessitate surgical intervention, we studied *fractalkine* expression and its relation to inflammation, pain and fibrosis in the chronically inflamed pancreas.

MATERIALS AND METHODS
Patients and Tissues
A total of 61 CP tissue samples were collected from patients undergoing pancreatic head resection (45 males, age 46 ± 2 years; 16 females, age 42 ± 3 years). The etiology of the pancreatitis was alcoholic CP ($n = 26$), biliary CP ($n = 5$), idiopathic CP ($n = 24$) and autoimmune pancreatitis ($n = 6$). Written informed consent was obtained from all patients. Normal pancreatic tissue samples (NP) were collected from

organ donors whenever there was no appropriate recipient for transplantation ($n = 21$; 13 males, 8 females; median age 40 years). During organ procurement, the pancreas, like the other organs (kidney, liver) used for solid organ transplantation, was perfused with a University of Wisconsin solution (UW) or with histidine-tryptophan-ketoglutarate solution. Only pancreata that were explanted in our department were taken for further research studies. The pancreata were removed and transported in the same conservation solution on ice under sterile conditions after tissue preservation. Therefore, the median time from cross-clamp to processing was approximately about 30 min. This study was approved by the Ethics Committee of the University of Heidelberg (Heidelberg, Germany).

Resected pancreatic tissues were divided into several parts and the aliquots were (a) frozen in liquid nitrogen and stored at $-80°C$ for protein extraction, (b) taken into RNAlater (Ambion, Huntington, UK) for RNA extraction, (c) fixed in 5% paraformaldehyde, and later embedded in paraffin for immunohistochemical analysis and (d) immediately processed for hPSC isolation.

Reagents
The following reagents were purchased: RPMI-1640, DMEM, Trypsin-EDTA and penicillin–streptomycin from Invitrogen (Karlsruhe, Germany); fetal calf serum (FCS) from PAN Biotech (Aidenbach, Germany); *fractalkine* and *CX3CR1* rabbit polyclonal antibodies from Abcam (Cambridge, UK); *Collagen-1* goat polyclonal antibody from Santa Cruz Biotechnology (Heidelberg, Germany); *CD45*, *CD68* and *α-SMA* mouse monoclonal and *PGP9.5* rabbit polyclonal antibody from DAKO Cytomation (Hamburg, Germany) and *γ-tubulin* goat polyclonal antibody from Santa Cruz Biotechnology. The DAKO Envision system was used for immunohistochemistry and the ECL immunoblotting detection reagents from Amersham Biosciences (Buckinghamshire, UK) were used for western blot analysis. Recombinant human *fractalkine* was purchased from R&D Systems (Wiesbaden, Germany) and *TNF-α* from Chemicon (Temecula, CA, USA). All other chemicals were from Sigma Chemical Company (Taufkirchen, Germany).

Real-Time Light Cycler® Quantitative PCR
Extraction of mRNA from normal and CP tissues and of hPSCs was carried out by automated isolation using the MagNA Pure LC instrument and isolation kits I (for cells) and II (for tissue) (Roche Applied Science, Mannheim, Germany). cDNA was prepared using the first-strand cDNA synthesis kit for RT-PCR (Roche Applied Science). Quantitative real-time (PCR) QRT-PCR was carried out with the Light Cycler Fast Start DNA SYBR Green kit. Primers for *fractalkine*, *CX3CR1* and *MCP-1* were obtained from Search-LC (Heidelberg, Germany). The calculated number of specific transcripts was normalized to the housekeeping genes, cyclophilin B and hypoxanthine guanine phospho-ribosyltransferase,

and expressed as amount per microliter of input cDNA, as described earlier.[28]

Western Blot Analysis

Protein extraction and western blot analysis of cell culture monolayers or tissues were carried out as described earlier.[29] A total of 20 μg of protein/well in the tissue and cell lysates and 15 μg in the supernatants were separated and electro-blotted, and the membrane was exposed to *fractalkine* and *CX3CR1* antibodies (dilution of 1:2500 and 1:1000) at 4 °C overnight. Signal detection was performed using an enhanced chemiluminescence reaction. After film scanning, densito-metric analysis was carried out using the ImageJ software provided by the National Institutes of Health. Specific signal intensity was calculated and was corrected for the matching equal loading densities.[30] To ensure the specificity of the fractalkine antibody, HUVECs were stimulated with 10 ng/ml *TNF-α* for 12 h. Equal loading was assured by stripping the blots and re-probing with anti-γ-tubulin (1:5000).

Immunohistochemistry

Paraffin-embedded tissue sections of normal pancreas and CP samples were analyzed by immunostaining, using the DAKO Envision system with DAB as chromogene, as described earlier.[29] *PGP9.5*, *fractalkine* and *CX3CR1* antibodies were used at a dilution of 1:1000, 1:300 and 1:500, respectively. Standardized color reaction time was used for *fractalkine* (1.5 min) and *CX3CR1* (45 s) in all consecutive slides. Non-immunized rabbit IgG (DAKO) was used as a negative control. Digital imaging was performed using the Zeiss AxioCam HR system (Carl Zeiss AG, Oberkochen, Germany).

Clinicopathological Analysis

Consecutive sections obtained from the pancreatic tissue of each patient were stained with hematoxylin and eosin for concomitant histomorphological examination. Histopatho-logical analysis was carried out by two independent observers blinded to QRT-PCR, pain, *CD45* and *CD68* data, followed by resolution of any differences by joint review and con-sultation with a third observer. Histomorphological evalua-tion of the specimens included the severity of pancreatic neuritis and the severity of fibrosis, as previously earlier.[31] The severity of pancreatic neuritis was scored as absent (0), perineural (score I) or endoneural (score II). The severity of fibrosis was graded according to a scoring system (Table 1). The severity of fibrosis was revealed by the addition of in-tralobular and perilobular fibrosis scores as mild 0 (0–4), moderate I (5–9) or severe II (10–12) fibrosis.

No pathomorphological abnormalities were detected within the group of normal pancreas.

Neural Immunoreactivity

Analysis of the *fractalkine* and *CX3CR1* neuro-immunoreactivity in intrapancreatic nerves was performed by an independent observer (MWM) blinded to patient diagnosis, disease etiology, histopathological changes and pain history. From each section, all stained and unstained visible nerves were identified, using a consecutive *PGP9.5*-immunostained section of the same tissue as reference. All intrapancreatic nerves on each tissue section were analyzed for *fractalkine* and *CX3CR1* immunoreactivity. The evaluation included the intensity (none = 0, mild = 1, moderate = 2 or strong = 3) and the area of neural im-munoreactivity (< 30% = 1, 30–60% = 2 or > 60% = 3) for *fractalkine* and *CX3CR1*. To calculate the neural immunoreactivity score, intensity and area of immunoreactivity were multiplied. The median of all analyzed nerves was set as the representative score for *fractalkine* and *CX3CR1* neuro-immunoreactivity of every single patient.

Quantitative Analysis of Nerve Number and Total Nerve Area in CP

Intrapancreatic nerves in tissue sections of normal pancreas and CP patients were immunolabeled using the pan-neuronal marker *PGP9.5*. To quantify the total number and the total nerve area, the entire pancreatic tissue sections were scanned at high magnification (× 100) and reconstructed into a digital mosaic using the Zeiss KS300 program, with analysis of all *PGP9.5*-immunoreactive nerves as shown before.[31]

Pain Scoring and Disease Duration

In all CP patients, the individual pain score was registered before operation, including pain intensity and frequency as described earlier.[31] The intensity of pain was graded by using the scale as follows: 0 = none, 1 = mild, 2 = moderate and 3 = strong pain. The frequency of pain was graded as 1 = monthly, 2 = weekly and 3 = daily. To calculate the pain severity, pain intensity and frequency were multiplied. According to the final pain score, the patients were divided into three groups, as described earlier:[31,32] Pain 0 (0), representing the group of patients who did not have any pain; Pain I (1–3), representing the group of patients who suffered from mild pain; and Pain II (4–9), the group of patients with moderate-to-severe pain. Accordingly, patients' individual time period of disease (CP) was asked. The patients' disease duration was recorded in weeks.

