Characterization of structural, metabolic and functional brain alterations in patients with chronic pancreatitis – A multimodal brain MRI study

Muthulingam, Janusiya A.

Publication date:
2019

Document Version
Publisher's PDF, also known as Version of record

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– A MULTIMODAL BRAIN MRI STUDY

BY
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DISSERTATION SUBMITTED 2019

AALBORG UNIVERSITY
DENMARK

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Dissertation submitted September 2019

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Dissertation submitted: 30th September 2019

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PhD Series: Faculty of Medicine, Aalborg University

Department: Department of Clinical Medicine

ISSN (online): 2246-1302
ISBN (online): 978-87-7210-508-6

Published by:
Aalborg University Press
Langagervej 2
DK – 9220 Aalborg Ø
Phone: +45 99407140
aauf@forlag.aau.dk
forlag.aau.dk

Printed in Denmark by Rosendahls, 2019

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2. Furman AJ, Meeker TJ, Rietschel JC, Yoo S, **Muthulingam J**, Prokhorenko M, et al. Cerebral peak alpha frequency predicts individual differences in pain sensitivity. Neuroimage. 2018 Feb;167:203–10.

3. **Muthulingam J**, Olesen SS, Hansen TM, Seminowicz DA, Burrowes S, Drewes AM, et al. Progression of Structural Brain Changes in Patients With

Chronic Pancreatitis and Its Association to Chronic Pain: A 7-Year Longitudinal Follow-up Study. Pancreas. 2018;47(10):1267–76.

4. **Muthulingam JA**, Olesen SS, Hansen TM, Brock C, Drewes AM, Frøkjær JB. Study protocol for a randomised double-blinded, sham-controlled, prospective, cross-over clinical trial of vagal neuromodulation for pain treatment in patients with chronic pancreatitis. BMJ Open [Internet]. 2019 Jul 1;9(7):e029546.

5. **Muthulingam JA**, Hansen TM, Olesen SS, Drewes AM, Frokjaer JB. Altered brain morphology in chronic pancreatitis patients and its association with pain and other disease characteristics. Eur J Gastroenterol Hepatol. 2019 Jun 31(9):1092-1098.

6. Hansen TM, **Muthulingam JA**, Drewes AM, Olesen SS, Frokjaer JB. Cingulate glutamate levels associate with pain in chronic pancreatitis patients. NeuroImage Clin. 2019;23:101925.

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Abbreviations

ACC	Anterior cingulate cortex
BOLD	Bold oxygen level dependent
BPI	Brief pain inventory
CP	Chronic pancreatitis
CNS	Central nervous system
DLPFC	Dorsolateral prefrontal cortex
DMN	Default mode network
EEG	Electroencephalography
fMRI	Functional magnetic resonance imaging
MRI	Magnetic resonance imaging
PNS	Peripheral nervous system
QoL	Quality of life
QST	Quantitative sensory testing
SAPE	Sentinel acute pancreatitis event
SBM	Surface based morphometry
SN	Salience network
VBM	Voxel-based morphometry

ENGLISH SUMMARY

Chronic pancreatitis (CP) is an inflammatory disease of the pancreas, manifested by abdominal pain, exocrine and endocrine insufficiency, and often leads to multiple hospitalizations leading to an immense burden on both patients and the clinicians. Particularly, treating chronic pain secondary to CP remains a difficult clinical problem to manage, as the current pain treatment is often unsatisfactory and accompanied with undesirable side effects. Hence, new specific treatments to control the CP pain are highly warranted. However, there are some difficulties in developing and trialing new treatments for CP pain i.e. the pathophysiology of CP pain is still incompletely understood.

To date, several studies have demonstrated that the pain is largely driven by central mechanisms rather than morphological changes of the pancreas organ. Therefore, quantitative sensory testing and electroencephalography techniques have been used to identify changes of the central nervous system (CNS), however, the full extent of the CNS involvement in CP pain still remains largely unknown and needs to be investigated in more detail. The purpose of this Ph.D. project was to perform a multimodal magnetic resonance imaging (MRI) brain assessment of structural, functional and metabolic properties to provide more objective information about the role of the CNS in the pathophysiology of chronic pain in CP.

Four papers compile this Ph.D. project. Paper I was based on a longitudinal study, assessing the brain morphology changes using structural MRI. This study demonstrated predominantly reduced gray matter volume and cortical thinning in pain related areas over a 7-year follow up period. Furthermore, gray matter volume loss in the thalamus was associated with pain scores.
The remaining papers (II, III and IV) were based on a cross-sectional study. Paper II aimed to assess the brain morphology in CP patients as well as identifying the most prominent risk factor associated with altered brain structure. This study confirmed that CP patients had reduced gray matter volume and cortical thinning. Interestingly, this study found that alcoholic etiology of CP is the most prominent factor associated with gray matter volume loss. Overall, Paper I and II provide evidence of structural reorganization of the brain in CP patients. Paper III used MR spectroscopy to assess brain metabolites changes in CP patients. This study showed that CP patients had increased glutamate/creatine level in the anterior cingulate cortex (ACC) and was associated with patients' pain symptoms. Additionally, Paper III revealed that patients with alcoholic etiology of CP had reduced N-acetyl-aspartate/creatine level, compared with CP patients without alcoholic etiology. Finally, Paper IV assessed functional

connectivity alterations in two brain networks (default mode and salience network). Paper IV showed that CP patients had hyper-connectivity in both networks. Furthermore, the communication between those networks were disrupted, given that the anti-correlation was reduced in CP patients compared with healthy controls. Most interestingly, Paper IV showed an association between altered ACC glutamate level in CP patients and altered default mode network, implicating that glutamate may modulate the function of the default mode network. Overall, Paper IV provides strong evidence of functional reorganization of the brain in CP patients.

