Neural alterations in surgical stage chronic pancreatitis are independent of the underlying aetiology

H Friess, S Shrikhande, M Shrikhande, M Martignoni, C Kulli, A Zimmermann, A Kappeler, H Ramesh, M Büchler

Background and aims: Among various causes, nerve alterations and neuroimmune interactions have been suggested to participate in the generation of pain in chronic pancreatitis (CP). In this study, we compared neural changes and the pattern of perineural inflammatory cell infiltrates in three different aetiological forms of CP (alcoholic, idiopathic, and tropical) and evaluated whether differences exist between these groups.

Patients and methods: A total of 35 patients with CP (12 tropical, 12 idiopathic, and 11 alcoholic) were included. Ten normal pancreatic tissues obtained from healthy organ donors served as controls. In all samples, the number of nerves, area of neural tissue, nerve size, and percentage of neural tissue and perineural inflammatory cell infiltrates were analysed histologically.

Results: The median number of nerves per 10 mm² tissue area was 2.3, 4.3, 4.4, and 2.6 in the normal pancreas, alcoholic CP, idiopathic CP, and tropical CP, respectively. Median area of neural tissue per 10 mm² was 2550, 21803, 18595, and 24666 µm² in the normal pancreas, alcoholic CP, idiopathic CP, and tropical CP, respectively. Median nerve diameter was 36.85 µm in the normal pancreas, 80.6 µm in alcoholic CP, 68.95 µm in idiopathic CP, and 93.05 µm in tropical CP. In comparison with normal controls, all of these parameters were significantly increased except the number of nerves in tropical CP. For all parameters there were no significant differences between alcoholic, idiopathic, and tropical CP. When the degree of perineural inflammation was evaluated, no differences were observed among the three CP groups.

Conclusions: Independent of the underlying aetiology, CP is associated with an increase in neural tissue, and neural alterations occur in a similar fashion irrespective of the type of initiating event.

Chronic pancreatitis (CP) is an inflammatory disease primarily of the exocrine pancreas which leads to persistent progressive morphological alterations and physiological dysfunction. In the past few years several aetiological factors of CP have been recognised, contributing to a better understanding of the disease. We have learnt that mutations in the cationic trypsinogen gene are present in patients with hereditary CP, and it has been reported that approximately 30% of patients with so-called idiopathic CP have mutations in the cystic fibrosis transmembrane conductance regulator gene. Although the morphological changes in CP have been recognised and described for many decades, the pathophysiology of CP is still poorly understood and it is not known whether the pathophysiological events in different CP aetiologies are similar or diverse.

In Western industrialised countries, the dominant aetiological factor of CP is alcohol abuse, accounting for approximately 80% of patients. In a small percentage of patients with CP alcohol abuse can be clearly ruled out as an aetiological factor, and if other known aetiological factors are absent, these patients are then classified as having idiopathic CP. In contrast with Western countries, in Southern India an alcohol independent form of CP—so-called tropical pancreatitis—is common. Here there is a chronic calcifying form of CP in a younger age group in a region of high incidence without any history of alcohol consumption. The aetiology of tropical CP is largely unknown, and nutritional and environmental factors have been propagated since the 1970s and 1980s as major causative factors in the aetiopathogenesis of this form of CP.

In alcoholic CP, changes involving neural proliferation with the resultant increase in number of nerves and nerve diameter, damage to the perineurium, increase in sensory neurotransmitters, and a neuroimmune interaction of inflammatory cells with altered nerves were reported recently. Furthermore, these changes were suggested to be involved in pain in alcoholic CP. However, idiopathic and tropical CP are also associated with pain. Therefore, if nerves are involved in the pathogenesis of pain in CP, similar neural alterations should be present in CP independent of the underlying aetiology. Similar neural changes would provide further evidence with regard to the significance of neural alterations in the pain pathogenesis of CP. Therefore, in the present study, neural changes were analysed and compared in three different aetiological forms of CP: alcoholic, idiopathic, and tropical CP. As inflammatory cell infiltrates are regularly found in alcoholic CP and a close interaction between inflammatory cells, pancreatic nerves, and the presence of pain was recently reported, perineural inflammatory cell infiltrates were also evaluated.