Quantitative Analysis of *CD45* and *CD68* Immunoreactivity in CP

To quantify the intensity of inflammatory cell and macro-phage infiltration, tissue sections of CP patients were immunostained using the pan-leukocyte markers *CD45* and *CD68* for macrophages. Subsequently, the entire pancreatic tissue sections were scanned and reconstructed into a mosaic image using the Zeiss KS300 program. The inflammatory cells, immuno-labeled for *CD45* and *CD68*, were auto-matically detected and evaluated by the KS300 software in the scanned tissue sections. The intensity of *CD45* and *CD68* was expressed as the percentage of area occupied by

immunoreactive cells within the total area of the analyzed section.

Isolation of hPSCs
hPSCs were isolated from freshly resected pancreatic tissue of CP patients using the outgrowth method described by Bachem *et al*.[5] HPSCs were cultivated in a 1:1 dilution of Ham's F-12 nutrient medium and low-glucose DMEM supplemented with penicillin, streptomycin, amphotericin and 20% of FCS, as described earlier.[5]

Fractalkine stimulation of hPSCs
HPSCs were grown in six-well plates for protein and RNA extraction. When the cells reached 70% confluence, fresh medium was supplemented with 1% of FCS containing fractalkine (10 and 100 ng/ml). PBS was used as a control. Changes in *Collagen-1* and *α-SMA* mRNA expressions in

hPSCs were analyzed in cell lysates after 12, 24 and 48 h using QRT-PCR. After an incubation time of 72 h, western blot analyses of *α-SMA* (1:5000) and *Collagen-1* (1:1000) were performed in the hPSC lysates. Equal loading was assured by stripping the blots and re-probing with anti-γ-tubulin (1:5000). Furthermore, the secreted *Collagen-1* (1:1000) was analyzed in the hPSC supernatants, by western blot analysis, as shown earlier.[30] For this, 20 000 hPSCs were placed per well after a 24-h stimulation with *fractalkine*.

All experiments were repeated thrice.

Statistical Analysis
Statistical analysis was carried out using the GraphPad Prism 4 Software. The Shapiro–Wilk test was used to evaluate data distribution. Results are expressed as mean ± s.e.m. The Mann–Whitney *U*-test was carried out in the comparisons of intrapancreatic *fractalkine* and *CX3CR1* mRNA expressions in CP samples with normal control patients. For multiple comparisons, analysis of variance for random measures followed by Bonferroni's *post hoc* test was used. The relationship between *fractalkine* and *CX3CR1* mRNA expression/neural immunoreactivity with severity pancreatic neuritis, fibrosis, pancreatic neuropathy, duration of CP-disease and *CD45/CD68* immunoreactivities were examined using the Spearman's ρ-test. Two-sided *P*-values were always computed, and an effect was considered statistically significant at a *P*-value ≤ 0.05.

RESULTS
Pathomorphological Features and Pain Related to CP etiology
Among the four subtypes of CP (alcoholic, biliary, hereditary and autoimmune), the features of inflammatory cell infiltration (*CD45* and *CD68*) and neural hypertrophy were

Table 1 Scoring system for the evaluation of fibrosis in chronic pancreatitis

	Severity of fibrosis			
	Mild	Moderate	Severe	Score
Perilobular				
Focal	1	2	3	1–3
Diffuse	4	5	6	4–6
Intralobular				
Focal	1	2	3	1–3
Diffuse	4	5	6	4–6

Table 2 Relationship between the etiology of chronic pancreatitis and mRNA expressions of *fractalkine* and *CX3CR1*

	Alcoholic CP (n = 26)	Biliary CP (n = 5)	Idiopathic CP (n = 24)	AIP (n = 6)	*P*-value
Fibrosis score	9.2 ± 0.6	3.2 ± 2.0	7.6 ± 0.8	7.5 ± 2.0	= **0.022**
CD45	2.6 ± 0.4	1.4 ± 0.7	2.5 ± 0.5	3.8 ± 1.6	= 0.43
CD68	0.4 ± 0.06	0.1 ± 0.05	0.7 ± 0.4	1.1 ± 0.4	= 0.49
Neural hypertrophy[a]	5725 ± 1577	586 ± 356	7794 ± 2442	1637 ± 572	= 0.35
Neural density[b]	0.77 ± 0.1	0.20 ± 0.09	1.2 ± 0.2	0.53 ± 1.4	= **0.047**
Neuritis score	1.6 ± 0.1	0.4 ± 0.2	1.2 ± 0.1	1.0 ± 0.4	= **0.016**
Pain score	6.0 ± 0.7	1.2 ± 1.2	4.6 ± 0.7	3.0 ± 1.3	= **0.031**
Fractalkine[c]	228 ± 41	131 ± 37	313 ± 53	222 ± 39	= 0.31
CX3CR1[c]	44 ± 7	26 ± 13	45 ± 12	100 ± 20	= 0.07

AIP, autoimmune pancreatitis; CP, chronic pancreatitis.
[a]Expressed as μm².
[b]Nerve number in 1 mm² tissue section.
[c]Expressed as mRNA transcripts/μl.

not significantly associated with CP etiology (Table 2). Neural hypertrophy tended to be highest in hereditary and alcoholic CP, but did not reach statistical significance, whereas increased neurite density was uppermost in patients with hereditary and alcoholic CP (Table 2). In patients with biliary CP, the severity of fibrosis was not as pronounced as in those with hereditary, autoimmune or especially alcoholic CP. Similar discrepancies were observed regarding pain severity, with highest pain scores determined in patients with alcoholic CP (Table 2).

Expression of Fractalkine and *CX3CR1* in CP

Quantitative RT-PCR analysis was carried out to investigate *fractalkine* and *CX3CR1* mRNA expressions in NP and CP tissues. *Fractalkine* mRNA level of 279 ± 31 transcripts/μl (mean $\pm$ s.e.m.) did not differ from that of NP, with 238 ± 27 transcripts/μl (Figure 1a). In contrast, the *CX3CR1* mRNA level increased significantly ($P < 0.0001$) up to nearly three-fold in CP (48 ± 7) compared with NP (13 ± 2; Figure 1b). Neither *fractalkine* nor *CX3CR1* mRNA expression was significantly altered among alcoholic, biliary, hereditary or autoimmune CP (Table 2). Western blot analysis revealed a

remarkable increase for both fractalkine (8.0-fold) and *CX3CR1* (6.9-fold) proteins in CP compared with NP (Figure 1c and d). Sensitivity of the *fractalkine* antibody was ensured indicating upregulated *fractalkine* protein in HUVECs when stimulated with *TNF-α* (Figure 1e).