This multimodal MRI study provides strong evidence of structural and functional reorganization in CP patients, in addition to metabolic brain alterations. Taken together, these brain alterations may arise from a combination of both sustained nociceptive effect of long-lasting pain and previous neurotoxic effect of alcohol. This knowledge contributes to improve insight into pain mechanisms and treatment in CP, suggesting that the treatment must also be aimed at the CNS. The first step in treating pain could be examination and profiling the sensory system in CP patients, enabling the clinicians to provide a more precise and effective (personalized) treatment based on the central pain mechanisms.

Additionally, this Ph.D. thesis also provides valuable knowledge to design future clinical studies with painful CP patients.

DANSK RESUME

Kronisk pancreatitis (KP) er en kronisk betændelsestilstand af bugspytkirtlen (pankreas) og er en relativ hyppig årsag til kroniske mavesmerter, der medfører betydelige personlige, samfundsmæssige og økonomiske problemer. KP er karakteriseret ved progressiv ødelæggelse af pankreas med atrofi, bindevævsdannelse, eksokrin insufficiens og endokrin insufficiens.

Kroniske mavesmerter er det vigtigste symptom hos KP patienter. Traditionel smertebehandling med morfinlignende lægemidler har ofte en begrænset og utilstrækkelig effekt på smerterne og har tit uønskede bivirkninger. Derfor er nye behandlingstiltag nødvendige. Tidligere har KP smertefysiologien fokuseret på selve pankreas organet, hvor man mente at anatomiske forandringer i pankreas medførte smerte, men nyere studier har vist, at der ikke er nogen klar sammenhæng mellem anatomiske forandringer i pankreas og smerterne. Samtidigt har man vist, at smerte ved KP involverer sensibilisering af centralnervesystemet med øget følsomhed for sensorisk påvirkning. Dette spiller en vigtig rolle i udviklingen af de kroniske smerter.

Den seneste udvikling indenfor radiologien har muliggjort kortlægning af hjernens struktur og funktioner med avancerede magnetisk resonans (MR) billeddannelse. Dette ph.d.-projekt har til formål at undersøge I) strukturelle, II) metaboliske og III) funktionelle hjerneforandringer hos patienter med KP sammenlignet med raske forsøgspersoner. Projektet indeholder to studier: Et longitudinelt studie og et tværsnitsstudie, som resulterede i fire videnskabelige artikler.

Artikel 1 undersøgte udviklingen af strukturelle MR hjerneforandringer over en periode på 7 år hos KP patienter. Denne artikel viste, at patienterne havde reduceret volumen af grå substans og nedsat tykkelse hjernebarken i smerte relaterede områder i hjernen som hænger sammen med udviklingen af smerterne.

Artikel 2 undersøgte strukturelle MR hjerneforandringer med henblik på at identificere de mest betydende risikofaktorer, som driver morfologiske hjerneforandringer hos patienter med KP. Denne artikel, bekræftede reduceret volumen af grå substans og nedsat tykkelse af hjernebarken. Endvidere, blev tidlige brug af alkohol (alkoholisk ætiologi for KP) identificeret som den mest betydende risikofaktor relateret til morfologiske hjerneforandringer hos KP patienter.

Artikel 3 undersøgte metaboliske forandringer i hjernen med MR spektroskopi. Dette studie viste, at patienterne har øget glutamat/kreatine koncentration i hjerneområdet anterior cingulate cortex (ACC). Patienterne med mest smerte havde ligeledes højst

glutamat/kreatine koncentration. Ydermere viste artikel 3, at patienterne havde signifikant reduceret N-acetyl-aspartat/kreatin koncentration i det parietale område i hjernen, som ligeledes var associeret til tidlige brug af alkohol.

Artikel 4 undersøgte hjernens funktionelle netværk hos patienter med KP og viste, at patienterne havde øget konnektivitet i to vigtige netværk (default mode og salience netværkene). Patienterne havde ydermere reduceret anti-korrelation mellem netværkene, hvilket indikerer en ubalance i kommunikation i hjernen hos patienter med KP, som også ses hos andre patienter med kroniske smerter. Afslutningsvis, viste artikel 4 en sammenhæng mellem forstyrret konnektivitet i default mode netværket og glutamat i ACC.

Den overordnede konklusion i dette ph.d. projekt er, at patienter med KP har både strukturelle, metaboliske og funktionelle hjerneforandringer, som tyder på at være drevet af både smerte og neurotoksisk effekt (tidligere alkohol misbrug). Idet, dette ph.d. projekt har påvist, at KP patienter har udtalte hjerneforandringer, bør fremtidige behandlinger være CNS målrettet og selve behandlingen bør tilrettelægges efter den enkelte patients karakteristika ved eksempelvis at fænotype patienternes sensoriske smerte system.

ACKNOWLEDGEMENTS

The process of earning a Ph.D. degree and writing a dissertation is long and arduous, and would never be possible without moral, technical, professional and personal support from my supervisors, colleagues and family.

First of all, I owe my most sincere gratitude to my main supervisor Professor Jens Brøndum Frøkjær for outstanding inspiration, for pushing me to advance my research skills, supervision and constructive feedback of my work. Especially, I thank you for fruitful discussions and for respectfully smiling with me over my mistakes and stepping in to help when I need it.

Furthermore, I would like to thank my co-supervisor Associate Professor Søren Schou Olesen for providing outstanding clinical support, for sharing your knowledge in research, especially regarding statistics and for always providing relevant and positive critics. Also, I would also like to extend my gratitude to my co-supervisor, Assistant Professor Tine Maria Hansen for sharing your knowledge in MRI and providing outstanding technical support whenever I needed it, for fruitful discussions and for daily assistance.

A special thanks to the director of Mech-Sense, Professor Asbjørn Mohr Drewes for important support and scientific contribution, for fast reply on my manuscripts, and for providing helpful advices. In my daily life, I have been blessed with a great research team of colleagues/friends at Mech-Sense. You have all supported me enormously and I cannot thank you enough for all the great ideas you gave me for my conference presentations at the lab-meetings, for fruitful discussions at the journal club meetings, for providing me feedback on my scientific posters and most importantly for creating a great working environment. Especial thanks to our research radiographers at the Department of Radiology: Kenneth Krogh Jensen and Louise Bach Jensen for their excellent work behind the scanner and all the technical support. Also thanks to our secretaries Karina Dybkær and Britta Lund Dalgaard for administrative support.