PATIENTS AND METHODS

Patients

Pancreatic tissue samples of 35 patients (25 males and 10 females; mean age 37 (11.8) years) with CP and pancreatic resection were included in the study. No duct samples were included and only acinar tissue was used in this study. Aetiology was alcohol in 11 patients (nine males, two females; mean age 43.8 (8.1) years) and idiopathic in 12 patients (six males, six females; mean age 33.5 (15.1) years). While all had

Abbreviations: CP, chronic pancreatitis; PGP 9.5, protein gene product 9.5; TBS, Tris buffered saline.
an inflammatory mass in the pancreatic head, there was some obstructive element with ductal dilatation in all patients except one. All patients with alcoholic and idiopathic CP were operated on at the Inselspital, University of Bern. Pancreatic tissue from 12 patients with tropical CP (10 males, two females; mean age 33.8 (9.2) years) who were operated on in India were also included. While eight tropical CP patients had a dilated duct disease, four had an inflammatory mass in the head of the pancreas. The degree of ductal dilatation was in the range 7–10 mm. The indication for surgery, both in Bern and in India, was uncontrollable pain and patients had either an inflammatory mass in the head of the pancreas leading to obstructive complications on pancreatic neighbouring organs, a dilated main pancreatic duct, or a combination of both. Overall, no major differences were observed between European and Indian subjects. For patients with idiopathic CP alcohol abuse and other known causes of CP were ruled out. The same was true for Indian patients with tropical CP. Additionally, 10 normal human pancreatic tissue samples, obtained through an organ donor programme, served as controls. There were five male and five female organ donors (mean age 44.4 (17.1) years). The studies were approved by the human subjects committee of the University of Bern.

### Table 1 Comparison of nerve related parameters in the different aetiological groups of chronic pancreatitis (CP)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal pancreas</th>
<th>Alcohol CP</th>
<th>Idiopathic CP</th>
<th>Tropical CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median tissue area analysed (mm²)</td>
<td>80.9 (61–125)</td>
<td>97.9 (57.9–149.7)</td>
<td>62 (42.3–85.9)</td>
<td>120.5 (77–174.7)</td>
</tr>
<tr>
<td>Median No of nerves per 10 mm² tissue area</td>
<td>2.3 (1.2–2.8)</td>
<td>4.3 (2.4–5.6)</td>
<td>4.4 (3.9–6.6)</td>
<td>2.6 (2.2–3.9)</td>
</tr>
<tr>
<td>Median nerve area (µm²)</td>
<td>2550 (1474-3506)</td>
<td>21 803 (10382-39554)</td>
<td>18 595 (11808-43263)</td>
<td>24 666 (14438-46589)</td>
</tr>
<tr>
<td>Median nerve diameter (µm)</td>
<td>36.8 (34.2–42.2)</td>
<td>80.6 (63.1–117.4)</td>
<td>68.9 (58.3–112.5)</td>
<td>93.0 (81.7–146.3)</td>
</tr>
<tr>
<td>Median (Mean area of perineural foci with density score 1 × No of perineural foci with density score 1 + No of perineural foci with density score 2 × No of perineural foci with density score 2 + No of perineural foci with density score 3 × No of perineural foci with density score 3) × 1000/Area of tissue evaluated (mm²).</td>
<td>0.025 (0.014–0.035)</td>
<td>0.21 (0.10–0.39)</td>
<td>0.16 (0.11–0.43)</td>
<td>0.24 (0.14–0.46)</td>
</tr>
<tr>
<td>Median percentage of neural tissue [%]</td>
<td>19</td>
<td>20</td>
<td>40</td>
<td>30</td>
</tr>
</tbody>
</table>

Values are median (lower and upper quartiles).