Immunolocalization of Fractalkine and *CX3CR1*

To define the localization of *fractalkine* and *CX3CR1* in CP and the normal pancreas, immunohistochemical analysis was carried out. In the normal pancreas, *fractalkine* showed a weak immunoreactivity in endothelial cells and moderate immunoreactivity in islets (Figure 2a and c). *CX3CR1* was detected only in inflammatory cells (Figure 2g–i, Table 3). In CP, the endothelial cells showed moderate *fractalkine* immunoreactivity (Figure 2d), which was also identified in tubular complexes and ductal cells, and strong immunoreactivity was identified in islets (Figure 2f, Table 3). More prominent changes were observed for *CX3CR1* in CP, with strong immunoreactivity in the smooth muscle of arteries, in tubular complexes and inflammatory cells (Figure 2j–l, Table 3). Regarding the acini in CP, both *fractalkine* and *CX3CR1* showed weak-to-moderate
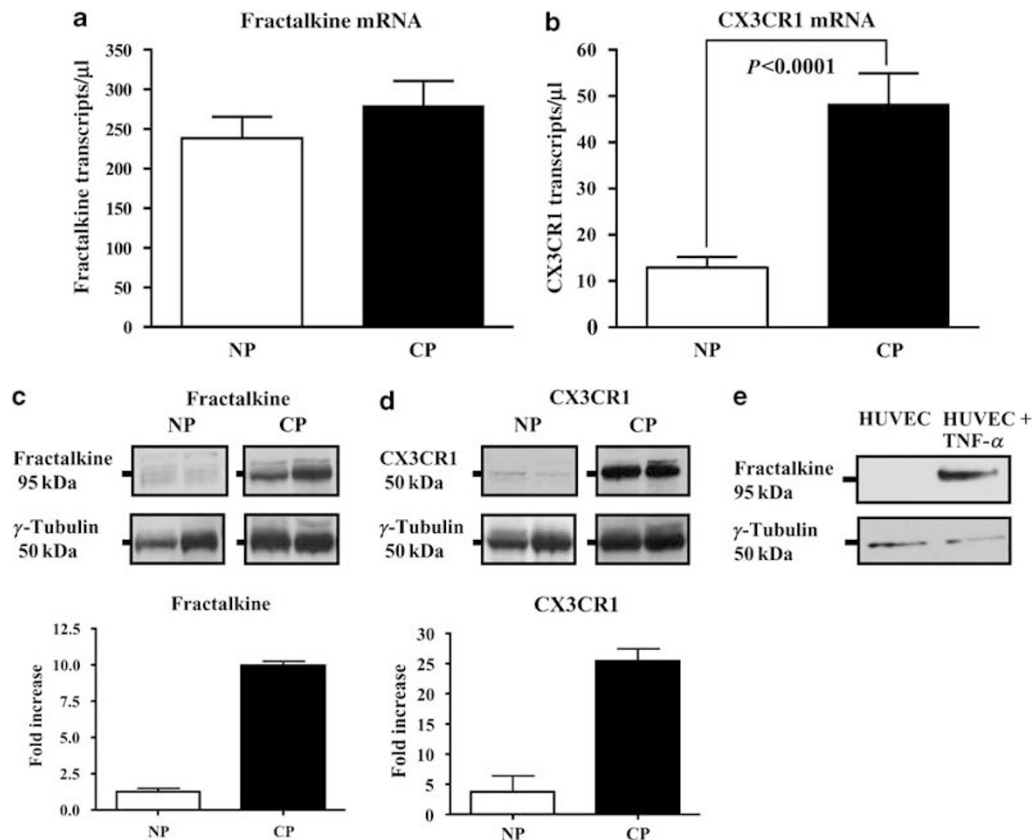

Figure 1 Quantitative RT-PCR analysis of *fractalkine* (**a**) and *CX3CR1* (**b**) in pancreatic tissue sections from patients with normal pancreas (NP) and chronic pancreatitis (CP). Western blot analysis of *fractalkine* (**c**) and *CX3CR1* (**d**) in NP and CP, showing an 8.0- and 6.9-fold increase, respectively, in CP compared with normal pancreas. (**e**) The sensitivity of *fractalkine* was ensured by the stimulation of HUVECs with 10 ng/ml of *TNF-α*, which is known to upregulate *fractalkine*. Equal protein loading is verified with γ-tubulin. Data are presented as mean $\pm$ s.e.m.
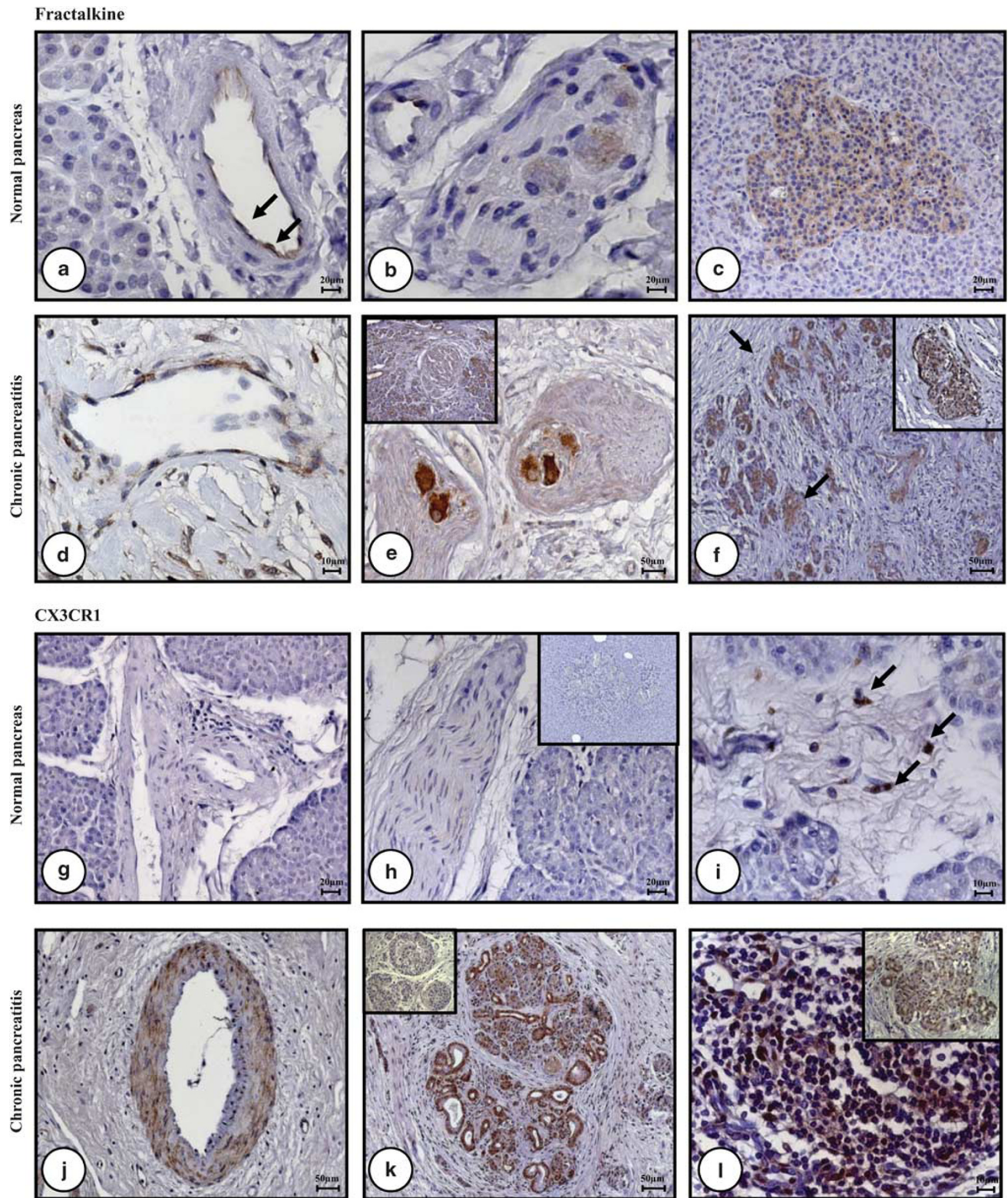
Figure 2 Immunolocalization of *fractalkine* and *CX3CR1*: In normal pancreas, *fractalkine* was weakly present in endothelial cells (see arrows in **a**) and ganglia, but was strong in islets (**a**–**c**). *Fractalkine* immunoreactivity in chronic pancreatitis (CP) showed moderate-to-strong accumulation in endothelial cells (**d**), in intrapancreatic ganglia and tubular complexes (**d**–**f**). Insets in panel (**e**) depict immunoreactivity of *fractalkine* in CP acini and islets (**f**). *CX3CR1* was only weak-to-moderate immunoreactive in inflammatory cells in the normal pancreas (**g**–**i**, inset in panel h shows islets in NP). In CP, *CX3CR1* immunoreactivity changed noticeably with strong immunoreactivities in arteries, tubular complexes and inflammatory cells (**j**–**l**, inset in panel k depicts islets and in panel l acini in CP).