This Ph.D. thesis is based on two studies. Without support from chronic pancreatitis patients and healthy subjects, I would not have conducted those studies. Therefore a special thanks to all participants enrolled in our studies and for your invaluable contribution.

Back in 2017, I had an external research stay for one month at the Department of Neural and Pain Sciences, University of Maryland in Baltimore, United States of America. I collaborated with Associate Professor David Seminowicz and Postdoc Shana Burrowes on Study I, and gained new knowledge regarding longitudinal MRI analysis. Thank you for providing me technical support and for having me in your Pain Imaging Lab. This research stay would not have been possible without financial support from the Augustinus Foundation and Aalborg University. Thank you for your financial support.

Furthermore, I would like to acknowledge the Obel Family Foundation, Independent Research Fund Denmark, Region Nordjyllands Research Foundation, and Muusfeldt & Sofus Emil Foundation. All contributors have been of great value.

Last and most importantly, I would like to thank my wonderful family and friends for always believing in me and for the never-failing support and encouragement.

Janusiya Anajan Muthulingam, September 2019, Aalborg

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CHAPTER 1. INTRODUCTION

Chronic pancreatitis (CP) is a progressive fibro-inflammatory disease of the pancreas and is a major source of morbidity with a prevalence of CP, varying between 120 and 140 per 100,000 population (1). CP has an essential clinical and economic impact, and the pain in CP represents the main challenge for primary care physicians and gastroenterologists (2).

Abdominal pain is the primary clinical manifestation in CP patients associated with a negative impact on the overall quality of life (QoL) (3). The pain mechanisms are incompletely understood and probably multifactorial, which often results in insufficient and empirical treatment. Previously, the understanding of pain in CP has been focused on the pancreas, assuming pain is caused by increased parenchymal or ductal pressure. However, there is a growing evidence that alterations of the central nervous system (CNS) also play an important role in the pathophysiology and symptom generation (4,5).

Thus far, quantitative sensory testing (QST) studies and electroencephalography (EEG) studies have made major progress in identifying key neural structures within the brain-gut axis involved in CP (5). Particularly, QST studies have shown that pain in CP and other visceral pain syndromes share similarities in pain mechanisms, with evidence of both peripheral and central sensitization (6). Moreover, EEG studies have demonstrated following characteristics in CP patients: 1) functional reorganization of the insular cortex, 2) thalamocortical dysrhythmia, and 3) cortical hyperexcitability (5,7). However, the full extent of the CNS involvement in CP pain still remains largely unknown and needs to be investigated in more detail to improve our understanding of the pain mechanisms in CP and potentially improve the pain treatment.

In addition to EEG, magnetic resonance imaging (MRI) is another valuable neuroimaging technique to study the brain non-invasively. Comparing to EEG, MRI has an excellent spatial resolution, specifically in the superficial layers of the brain (8). Hitherto only few MRI studies have investigated the CNS in CP patients (9,10), reporting that CP patients have cortical thinning in brain areas involved in pain processing (9), as well as microstructural white matter changes (11). Although, these studies have contributed to our knowledge of the brain structure in CP patients, they have some limitations i.e. only few pre-selected areas of the brain were investigated. Hence, there is a need to study the whole brain anatomy thoroughly in CP patients.

MRI can provide important structural features such as brain volumetry (i.e. gray matter volume), and cortical thickness (12). Furthermore, MRI provides functional characteristics of the brain using blood-oxygen-level dependent (BOLD) signal. This approach is the most widely used technique in functional MRI (fMRI) (13). Additionally, neurochemical activities, i.e. concentration of neurotransmitters, can be assessed using MR spectroscopy (14). Characterization of the structural, metabolic and functional brain with multimodal MRI modalities, will greatly improve our understanding of CP pathophysiology.

CHAPTER 2. BACKGROUND

2.1 CHRONIC PANCREATITIS: PATHOPHYSIOLOGY, ETIOLOGY AND SYMPTOMS

Chronic pancreatitis is a multifactorial disease, seen in both males and females, however it is more common in men with a gender ratio (male/female) of 4.6 (15). The prevalence of the disease increases with age (15), and moreover CP has been shown to be associated with approximately 50% mortality rate within twenty years of diagnosis (16).

Acute pancreatic, recurrent acute pancreatitis, and CP are interconnected in a disease continuum and share some common features i.e. progressive inflammation of the pancreas (17,18). Acute pancreatitis is characterized by acute and partly reversible pancreatic injury, whereas CP is characterized by irreversible pancreatic injury resulting in loss of pancreatic parenchyma and functions (19). It is widely acknowledged that CP is the end result of repeated attacks of acute pancreatitis and chronic inflammation, leading to clinical manifestations such as exocrine and endocrine insufficiencies (18).

During the last couples of years, several hypothesis have been proposed explaining the pathophysiology of the CP. At the moment, sentinel acute pancreatitis event (SAPE) hypothesis model (18) is the most prevailing hypothesis proposed to be involved in the development of CP (16,20). SAPE-model, contains several elements such as 1) the necrosis-fibrosis hypothesis, 2) the toxic-metabolic hypothesis, and 3) the oxidative stress hypothesis (21).
In general, SAPE model is based on a “two-hit” hypothesis and can be divided into three stages (Figure 1) (21). The first stage is called, pre-acute pancreatitis stage, in which the risk factors (genetic, metabolic and environmental) will be recognized, and then a first (sentinel) episode of acute pancreatitis activates the immune system (including proinflammatory and anti-inflammatory response) (18,21). The latter is the second stage, named the initial attack of acute pancreatitis stage, which is also considered as the “first hit” (18,21). The outcome of acute pancreatitis can either be complete recovery, or pathologically progression towards CP, which is the third stage, called progression stage (18,21). The third stage is highly dependent on factors that drive the immune system, thru several stressors (alcohol, smoking) and remains the “second hit” (Figure 1) (21). For example, patients with minimal ongoing stress to the immune system and no major changes to the immune system, appears to return to normal pancreas, already after the first attack of acute pancreatitis and becomes completely recovered. In contrast, patients with continued stress to the immune

system (i.e. alcohol/smoking), progress towards CP (21). The pathological progression towards CP may be triggered by several mechanisms i.e. oxidative stress, progressive inflammation, or repeated episodes of acute inflammation, which may or may not be clinically apparent (18,21,22).