**Quantitative analysis of neural tissue**

Analysis of pancreatic nerves was carried out by two independent observers with the help of digital image analysis and the Image Pro-plus software (Media Cybernetics, Silver Spring, Maryland, USA). A charge coupled device video camera module and PROSCAN electronics 5000 (Hama, Germany) connected to the Image Pro-plus version 3.1 software (Media Cybernetics) were used to measure the entire area of each tissue section, number of nerves, and nerve area. From the recorded measurements, average area of nerves per 10 mm² tissue area and the percentage of tissue occupied by nerves were calculated. Also, innervation area per nerve and number of nerves per 10 mm² tissue area were calculated.

**Evaluation of inflammatory cell infiltrates**

The tissue sections were also evaluated for perineural inflammatory cell infiltrates by two independent observers blinded to patient status, followed by joint resolution of any differences. Furthermore, infiltrate density was graded as mild (score 1), moderate (score 2), or severe (score 3). For the purposes of comparative evaluation between the different groups, the area of inflammatory foci was measured with a video image system. To summarise the degree of perineural inflammation in CP tissues, a perineural inflammation number score and a perineural area score were calculated, taking into account the total number of perineural inflammatory foci, density of the cell infiltrates, area of foci, and area of the tissue sections evaluated. Hence the perineural inflammation number score was defined as:

\[
\text{Perineural inflammation number score} = \text{(No of perineural foci with density score 1 × 1) + (No of perineural foci with density score 2 × 2) + (No of perineural foci with density score 3 × 3)} \times 1000/\text{Area of tissue evaluated (mm²)}.
\]

The perineural inflammation area score was defined as:

\[
\text{Perineural inflammation area score} = \text{(Mean area of perineural foci with density score 1 × 1) + (Mean area of perineural foci with density score 2 × 2) + (Mean area of perineural foci with density score 3 × 3)} \times 1000/\text{Area of tissue evaluated (mm²)}.
\]
(Mean area of perineural foci with density score 2×2) + (Mean area of perineural foci with density score 3×3)×10^6
Area of tissue evaluated (mm^2)) where mean area of perineural foci was measured in mm^2.

Also, the percentage involvement of the perineural infiltrate was evaluated and summarised for each patient group.

Evaluation of pancreatic fibrosis
The degree of pancreatic fibrosis was evaluated on haematoxylin-eosin stained tissue sections by the use of digital image analysis and the Image Pro-plus software (Media Cybernetics). For further analysis, fibrosis was grouped as mild (<25%), moderate (≥25–50%), severe (≥50–75%), and marked (≥75%).

Statistical analysis
Statistical analysis was performed with the SPSS 9.0 statistical program. Data are given as mean (SD) or median (upper/lower quartiles). For statistical analysis, the Kruskal-Wallis test, Mann-Whitney U test, and Spearman correlation analysis were used. A p value <0.05 was taken as significant.

RESULTS
The pain history of patients with alcoholic, idiopathic, and tropical CP was similar (p=0.13).

In alcoholic CP, four patients had severe pain, four had moderate pain, and three had mild pain. While two patients had daily pain, five had weekly pain, and four had monthly pain. The median (25th–75th percentiles) global pain score in alcoholic CP was 6 (6–7)/patient.

In idiopathic CP, two patients had severe pain, six had moderate pain, and four had mild pain. While four patients had daily pain, five had weekly pain, and three had monthly pain. The median (25th–75th percentiles) global pain score in idiopathic CP was 6 (6–7)/patient.
In tropical CP, 11 patients had severe pain and one had moderate pain. While three patients had daily pain, four had normal pain, and five had monthly pain. The median (25th–75th percentiles) global pain score in tropical CP was 7 (7–8)/patient.

**Evaluation of pancreatic nerves**

The median (upper/lower quartiles) analysed tissue area, median number of nerves per 10 mm² of tissue area, median innervation area per nerve, median area of nerves per 10 mm² area of tissue, median nerve area, median nerve diameter, and median percentage of neural tissue in normal pancreas, alcoholic, idiopathic, and tropical CP are given in table 1.