Table 3 Localization of *fractalkine* and *CX3CR1* in NP and CP

	Acini	Ductal cells	Tubular complexes	Ganglia	Inflammatory cells	Arteries	Islets
NP							
Fractalkine	−	−		−/+	−	−/+	++
CX3CR1	−	−		−	+/++	−	−
CP							
Fractalkine	(+/++)	++	++	+++	+	++	+++
CX3CR1	(+/++)	+++	+++	++/+++	++/+++	++/+++	−

CP, chronic pancreatitis; NP, normal pancreas.

Semi-quantitative analysis of *fractalkine* and *CX3CR1* immunoreactivity in NP and CP.

−, absent; +, weak; ++, moderate; +++, strong immunoreactivity.

immunoreactivity only in the damaged acini (Figure 2f and k, inset in panel l; Table 3). The unaffected pancreatic acini showed neither *fractalkine* nor *CX3CR1* immunoreactivity.

Neural Immunoreactivity of *Fractalkine* and *CX3CR1*

In normal pancreas, axons, perineurium and endoneurium were *fractalkine*-negative with only weak immunoreactivity in intrapancreatic ganglia, whereas *CX3CR1* did not show any neural immunoreactivity at all (Figure 2b and h and Table 3). The staining pattern changed in CP with strong *fractalkine* and moderate-to-strong *CX3CR1* immunoreactivity in the intrapancreatic ganglia (Figures 2e and 3h). As shown in Figure 3a–h, the immunoreactivity of nerve fibers showed an assortment of diverse intensities for *fractalkine* and *CX3CR1* in different CP patients. It was remarkable that in nerves without neural infiltration of inflammatory cells, no or weak immunoreactivity of *fractalkine* and *CX3CR1* was detected (Figure 3a, c, e and g), whereas in nerves with perineural or endoneural mononuclear cell infiltration, the neural immunoreactivity for both *fractalkine* and *CX3CR1* was noticeably higher (Figure 3b, d, f and h).

Fractalkine/CX3CR1 and Pancreatic Neuritis

The severity of pancreatic neuritis was significantly associated with higher pain scores of the CP patients (Figure 4a). Patients with endoneural inflammatory cell infiltration revealed higher pain scores (II/5.4 ± 0.5) than those without any pancreatic neuritis (0/2.8 ± 0.8; Figure 4a). To investigate the possible influence of *fractalkine* on pancreatic neuritis, we related the severity of pancreatic neuritis to the neural immunoreactivity of *fractalkine*. *Fractalkine* neuro-immunoreactivity scores (II/3.9 ± 0.5) were significantly ($P < 0.05$) stronger in patients with endoneural pancreatic neuritis than those with no pancreatic neuritis (0/1.2 ± 0.6; Figure 4b). Correspondingly, increased tissue *fractalkine* mRNA expression was linked to severe pancreatic neuritis (341 ± 54 transcripts/μl compared with no neuritis (141 ± 27

transcripts/μl; Figure 4d). Both neural immunoreactivity and mRNA expression of *CX3CR1* tended to increase with the severity of pancreatic neuritis, but did not reach statistical significance (Figure 4c and e).

Fractalkine/CX3CR1 and Pancreatic Pain

In the investigated collective, 15 patients had no pain (Pain 0), 8 patients had mild pain (Pain I) and 38 patients had moderate-to-severe pain (Pain II). *Fractalkine* mRNA expression was positively related to the severity of pain in CP and was significantly ($P < 0.05$) increased in patients with severe pain (Pain II, 325 ± 42 transcripts/μl) compared with those without pain (Pain 0, 147 ± 21 transcripts/μl; Figure 5a). *CX3CR1* was also increased in the Pain II group compared with the Pain 0 group, but failed to reach statistical significance (Figure 5b). These results were confirmed on the protein level: the *fractalkine* protein level was positively related to the severity of pain in CP and was increased in patients with severe pain (Pain II, 4.7-fold) compared with those without pain (Pain 0, Figure 5c). Similarly, the *CX3CR1* protein content was also increased in the Pain II group compared with the Pain 0 group (3.5-fold, Figure 5d). Furthermore, the duration of CP-associated complaints was significantly related to the neural immunoreactivity of fractalkine (Table 4). Patients with the longest history of CP showed the strongest *fractalkine* neuro-immunoreactivity. This phenomenon was not observed with *CX3CR1* (Table 4).