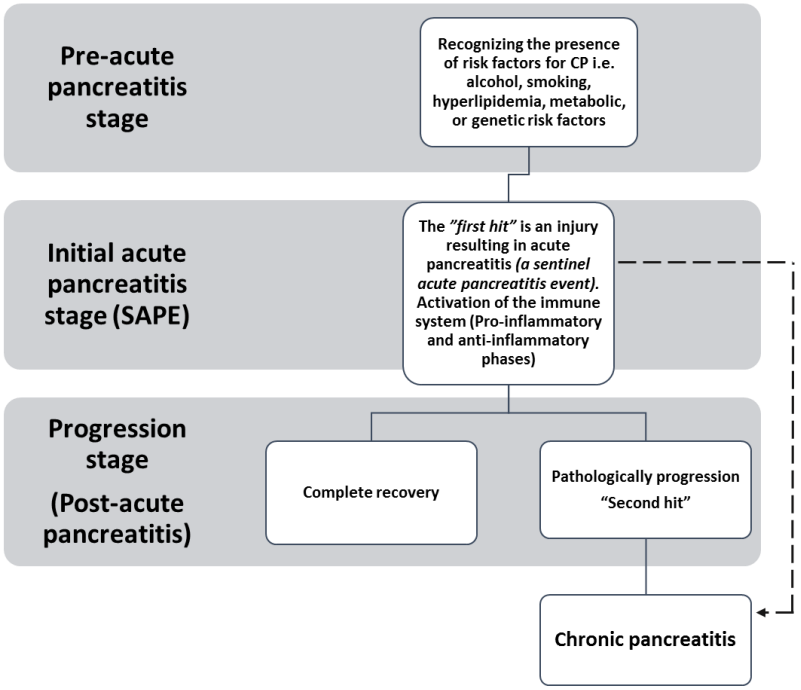

Figure 1: Overview of the sentinel acute pancreatitis event hypothesis. This hypothesis proposes that an initial episode of acute pancreatitis sensitizes the pancreas and is the very first step in a series of events resulting in CP. Abbreviations: CP: Chronic pancreatitis. SAPE: sentinel acute pancreatitis event.

To date several classification systems of the etiology behind CP exist, i.e. The American classification system, TIGAR-O, is based on various risk factors, and can be categorized into six groups (1) Toxic-metabolic, (2) Idiopathic, (3) Genetic, (4) Autoimmune, (5) Recurrent and severe acute pancreatitis or (6) Obstructive (15).

The European classification system, M-ANNHEIM, is another widely used classification system, describing the heterogeneity of the pathology in detail based on etiological risk factors such as: alcohol consumption, nutritional factors, hereditary factors, efferent duct factors, immunological factors and miscellaneous including metabolic factors (23). Additionally, M-ANNHEIM classification enables to classify the patients according to clinical stage, and severity of the CP disease (24). Even

though, the TIGAR-O and M-ANNHEIM classifications are two different classification system, they share same features and overlap greatly.
In Western countries, it is generally accepted that excessive consumption of alcohol over many years, is the most common cause for developing CP. Current literature suggests that approximately half of the patients have alcoholic etiology (3,16). Together with alcohol, smoking remains a common co-existing risk factor, contributing to the development of CP. Smoking does not only increase the risk factor for CP, but does also increase the risk factor for pancreas cancer (16).

The most significant clinical symptom in CP is abdominal pain, presenting up to 90% of patients and pain is the primary cause for patients seeking medical care (3). Furthermore, chronic abdominal pain causes impairment of QoL, disability and unemployment resulting in major healthcare costs (3,25,26). CP pain often originates in the epigastrium and radiates to the back in a belt-like manner and is highly fluctuating (27). Furthermore, the pain may often manifests as postprandial pain, associated with nausea and vomiting. The pain pattern in CP patients can be divided into three groups: A) Intermittent pain, B) constant pain, and C) constant pain with acute exacerbations (28). The causes of pain have previously been explained by structural changes of the pancreas and increased pressure in the ductal system or tissue (25,29). However, in cases of more long-lasting pain, alterations of the CNS have also been found to play a role, indicating that the chronic pain in CP probably also has a neuropathic origin (19). Among other, chronic visceral pain is considered to be the most severe complication of CP, mainly as it is poorly understood and challenging to treat (3). In addition to pain, CP is often accompanied by other symptoms arising from the autonomic and enteric nervous system that may need specific management, including exocrine and endocrine insufficiency accompanied with weight loss, steatorrhea, and other gastrointestinal disturbances (30).

Currently, analgesics are the cornerstone of CP pain management, but when opioids are used, it may consequently result in dependency, opioid-induced hyperalgesia and other side-effects such as opioid-induced constipation, and cognitive dysfunction (31). Most despondently, CP patients are treated with opioids, despite the lack of clear evidence that they are effective for long-term pain management (31). Finally, if the analgesic therapy fails, invasive treatments including endoscopic pancreatic duct clearance, stenting, and surgery can be considered. However, invasive treatments are not always effective for pain relief, probably reflecting that many CP patients suffer from chronic visceral pain which may likely be due to abnormal central pain processing (31).