**Comparison of neural changes between normal and alcoholic, idiopathic, and tropical CP (fig 1)**

Comparison of alcoholic CP with normal controls revealed that while the median number of nerves/10 mm² of tissue area was marginally higher (p=0.051) than in normal controls, the median area of nerves/10 mm² of tissue area (p<0.001), median nerve area (p<0.001), median nerve diameter (p<0.001), and percentage of tissue area occupied by nerves (p<0.001) were significantly higher in alcoholic CP than in normal controls.

Comparison of idiopathic CP with normal controls revealed that the median number of nerves/10 mm² tissue (p=0.003), median area of nerves/10 mm² of tissue area (p<0.001), median nerve area (p<0.001), median nerve diameter (p<0.001), and percentage of tissue area occupied by nerves (p<0.001) were significantly higher in idiopathic CP than in normal controls.

Comparison of tropical CP with normal controls revealed that while the median number of nerves/10 mm² tissue (p=0.283) was nearly the same as in normal controls, median area of nerves/10 mm² of tissue area (p=0.001), median nerve area (p<0.001), median nerve diameter (p<0.001), and percentage of tissue area occupied by nerves (p<0.001) were significantly higher in tropical CP than in normal controls.

**Comparison of neural findings in different aetiologies of CP**

Median innervation area per nerve was significantly higher in tropical CP compared with idiopathic CP (p=0.02). Therefore, the number of nerves/10 mm² of tissue area was significantly higher in idiopathic CP in comparison with tropical CP (p=0.02).

For all other parameters—including median number of nerves/10 mm² tissue area, median area of nerves/10 mm² of tissue area, median nerve area, median nerve diameter, and percentage of tissue occupied by nerves—no significant differences were observed between alcoholic, idiopathic, and tropical CP.

**Evaluation of perineural inflammatory cell infiltrates in different aetiologies of CP**

All tissue sections of the normal pancreas had no perineurial inflammatory cell infiltrates. In contrast, in alcoholic, idiopathic, and tropical CP various degrees of perineural cell infiltrates were present. However, comparison of the perineural inflammation number score revealed no differences between alcoholic and idiopathic CP (p=0.26), between alcoholic and tropical CP (p=0.928), or between idiopathic and tropical CP (p=0.29) (table 2). When the perineural inflammation area scores were compared in the different aetiological groups, again no differences were present between alcoholic and idiopathic CP (p=0.08), between alcoholic and tropical CP (p=0.88), or between idiopathic and tropical CP (p=0.18) (table 2).

The percentage involvement of perineural foci was also evaluated in the different aetiological groups and no significant differences between the three groups were found (p=0.37).

**Comparison of pancreatic fibrosis in different aetiologies of CP**

There was no relationship (p>0.05) between the degree of pancreatic fibrosis and pain in any aetiological CP group.

**DISCUSSION**

A common dominant feature of CP, independent of the underlying aetiology, is abdominal pain. In the past few years, various hypotheses have been implicated in the pathogenesis of pain in CP; however, none has firmly stood the test of time. Increased intraductal pressure as a result of strictures and/or calculi is believed to be the most common cause of pain for patients with dilated main pancreatic duct disease, and relief from pain by drainage of a dilated main pancreatic duct is often seen.21 22 The other suggested causes of pain are duodenal and common bile duct stenosis due to ongoing pancreatic inflammation and subsequent scarring.23 Additionally, pancreatic fibrosis, interstitial hypertension, and pancreatic ischaemia have all been implicated either as a sole or additional factors involved in the pathogenesis of pain.24 25