To study, whether neural *fractalkine* immunoreactivity was interfering with the pain sensation, *fractalkine* and *CX3CR1* were related to the severity of pain. *Fractalkine* and *CX3CR1* neural immunoreactivity revealed a significant association with the pain sensation in CP (Figure 5e and f). *Fractalkine* ($P < 0.002$) and *CX3CR1* ($P < 0.001$) neural immunoreactivity was significantly increased in patients with severe pain (Pain II) when compared with those without pain (Pain 0) (Figure 5e and f). *CX3CR1* immunoreactivity even differed significantly ($P < 0.05$) between the moderate Pain I and severe Pain II group (Figure 5f).
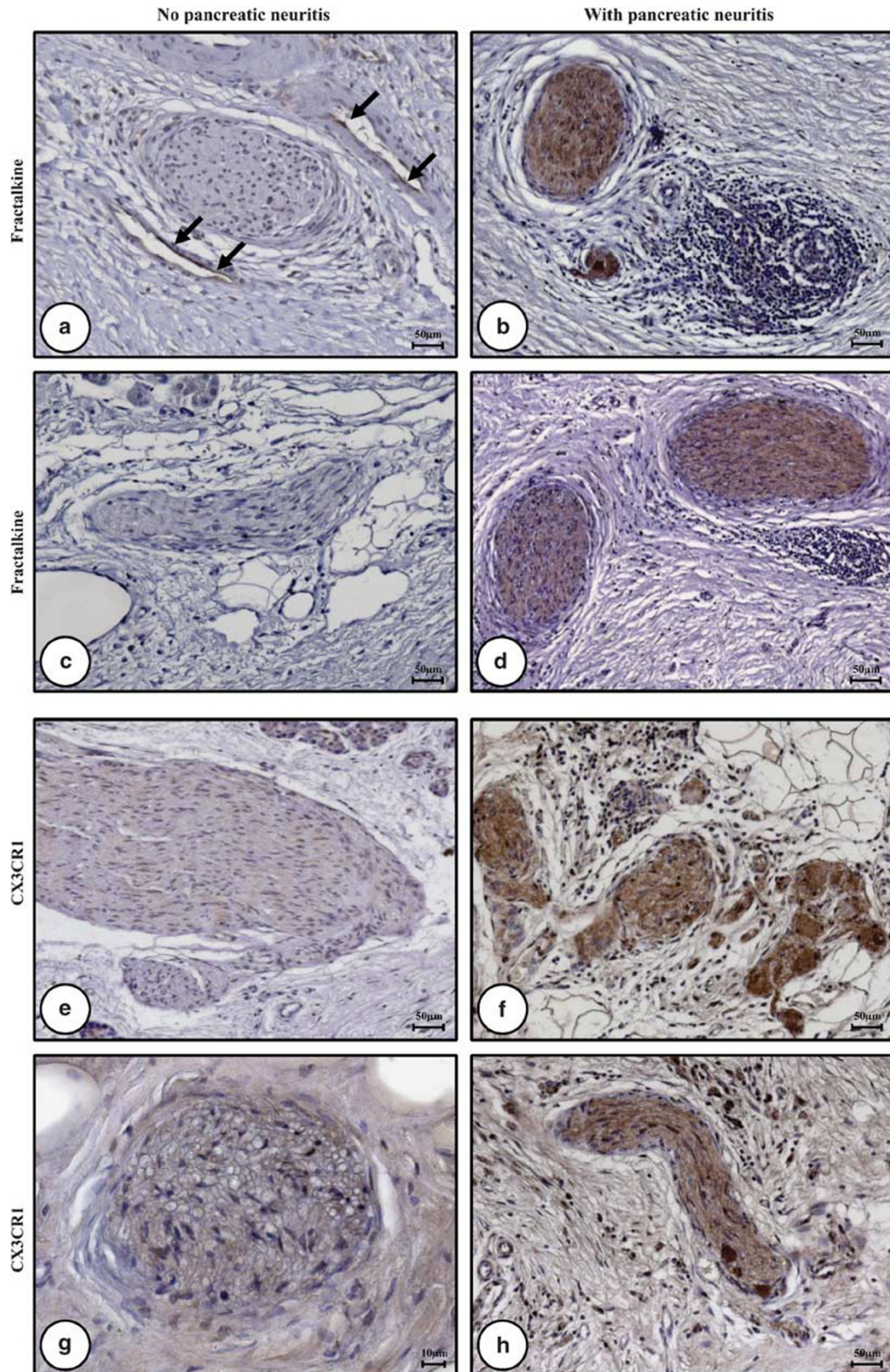
Figure 3 In CP, immunoreactivity of *fractalkine* (**b** and **d**) and *CX3CR1* (**f** and **h**) was markedly increased in intrapancreatic nerves with inflammatory cell infiltration (pancreatic neuritis) than without (**a**, **c** and **e**, **g**, respectively). (**a**) Endothelial cells immunoreactive (see arrows) for *fractalkine*, but missing neural immunoreactivity.
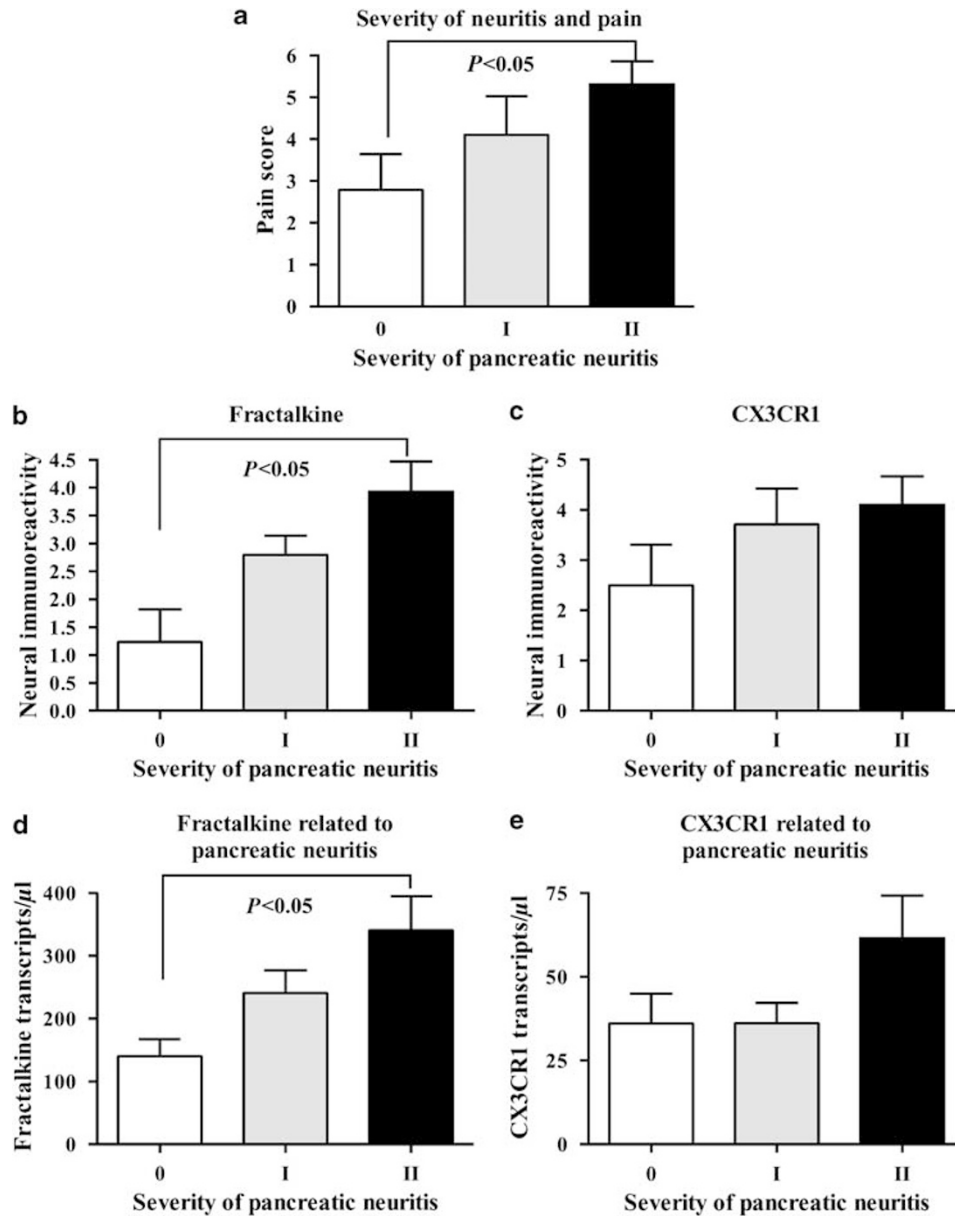
Figure 4 (**a**) The severity of pancreatic neuritis was significantly associated with pain sensation in CP. (**b**) Neuro-immunoreactivity of *fractalkine* was closely linked to the severity of pancreatic neuritis, whereas (**c**) *CX3CR1* neuro-immunoreactivity tended to increase with enhanced pancreatic neuritis, but failed to reach statistical significance. Correspondingly, tissue (**d**) *fractalkine* mRNA expression was also associated with the severity of pancreatic neuritis, and again (**e**) *CX3CR1* transcripts showed the same trend, but was again not significant.

Fractalkine/CX3CR1 and Neural Alterations in CP

Owing to the prominent neural activity of fractalkine and *CX3CR1* in CP, we studied their possible influence on CP-associated neuropathic changes, characterized by increased nerve fiber density and hypertrophy. A quantitative analysis revealed an evident increase in the nerve fiber density and total nerve area in CP compared with NP, as reported before ($P < 0.001$, data not shown).[31,33] However, *fractalkine* and *CX3CR1* mRNA expressions and neural immunoreactivity did not correlate with changes in nerve fiber density and hypertrophy in CP (data not shown).