2.2 PHYSIOLOGICAL PAIN PROCESSING

According to Woolf et al (32), pain can be classified into 1) nociceptive pain (the physiological protective system), 2) inflammatory pain (tissue damage), and 3) pathological pain (abnormal functioning of the nervous system). In order to understand the pathological pain in patients with CP, we first need to understand the physiologic pain. Therefore a brief section of pain physiology will be addressed.

According to the International Association for the Study of Pain (IASP), pain is defined as *"an unpleasant sensory and emotional experience associated with actual or potential tissue damage in terms of such damage",* indicating that pain has objective, physiological sensory components in addition to subjective emotional and psychological components (33). Although pain is associated with unpleasantness and negative thoughts, it is an indispensable mechanism for the human body, preventing and minimizing tissue damage from the environment, using the neural mechanisms for pain and nociception (34).

Pain physiology (nociception) involves four neural processes; A) transduction, B) transmission, C) modulation and D) perception (Figure 2) (35).

The *transduction* refers to the process that turns the noxious stimulus (mechanical, thermal or chemical) into electrical signal by nociceptors (free afferent nerve ending) (36). Then, *transmission* is the second step, in which the nociceptive information is transmitted from the peripheral nervous system to the CNS in several phases: 1) from periphery to spinal cord and 2) from spinal cord to the brain (36). From the periphery, the nociceptive inputs travel to the dorsal horn of the spinal cord. Here, excitatory neuropeptides i.e. glutamate is capable to facilitate and amplify the pain signals, while inhibitory amino acids i.e. GABA and neuropeptides reduce the nociceptive response through descending inhibitory system (36,37). These processes are covered by *modulation* and occurs at several levels i.e. peripheral, spinal, and supraspinal level. Thus, modulation takes place simultaneously, so the brain can alter the transmission of a nociceptive signal (36–38). Phase two in transmission (from spinal cord to the brain), projects the nerve processes of the dorsal horn to the brain in bundles termed ascending tract, transmitting the nociceptive inputs to the thalamus through spinothalamic tract and is further processed to the somatosensory cortex (36). Finally, *perception* is the cerebral cortical response to the nociceptive signals involving the limbic system. Perception includes several processes i.e. attention, expectation, emotions, and interpretation (35,36).

These four steps of the pain pathway are the fundamental basis for the nociceptive signaling and processing. The above four neural processes can be assessed using methods i.e. QST (33). QST provides clinicians and researchers the opportunity to investigate abnormalities in the sensory system as well as characterizing mechanisms underlying pathological pain disorders (25).

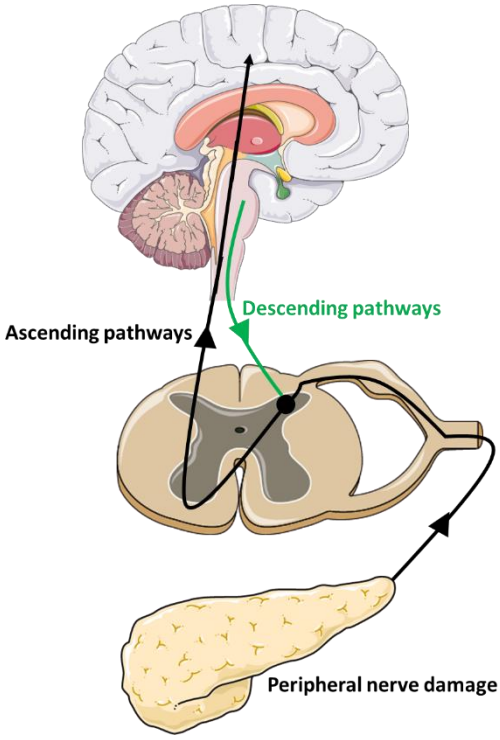

Figure 2: Conceptual model for pain mechanisms involved in chronic visceral pain. Nociceptive pathway consists: A) Transduction, B) Transmission, C) Modulation, and D) Perception. Examples of altered pain mechanisms in CP: central sensitization (incl. hyperalgesia and allodynia), cortical reorganization in the brain and defect in descending pain pathways.

2.3 PATHOLOGICAL PAIN IN PATIENTS WITH CHRONIC PANCREATITIS

Pain originated from visceral organs (i.e. heart, lungs, gastrointestinal tract, pancreas etc.), is called visceral pain, whereas pain arising from tissues such as skin, muscle, join capsules and bone is called somatic pain (39). Although visceral pain, as seen in

CP patients, share some mechanisms with somatic pain, there are some major differences in central nociceptive processing of pain (40), that should be considered when initiating analgesic treatment (33,41). For instance, visceral pain is described as diffuse and difficult to localize (30,42).

For many years, the CP pain theories were focused on the pancreatic gland, and it was believed that the pain in most patients was caused by anatomical changes such as increased ductal and intestinal pressure (29). This was the basis for the mechanistic understanding of pain, called "the plumbing theory" and is the rationale for most interventions i.e. surgical and endoscopic procedures. However, recent studies have provided evidence of no association between microstructural and macrostructural findings and the pain characteristics (43,44). Currently, the literature suggest that the peripheral and central pain processing is abnormal in CP patients, mimicking neuropathic pain disorders (3,45). Particularly, studies have provided evidence of pancreatic neuropathy and neuroplasticity at the peripheral level and at the central level of the sensory system (3).

The mechanisms that involve peripheral nervous system can be grouped into morphological and functional changes (46). Among others, morphological changes include enlarged neural density, neural hypertrophy and pancreatic neuritis. Functional changes include reduced sympathetic innervation.

The mechanisms that involve changes in the CNS are central sensitization, impaired pain modulation, as well as cortical reorganization and hyperexcitability (46). Normally, central pain processing is balanced between descending excitatory and inhibitory drives, however in CP patients, it has been observed that an imbalance between inhibitory and facilitatory descending pain modulatory systems occurs, resulting in greater gain of incoming nociceptive signals (19,46).
Sensitization in CP is characterized by hyperalgesia (an increase in responsiveness and prolonged aftereffect to noxious stimuli) and allodynia (a reduction in pain thresholds with pain in response to stimuli which normally does not induce pain) (33). The hyperalgesia can manifest either locally as seen in peripheral sensitization or generalized as observed in central widespread sensitization (Figure 2) (3).