The most recent concept of pain pathogenesis in alcoholic CP postulates that alterations in pancreatic nerves and neuroimmune interactions are crucial factors in the pain syndrome.26 27 Pancreatic nerves are preferentially retained while the parenchyma degenerates and is replaced by fibrotic tissue. Increased diameter of pancreatic nerves, damage to the perineurium,28 and increased presence of neurotransmitters such as substance P and CGRP in enlarged pancreatic nerves in CP suggests that pancreatic nerves might be responsible for or are at least one of the main players in the long lasting pain syndrome in these patients.29 However, if nerves are involved in the pathogenesis of pain in alcoholic CP similar neural alterations should also be present in other types of CP associated with pain, independent of the underlying aetiology. Inasmuch as pain is a dominant feature of alcoholic, idiopathic, and tropical CP the presence of similar neurological alterations and their relationship to pain would support the significance of neural alterations in the pain pathogenesis of CP. In our study, patients of different aetiological groups had a comparable pain syndrome with regard to pain intensity, frequency, and duration, and exhibited similar neural alterations with an increase in neuronal tissue, supporting the hypothesis that changes in innervation are of importance in the pain syndrome of CP. The only statistically significant difference was noted between non-tropical CP and tropical CP in terms of median number of nerves per tissue area and consequently median innervation area per nerve. In tropical CP the

### Table 2 Perineural inflammation number score and perineural inflammation area score in different aetiological groups of chronic pancreatitis (CP)

<table>
<thead>
<tr>
<th>Aetiology</th>
<th>Inflammation No score</th>
<th>Inflammation area score</th>
<th>Intensity of pain</th>
<th>Frequency of pain</th>
<th>Global pain score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiopathic CP</td>
<td>80 (0–113)</td>
<td>300 (0–1340)</td>
<td>2 [1–2]</td>
<td>2 [1.25–3]</td>
<td>6 [6–7]</td>
</tr>
</tbody>
</table>

Values are median (25th and 75th percentiles).
median number of nerves per tissue area was significantly less compared with that in idiopathic CP and was also less compared with alcoholic CP although this was not statistically significant. As a natural consequence, median innervation area per nerve was more in tropical CP than in other forms of CP. A possible explanation could be that the individual nerve diameter in tropical CP, although not statistically significant, showed a definite trend towards being larger than in other forms of CP. Thus nerves appear to enlarge rather than proliferate in tropical CP. The final outcome being the amount of neuronal tissue, it is possible that either nerve enlargement or proliferation or likely a combination of both are responsible for the pain syndrome of CP.

In 1985, attention was focused on immune cell infiltrates which are frequently located close to pancreatic nerves in alcoholic CP. This histopathological study suggested that neuroimmune mechanisms are probably involved in the pain of CP. The study stimulated further research on the combined role of nerves and the immune system in the pathogenesis of this disorder. More recently, it was demonstrated that interaction of immune cells with pancreatic nerves and a marked increase in neuronal plasticity are likely additional pathogenic factors for the generation of pain in CP whereas the degree of pancreatic fibrosis has no major impact on the pain syndrome in these patients. Therefore, as inflammatory cell infiltrates are regularly found in alcoholic CP and a close interaction between inflammatory cells, pancreatic nerves, and the presence of pain has been observed, we also evaluated and compared the pattern of perineural inflammatory cell infiltrates in other aetiological groups of CP. Again we found a similar pattern of perineural inflammatory cell infiltrates, irrespective of the aetiological initiation of the disease. These findings, considered together, indicate that comparable neural alterations occur in painful CP independent of the underlying aetiology and possibly explain the similar course of pain observed in alcoholic and non-alcoholic CP.

A limitation of our study is that all pancreatic tissues were obtained from CP patients with “surgical stage CP”—the only stage when surgical intervention is considered necessary. Due to the lack of non-surgical stage human CP tissue and the unavailability of relevant experimental models of CP that mimic the aetiology and other features associated with human CP, the development of neural alterations and the relationship with perineural inflammatory cell infiltrates cannot be studied in a time-dependent fashion. Nevertheless, while there are reasons to believe that different forms of CP follow different courses depending on the initiating aetiological event, our findings suggest that as the disease seems to approach a common stage where the neural alterations and the pattern of perineural inflammation are similar irrespective of the underlying aetiology, the activated pathobiological pain pathways may ultimately follow a distinctive, aetiology-independent pattern in all forms of CP.

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