Fractalkine/CX3CR1 and Inflammatory Cell Infiltration in CP

To investigate the possible influences of *fractalkine* and *CX3CR1* expressions on lymphocyte and macrophage infiltration into the damaged CP tissue, mononuclear cells were labeled with *CD45* and *CD68*, respectively. The area of infiltrating *CD45*-positive lymphocytes in CP correlated positively with both *fractalkine* ($P < 0.001$, Figure 6a) and *CX3CR1* mRNA expressions ($P < 0.02$, Figure 6b). *Fractalkine* mRNA levels did not correlate with the area of *CD68*-positive cells ($P = 0.1$, Figure 6c), whereas *CX3CR1*
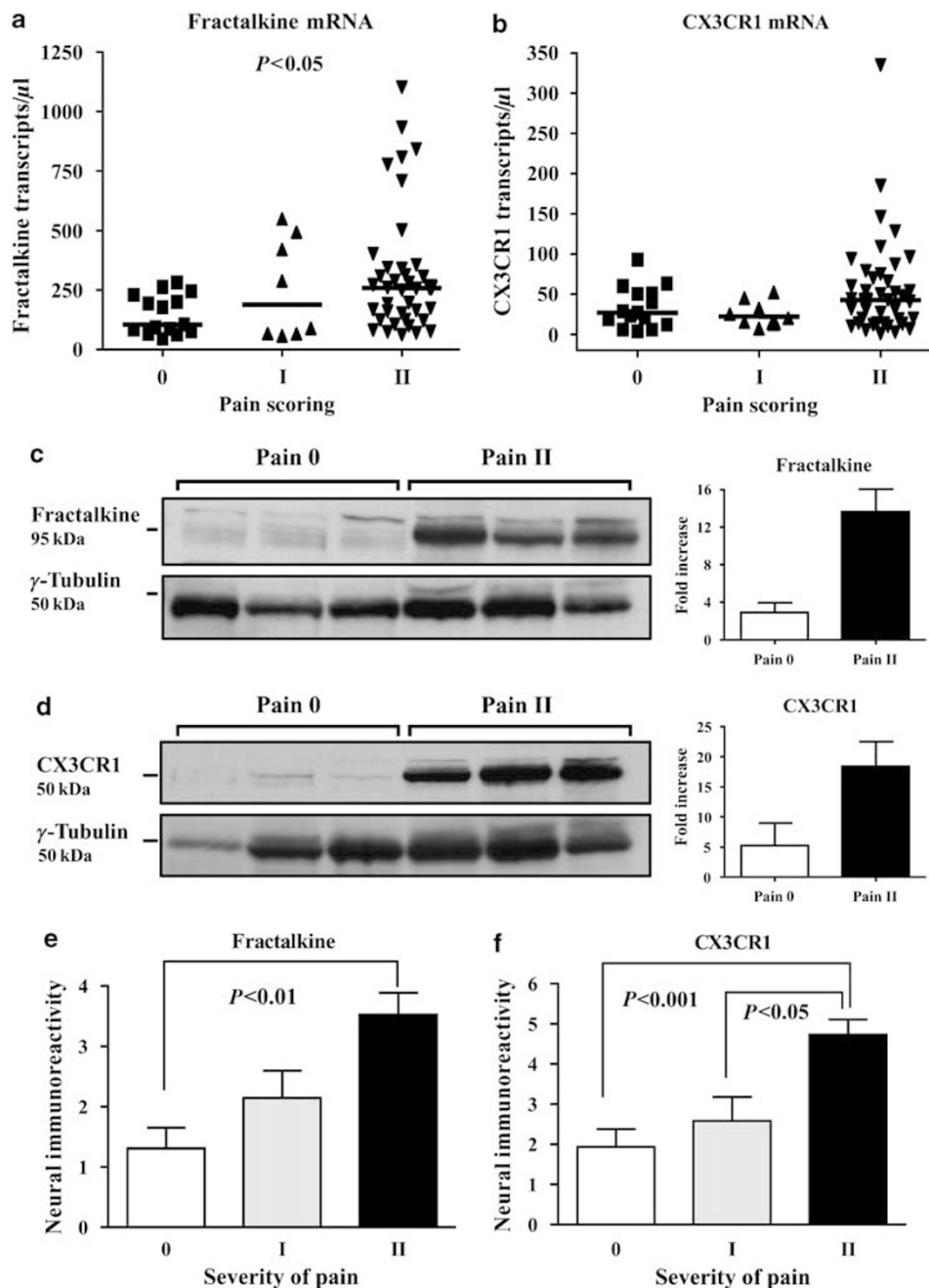
Figure 5 (**a**) *Fractalkine* mRNA expression was positively related to the severity of pain in CP ($P < 0.05$), whereas *CX3CR1* tended to increase in CP, but failed to reach statistical significance (**b**). Western blot analysis identified an increase in *fractalkine* (**c**) and *CX3CR1* (**d**) in patients with moderate-to-severe pain (Pain II; $n = 3$) compared with those without pain (Pain 0; $n = 3$). Equal protein loading is verified with γ-tubulin. Neural immunoreactivity of *fractalkine* (**e**) and *CX3CR1* (**f**) was significantly related to the severity of pain in patients with CP. Data are presented as mean ± s.e.m.

mRNA expression ($P < 0.0001$, Figure 6d) presented a highly significant correlation with the infiltration area of macrophages in CP. To evaluate whether the intrapancreatic infiltration of lymphocytes and especially macrophages are involved in pain sensation, the immunoreactivity of *CD45* and *CD68* was related to pain severity in CP patients. The percentage of *CD45*-positive lymphocytes was positively related to the severity of pain in CP, whereas *CD68*-positive

macrophages did not show any significant association with pain sensation (Figure 6e and f).

Fractalkine and Fibrosis in CP
The degree of fibrotic tissue remodeling in CP was closely associated with pain sensation in CP patients (Figure 7a). Patients with severe and frequent pain (9.2 ± 0.5) showed the worse fibrotic tissue reactions when compared with CP

Table 4 Correlation analysis of *fractalkine/CX3CR1* neuro-immunoreactivity with disease duration of CP

	CP disease duration	
	r-value	*P*-value
Fractalkine	**0.65**	**0.002**
CX3CR1	0.15	0.45

CP, chronic pancreatitis.

r-value: Spearman's correlation coefficient.

patients with no pain sensations at all (4.6 ± 1.2; Figure 7a). *Fractalkine* mRNA expression was associated with the severity of fibrosis in CP, whereas *CX3CR1* failed to reach statistical significance (Figure 7b and c). *Fractalkine* expression showed a significant ($P < 0.01$) increase in the fibrosis group II (346 ± 51 transcripts/μl) compared with group 0 (114 ± 75 transcripts/μl, Figure 7b). To evaluate the putative impact of *fractalkine* on fibrogenesis in CP, isolated hPSCs were incubated with different concentrations of *fractalkine*. Stimulation with *fractalkine* for 24 h decreased the expression of α-SMA and *Collagen-1* mRNA in isolated hPSCs in a nonsignificant manner (data not shown). In the hPSC lysates, the α-SMA and *Collagen-1* content did not change (Figure 7d). Corresponding to the mRNA data, *Collagen-1* in the hPSC supernatants gradually decreased with higher concentrations of *fractalkine* (Figure 7e).

DISCUSSION

The currently accepted definition for *neuropathic pain* is 'pain arising as a direct consequence of a lesion or disease affecting the somato-sensory system.'[34] Although pain in CP would traditionally be classified as inflammatory pain, recent studies pointed to the simultaneous presence of structural changes in axons and ganglia in CP, thus implying the involvement of a concomitant neuropathic component.[35,36,58] Previous studies have determined that the 'inflammatory' and the 'neuropathic' component of pain in CP may not be independent but instead closely linked: The 'neuro-immune crosstalk' through pancreatic neuritis, and changes in intrapancreatic neural plasticity were all associated with the severity of pain experienced by CP patients.[8,10,33,58] In addition, recent studies support the idea that the peripheral immune system and spinal microglia are crucial in the generation of neuropathic pain.[35,37,58]

The contribution of *fractalkine* to general neuropathic pain was shown earlier: In several experimental models of neuropathic pain, it was shown that endogenous *fractalkine* is released by DRG and is accompanied by the upregulation of *CX3CR1* in the spinal microglia and neuropathic pain behavior.[12,14,23,24,38,39] Similar effects were observed when *fractalkine* was administered exogenously.[40] In both cases,

neutralizing antibodies to *CX3CR1* blocked and attenuated neuropathic pain.[23,24,39–42] A recent spared nerve injury model of neuropathic pain in mice showed attenuation of neuropathic pain-related behavior after intraneural injection of *fractalkine* into the sciatic nerve.[27] Although a discrepancy between the central pro-algesic and peripheral anti-algesic effect of *fractalkine* is a possibility, these studies once again underline the pain-modulatory feature of *fractalkine*.