Cortical reorganization has been demonstrated in CP patients with chronic visceral pain using EEG studies, and hyperalgesia has been observed to be associated with a functional reorganization of the cerebral cortex (5). Particularly, it has been shown that CP patients show reorganization of brain areas involved in visceral pain processing including the insula, secondary somatosensory cortex and cingulate cortex compared to healthy controls (7,9). The patients had not only reorganization of the brain in the visceral pain processing regions, but showed also abnormal excitability

of these neural networks, reflecting cortical neuronal hyper-excitability (25). Another major finding from previous EEG studies is that disturbance of the thalamo-cortical interplay has been observed in CP patients with pain (5). Also, structural cortical reorganization has been demonstrated in CP patients using MRI techniques (9,11). Particularly, microstructural white matter changes in the insular and frontal brain areas as well as cortical thinning were found in CP patients (9,10). To date, neuroimaging studies have made a major contribution in understanding the pain related alterations occurring in CNS in general (47,48). However, there still is a knowledge gap regarding the involvement of the CNS in CP patient with chronic visceral pain. Particularly, it is unknown whether the gray matter volume is affected by the CP disease, and how clinical manifestations are related to brain changes over time, as well as, which factors drive the alterations in the CNS. In attempt to address the above mentioned knowledge gaps, this Ph.D. study uses advanced multimodal MRI assessments to assess the CNS changes in CP patients.

2.4 ASSESSMENT OF CHANGES IN THE CNS WITH NEUROIMAGING TECHNIQUES

The majority of the information we have regarding brain anatomy came from the study of thin histological sections that have been stained to show nerve cells and their process. However, during the last decades, a new generation of non-invasive imaging techniques has allowed us to in-vivo study the anatomy, physiology and pathophysiology in the human brain (8).

Neuroimaging techniques can be grouped into following categories (8,14):

- Functional neuroimaging: Functional MRI, magnetocencephalography (MEG), and EEG
- Structural imaging: MRI
- Biochemical neuroimaging: MR spectroscopy and positron emission tomography (PET)

This Ph.D. project will only focus on functional MRI, structural MRI and MR spectroscopy.

Functional MRI typically measures the BOLD signal based on the consumption of oxygen, induced by the neuronal activity. More precisely the fundamental basis of the BOLD signal relies on the magnetic properties of hemoglobin (Hb) (49). Hb is the protein molecule in red blood cells, carrying the oxygen and exists in two different forms, each with different magnetic properties (49). When the Hb is carrying the

oxygen, the Hb is in an oxygenated form (oxy-Hb) and has diamagnetic properties. In contrast, when the Hb is not carrying the oxygen, the Hb is in a deoxygenated form (deoxy-Hb) and has paramagnetic properties (49). Due to the different magnetic properties of Hb, different local magnetic fields are generated, i.e. deoxy-Hb suppresses the MR-signal because of its paramagnetic properties. Hence, as the concentration of deoxy-Hb decreased, the fMRI signal increases (49). Overall, changes in the ratio of oxygentated and deoxygentated blood can be inferred with fMRI by measuring the BOLD response. However it is important to note that the, BOLD signal is not directly measuring the neuronal activity, instead it measures the metabolic demands (oxygen consumption) of active neurons (49,50).

Structural neuroimaging enables to macroscopically study gray matter volume (voxel-based morphometry, VBM), cortical thickness (surface-based morphometry, SBM), fiber integrity (diffusion tensor imaging, DTI), and fiber tract density (tractography), which can reveal information on neuroplastic reorganization of the brain (48,51). MR spectroscopy remains another powerful tool providing biochemical information of the brain, including brain metabolites e.g. glutamate, N-acetyl aspartate, etc. (52).

With combination of three different MRI techniques, enormous knowledge of pain processing in the brain can be achieved, and furthermore, a profound understanding of pain mechanisms can be obtained to enhance clinical intervention as well as improve pain treatment.

2.5 REPRESENTATION OF PAIN IN THE BRAIN

MRI studies have improved our understanding of how the brain alters in patients with chronic pain or responds to pharmacological treatments (53–55) by providing novel information into structural, functional and neurochemical changes in several chronic pain conditions (56). Across multiple chronic pain conditions, structural changes in gray matter volume, and white matter volume have been demonstrated, indicating chronic pain to be a neurodegenerative disease (47). Furthermore, fMRI studies have shown that insular, anterior cingulate cortex, primary sensory cortex, thalamus and prefrontal cortex, and dorsolateral prefrontal cortex (DLPFC) are the most consistently activated brain regions functionally related to pain processing (47,48). Finally, MR spectroscopy studies have shown changes in neurotransmitters in chronic pain patients, by identifying neuronal and axonal markers, including glutamate, asparate, glycine, and GABA (47). Despite the enormous neuroimaging research in chronic pain, only limited research have been conducted on pain in CP patients using EEG and MRI. Although EEG is a valuable neuroimaging technique, it has some weakness i.e. poor spatial resolution. With MRI it is possible to non-invasively assess the brain with good spatial resolution and relatively good temporal resolution (51).

CHAPTER 3. HYPOTHESES & AIMS

The overall aim of this Ph.D. thesis was to perform multimodal MRI brain assessment of structural, functional and metabolic properties to provide objective information about the role of the CNS in the pathophysiology of chronic pain in CP. This knowledge could contribute to improve insight into pain treatment in CP. Thus, we hypothesized that structural, metabolic and functional alterations of the brain are present in patients with CP and that those alterations were associated with the disease characteristics (including risk factors and clinical outcomes i.e. chronic pain). Taken together, a multimodal objective assessment approach would likely provide complementary information in understanding the complex pain mechanisms in these patients. The thesis is based on three peer-reviewed papers and an additional paper, which is under review. Those four papers are compiling data from two studies; a longitudinal study (study I) and a cross-sectional study (study II). The four papers will from hereon be referred to as indicated in Figure 3.