Fractalkine is released by neurons after a nerve injury, attracts neutrophilic granulocytes to the sites of damage and leads to macrophage infiltration.[23,37,43] One can imagine that *fractalkine* might be inducing neural inflammatory cell infiltration in CP after its release from intrapancreatic nerves, and thus be a key mediator of pancreatic neuritis. The participation of a chemokine as the mediator in this process is supported by the fact that increasing inflammatory cell infiltration around sympathetic nerve fibers is in part triggered by cytokine and chemokine release by macrophages.[44] On the basis of our results, in CP, *fractalkine* seems to be involved in the recruitment of mononuclear cells to the pancreas. However, without the presence of a mediator between the intraneural and extraneural milieu, the sole presence of mononuclear cells in the pancreatic tissue may not be sufficient to both sensitize nociceptors and to induce neuropathic pain. Although *fractalkine* originating from intrapancreatic nerves might well be such a mediator, it would not be able to reach the extraneural milieu unless the barrier function of the perineurium is lost. Here, one should note that damage to the perineurium of intrapancreatic nerves is a frequently observed phenomenon in CP.[8] One can imagine that once this barrier function is lost, neural *fractalkine* would exit the intraneural space and enhance mononuclear cell infiltration by acting as a chemoattractant ('inside-out effect'). On the other hand, inflammatory mediators released by mononuclear cells, such as *TNF-α* or *IL-1*, can gain access to the axons through the damaged perineural barrier ('outside-in effect') and induce neuropathic pain.[45,46] Considering the presented data, it does not seem to be surprising that the severity of pancreatic neuritis and the severity of pain are closely related to neuronal *fractalkine* expression. Our results suggest that neuro-immune interactions are also of major importance for the development and maintenance of visceral pain in CP as proposed earlier for neuropathic pain after somatic nerve damage.[47] The lack of a correlation between *CD68* and pain sensation does not necessarily exclude a role for macrophages in pain in CP. In fact, the levels of *pituitary adenylate cyclase-activating polypeptide (PACAP)* secreted by macrophages was earlier shown to correlate with pain sensed by CP patients.[48] Similarly, there was a strong immunoreactivity for *PACAP* in the perineurium of hypertrophic nerves and in the areas of high immune cell infiltration. In this regard, future studies should aim at determining the distribution of immune cell subtypes infiltrating the nerves, that are neuritis-associated immune cells.
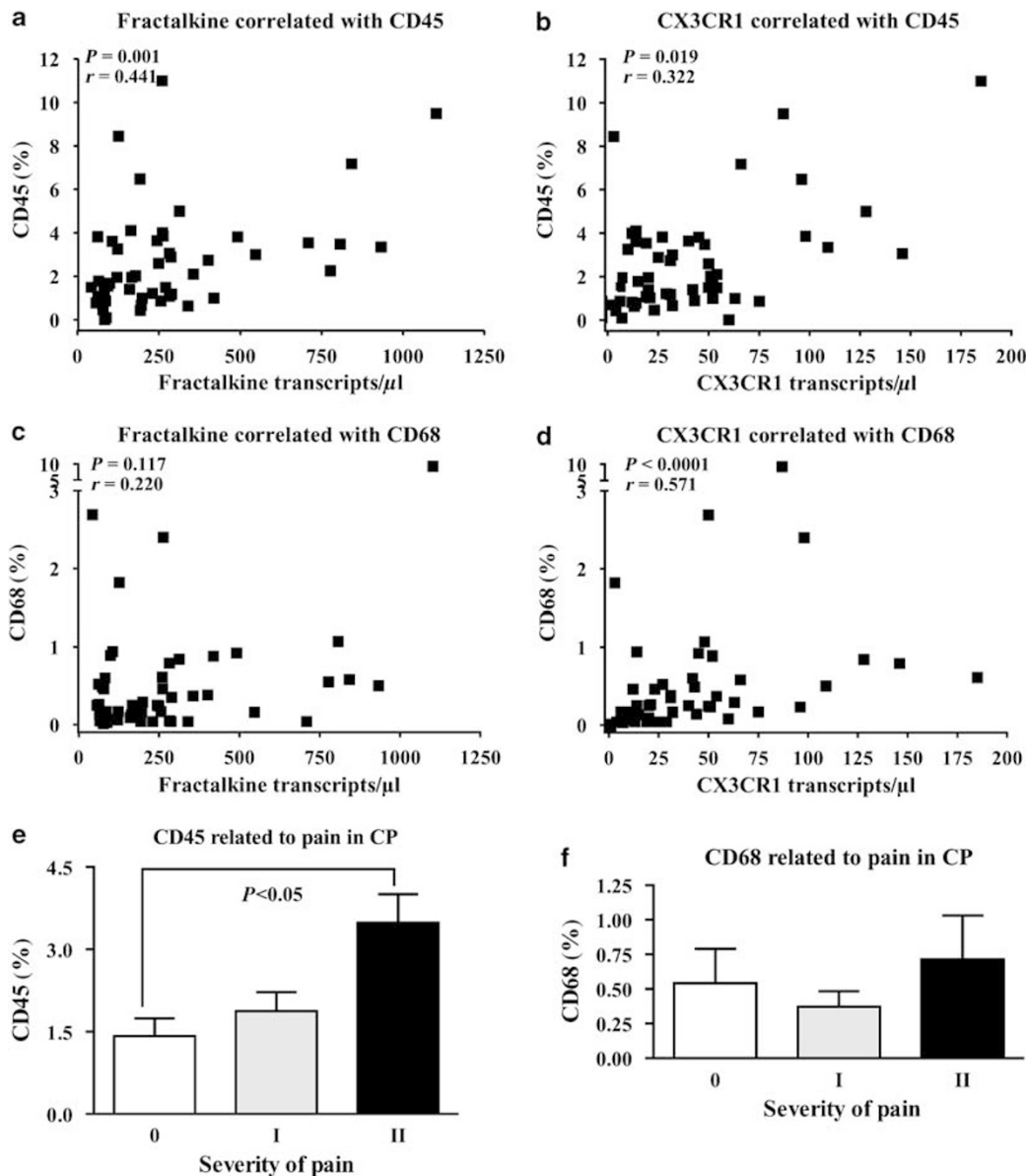
Figure 6 *Fractalkine* and *CX3CR1* mRNA expressions correlated with inflammatory cells immunopositive for *CD45* and CD68. The number of infiltrated CD45-positive cells in CP correlated positively with both *fractalkine* (**a**) and *CX3CR1* (**b**). The number of infiltrating macrophages correlated significantly higher with *CX3CR1* mRNA expression (**d**), but not with *fractalkine* (**c**). (**e**) *CD45*-positive inflammatory cell infiltration was positively related to the severity of pain in CP, but (**f**) CD68-positive macrophages did not show any relation to pain. Data are presented as mean ± s.e.m.

There is increasing body of evidence that pain in CP is of neuropathic origin.[58] Although several components of pancreatic neuropathy, including increased neural density and hypertrophy, were shown to be linked to pain sensation in CP, the role of another possible contributor, namely glia cells in CP, remains uninvestigated. In CP, increased neural and tissue fractalkine did not account for all the mechanisms of pain in CP, as changes in intrapancreatic neural plasticity were found as *fractalkine*-unrelated correlates of pain. The key role of glia cells, including Schwann cells in the peripheral nervous system, is well established in several neuropathic pain states.[37] In this regard, the very recently recognized role

of *fractalkine* in neuropathic pain through microglial activation deserves special consideration.[49] Indeed, although our study lacks a correlation between *fractalkine* expression and the morphological correlates of neural density and hypertrophy, *fractalkine* may well be exerting its neuropathic pain-modulatory effect through the activation of intrapancreatic Schwann cells, a possibility that certainly deserves to be a subject of future studies. With this study, we show, for the first time, *fractalkine* as an additional key player in clinical visceral pain. Moreover, the higher average *fractalkine* neural immunoreactivity in patients with a longer standing history of CP brings up the idea that there might be an accumulation
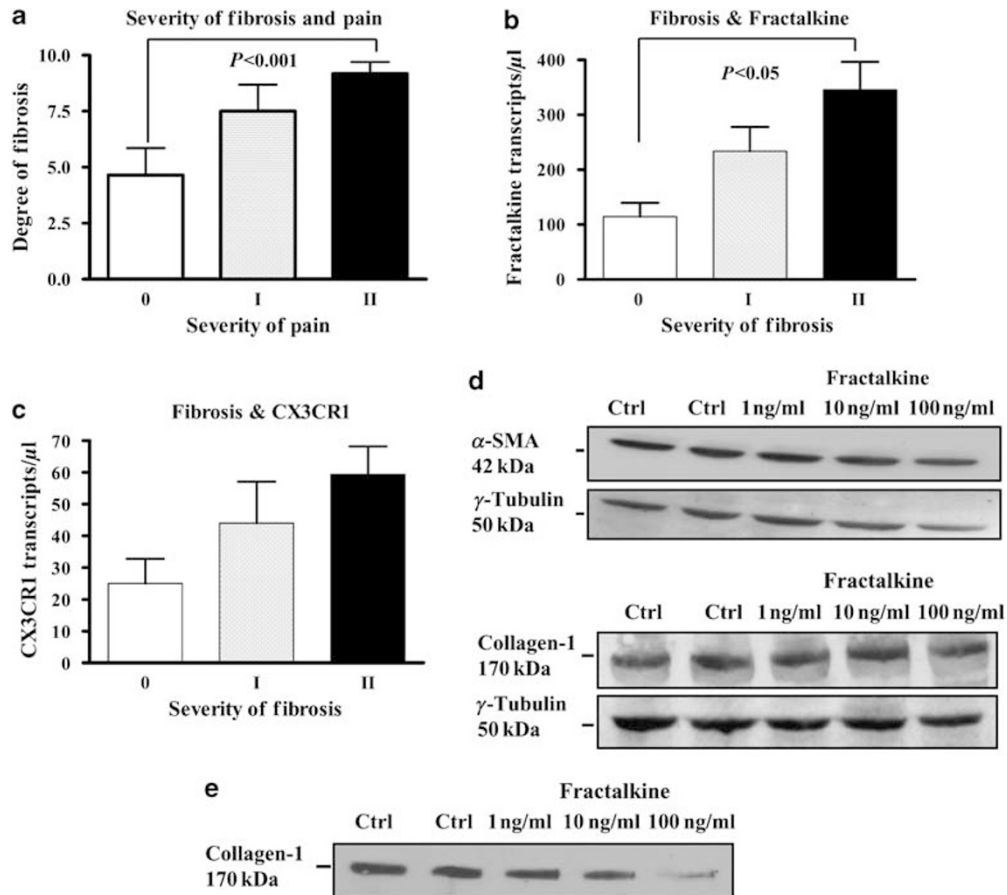
Figure 7 (**a**) The severity of fibrosis was significantly linked to the pain sensation of CP patients. (**b**) *Fractalkine* mRNA expression was associated with the severity of fibrosis in chronic pancreatitis, whereas (**c**) *CX3CR1* failed to reach statistical significance. Isolated human pancreatic stellate cells (hPSC) were incubated with different concentrations of fractalkine at different time points, followed by *α-SMA* and *Collagen-1* mRNA, and protein detection. (**d**) In the hPSCs lysates, no changes on protein level were detected for *α-SMA* and *Collagen-1* after fractalkine stimulation. Equal protein loading was verified with *γ*-tubulin. (**e**) Corresponding to the mRNA data, *Collagen-1* in the hPSC supernatants gradually decreased with higher concentrations of fractalkine.

of *fractalkine* in intrapancreatic nerves of CP patients over time. This contribution of *fractalkine* seems to distinguish it from our control chemokine, the *MCP-1*, which is secreted by macrophages at sites of neural injury.[50] Contrary to *fractalkine*, *MCP-1* expression did not show any significant association with pain sensation in our study (data not shown). However, whether this upregulation of *fractalkine* and *CX3CR1* in CP is a pro-algesic or a counter-regulatory anti-algesic mechanism remains unknown, and should be a subject for future investigations.

Cytokines, such as *TNF-α*, and chemokines, such as *IL-1*, *IL-6* and *MCP-1*, are not only related to nociceptor sensitization but also involved in the development of fibrosis in CP by interfering in hPSCs proliferation and ECM metabolism.[51–53] As a result of increased recruitment of inflammatory cells, activated mononuclear cells release profibrotic cytokines and chemokines, such as *TGF-β*, *TNF-α* and *MCP-1*, which in turn activate hPSCs and promote fibrosis in CP.[4,52,53] The chemokine *MCP-1*, one of the major monocyte chemoattractants, is highly expressed in fibroblasts

in CP, induces *TGF-β* upregulation and is associated with the progression of pancreatic fibrosis.[52,54] Blocking of *MCP-1* attenuates the degree of fibrosis in experimental CP.[52] Regarding *fractalkine* and fibrosis, *CX3CR1* was identified to have a genetic and functional importance in higher stages of liver fibrosis in chronic hepatitis C, and was noticeably upregulated in patients with enhanced renal fibrosis.[21,22] In experimental post-ischemic renal fibrosis, *fractalkine* and *CX3CR1* expression levels were associated with enhanced inflammation and fibrosis, which could be diminished by blocking antibodies to *CX3CR1* through the reduction of *α-SMA* and *Collagen-1*.[55] In human CP, this interaction appears to be different. Although *fractalkine* was associated with the severity of fibrosis, *fractalkine* had no significant impact on the activation of hPSCs, and even tended to reduce the activation of isolated hPSCs by decreasing the mRNA expression of *α-SMA* and especially that of *Collagen-1*. Regarding our presented data, *fractalkine* does not seem to be directly involved in the fibrogenesis processes in CP, as it was observed in renal and liver fibrosis.[21,22] In all likelihood, it

promotes tissue fibrosis indirectly by increasing the mono-nuclear cell infiltration and not through the activation of hPSCs. This hypothesis is supported by recent reports: (1) Ishida and colleagues[56] showed that *CX3CR1* depletion induced a marked reduction in the number of macrophages and in the release of *TGF-β1*, and observed reduced α-*SMA* and *Collagen-1* depositions in the skin from wounded *CX3CR1⁻/⁻* mice. (II) Considering that macrophages activate stellate cells and that suppression of macrophage infiltration inhibits hepatic stellate cell activation and liver fibrosis, the *fractalkine/CX3CR1*-triggered macrophage tissue infiltration might represent another possible contributor to fibrogenesis in CP.[57]

In conclusion, as a result of intrapancreatic neuropathic changes in CP, *fractalkine* may be involved in pain modulation through neural activation and through the attraction of inflammatory cells to intrapancreatic nerves. *Fractalkine* is exceptional among other chemokines because it binds selectively to the *CX3CR1*, and this receptor binds only to *fractalkine*.[13,15,33] Thus, it is an interesting idea that blocking a single chemokine receptor might influence abdominal pain sensation, inflammation and may be also fibrosis in CP. Therefore, further studies are needed to elucidate the particular role of *CX3CR1* in CP and to assess its potential role as a novel therapeutic target.

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