Subsequently, to fulfill the overall aim, the thesis contains following 3 aims:

- Aim I: To investigate changes in the brain structure over time using structural MRI in painful CP patients compared with controls (Paper I) and potential associations to pain related symptoms (Paper I) and to explore associations between morphological brain changes and disease characteristics and risk factors (Paper II).

  The hypothesis was that CP patients, in comparison to healthy controls, had a) progression of reduced gray matter volume and cortical thickness in areas involved in visceral pain processing, and b) that brain changes were associated with the disease characteristics including pain, and risk factors for CP.

- Aim II: To assess brain metabolites using MR spectroscopy in CP patients, compared with healthy controls, in pain related areas (ACC, insula and prefrontal cortex) and in a parietal control region, as well as to compare brain metabolite levels in subgroups with different disease characteristics including pain (Paper III).

The hypothesis was that CP patients had increased glutamate levels in pain related brain areas and that metabolites changes were associated to disease characteristics and patient outcomes.

- Aim III: To assess the resting state networks (default mode and salience mode networks) using functional MRI in CP patients compared with healthy controls, and to explore the association between altered brain network connectivity and the clinical parameters as well as brain metabolites (Paper IV).

  The hypothesis was that the CP patients demonstrated reduced anti-correlation between default mode network and salience network connectivity compared to healthy controls, and that the altered brain connectivity was associated to the pain related clinical parameters and levels of brain metabolites.

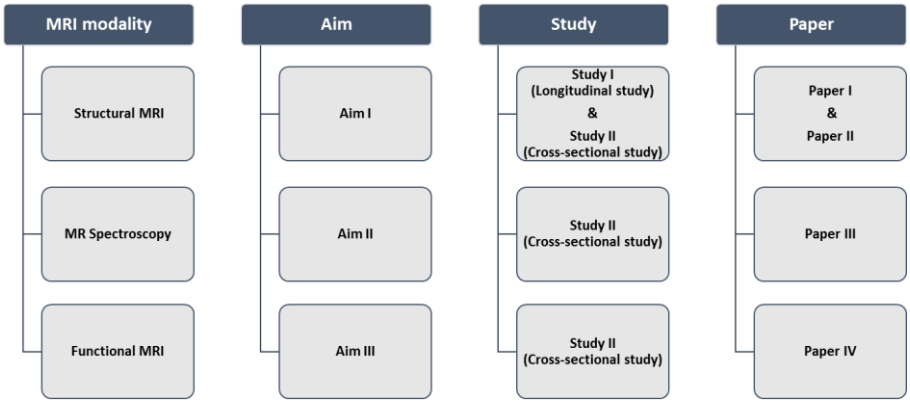

Figure 3: Overview of MRI modalities used in different aims which are investigated in study I and study II, presented in papers I-IV. Chronic pancreatitis patients and healthy controls were included in both studies. Abbreviations: MRI: magnetic resonance imaging.

CHAPTER 4. MATERIALS AND METHODS

4.1 MATERIALS

This Ph.D. thesis is based on two MRI studies conducted at the Departments of Radiology and Gastroenterology, Aalborg University Hospital: 1) a longitudinal study (study I) (presented in Paper I) and 2) a cross-sectional study (study II) (presented in Papers II+III+IV). Both studies were approved by the Ethics of Committee of Northern Jutland. For the longitudinal study the reference numbers were: N-20080028MCH and N-20090008. For the cross-sectional study, the reference number were: N-20090008, N-20130040, and N-20170023. Both studies were carried out in accordance to the Declaration of Helsinki. Screening sessions and physical examinations were conducted at the Department of Radiology, Aalborg University Hospital. The screening and the inclusion of the patients were performed by a medical doctor according to the Research Ethics Committee's guidelines. In order to participate in the respective studies, patients fulfilled the inclusion/exclusion criteria. The inclusion/exclusion criteria were identical for both studies. The inclusion criteria were as follows: (1) patients from the age of 18 years with a diagnosis of CP using the Mayo Clinic diagnostic criteria (57), (2) either chronic abdominal pain lasting for more than 3 days per week for the last 3 months or CP patients without ongoing pain, and (3) stable pain medication, including opioids/non-opioids. The exclusion criteria were as follows: (1) incapability to undergo MRI, (2) major illness i.e. cancer, (3) patients must not suffer from significant painful conditions other than CP, which make them unable to distinguish the pain related with CP from pain in other regions, (4) continuous and ongoing alcohol or drug abuse, and (5) had previously diagnosed depression or other significant mental illness.

For both studies, healthy controls were age and sex matched with the CP patients. Healthy controls had no previous history of chronic pain conditions nor gastrointestinal disease. Additionally, they were allowed to take their ordinary medication i.e. blood pressure medication. However, they were excluded if they used CNS active medication.

4.2 STUDY I

Study I was a 7-year prospective study consisting of CP patients and healthy controls. Both groups were seen on two visits: A baseline visit, which took place from 2009 to

2010 and a 7-year follow-up visit which took place from 2016 to 2017. Twenty-three CP patients and 14 healthy controls participated in the baseline visit, while only 11 CP patients and 12 healthy controls continued the study and completed the follow-up visit. At both visits, all participants underwent a single MRI scan session consisting of a high-resolution T1-weighted anatomical scan using exactly the same 3T MRI scanner. Furthermore, diffusion tensor image (DTI) was also acquired, however these data were not included in the Ph.D. study. Additionally, all patients fulfilled the BPI questionnaires and pain diary, and clinical characteristics were obtained from the patient medical records.

4.3 STUDY II

Study II was a cross-sectional study with 35 CP patients and 23 healthy controls. All participants underwent a single scan session consisting of a high-resolution 3D T1-weighted anatomical scan, resting state fMRI scan, and MR spectroscopy. In addition, DTI was also acquired, however these data were not included in the Ph.D. study. Patients also fulfilled QoL questionnaire, BPI questionnaires, and furthermore clinical characteristics were obtained from the patient medical record.

4.4 METHODS

As outlined above, both subjective and objective techniques were used in study I and study II (Table 1). Subjective techniques utilized in both studies were pain diary, BPI questionnaire, and QoL questionnaires. Those questionnaires were used to assess the subjective experience of pain perception as well as the health-related QoL in CP patients. A standardized pain scale called numeric rating scale (NRS) was used in the pain diary. Multimodal MRI techniques were used for objective brain assessment. Particularly, structural MRI, fMRI and proton MR spectroscopy were used to assess the brain changes. Both subjective and objective techniques are described in details in the following sections.

Subjective assessments	Objective assessments
- Pain diary (study I) - BPI questionnaire (study I+II) - Quality of life questionnaire (study II)	- 3D T1-weighted anatomical scan (study I and II) - MRI spectroscopy (study II) - Functional MRI (study II)

Table 1. Overview of methods used in study I, and study II. Abbreviations: BPI: brief pain inventory. MRI: Magnetic resonance imaging.

4.4.1 SUBJECTIVE ASSESSMENTS

In study I, CP patients were asked to complete the pain diary one week prior to MRI scan, both at the baseline and follow-up visits. Pain diary consisted to two questions. First, the average of the patients' level of pain during the respective day and second, the maximum intensity of the patient's level of pain during that day were asked. For this purpose a NRS for pain was used, which is a scale of 0 (no pain) and 10 (worst pain imaginable). Among others, NRS is one of the most commonly used rating scales for assessing pain and has an excellent test-retest reliability (58). NRS has been validated in patients in rheumatic and other chronic pain condition, and is widely used in the clinic and CP research (59,60).

BPI questionnaire was used to assess the clinical pain in CP patients in both study I and study II. The BPI questionnaire assesses pain at its "worst", "least", "average" level/intensity during the last 24 hours and "now". Based on the four items, the average BPI pain score was calculated. Moreover, the BPI questionnaire assesses to which degree the pain has interfered with daily lives. Based on seven interference items, the average BPI interference was calculated (61). The BPI questionnaire has been validated in several pain conditions such as cancer pain, fibromyalgia, and neuropathic pain (61). Even though BPI questionnaire has not been validated in CP patients, it is commonly used in CP research (62).
In study II, the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire Core 30 (EORTC-QLQ-C3O) was used to assess the health related QoL. Quality of life questionnaire is validated in several chronic pain conditions (63), and is widely used in CP patients (28). Recently a study evaluated QoL in CP patients, recommending to use QoL questionnaire to evaluate the well-being of CP patients (64). This questionnaire contains three main domains: Global health status, functional scales (physical, emotional, role, cognitive, social functioning), and symptom scales. According to the reference manual, all three domains were analyzed and presented as mean ± standard deviation. All three domains measure range in score from 0 to 100. A high score of global health status and a high score for the functional scale, indicate a high QoL and a healthy functioning, respectively. In contrast, a high score for the symptom scale, indicates a high level of symptomatology (63).
Other relevant clinical characteristics i.e. age, gender, etiology, diabetes status, use of opioids were obtained from the patient medical record.

4.4.2 OBJECTIVE ASSESMENTS

A detailed description of the three MRI techniques used in study I and study II, will be elucidated in this section. Please refer to Paper I-IV, for more technical information regarding sequence settings for each MRI technique.

4.4.2.1 STRUCTURAL MRI

T1-weigted images are the most commonly collected structural images, providing a basic anatomical image of the cerebrum (51). Using T1-images, voxel-based morphometry analyses (VBM) can be performed to assess the gray matter volume and surface-based morphometry analyses (SBM) to investigate the cortical thickness (12,65). In brief VBM technique uses statistical analysis approach to characterize changes in brain anatomy among subjects, enabling to study the presence of atrophy (66). Data preprocessing is an essential part of the MRI analysis. Preprocessing occurs before conducting the statistical analyses (67). The rationale behind preprocessing is a) to diminish the influence of data acquisition and physiological artifacts, b) to check statistical assumptions as well as transforming the data to meet these assumptions, and finally c) to standardize the location of brain regions across subjects to achieve validity and sensitivity in group analysis (67).

When working with T1 images, the preprocessing pipeline consists of segmentation (identifying gray, white matter and cerebro-spinal fluid), normalization (image transformation into a standard anatomical reference space using DARTEL), modulation (involving scaling by the amount of contraction, meaning that the modulated gray matter remains the same as it would be in the original volumes), and finally spatial smoothing (blurring anatomical differences as well as meet the assumption of random field of theory) (67) (Figure 4). After, preprocessing, the statistical analysis will be performed in order to assess the group differences. The statistical analysis will be described later (section 4.5).

4.4.2.2 FUNCTIONAL MRI

Similar to the structural image, the very first step in fMRI data analysis is preprocessing (67,68). In order to perform the preprocessing of the fMRI data, both the T1 structural scan and gradient EPI scan (fMRI) are required. The first step in preprocessing is slice-timing (67). Normally, fMRI data is acquired in slice-wise manner, meaning that some slices are collected later than other. Hence, the voxels do not have the same time points (67). Slice-timing correction enables to shift each voxel's time series so that all the voxels appear to have been sampled simultaneously.

The second step in fMRI preprocessing is motion correction (67). When analyzing fMRI data, it is pre-assumed that the voxels depicts the same region of the brain at every time point. Conversely, if the head moves between acquisitions, the voxels' signal intensity becomes "contaminated" (67). Thus, motion correction will be performed to compensate for the subjects' head movement by rotating and translating each individual image. The third step is co-registration, in which the high resolution 3D T1 image is aligned with the functional images (67). Finally, segmentation, normalization and smoothing are performed as part of the preprocessing, which will be performed identical to the structural image preprocessing (Figure 4) (67). After completion of preprocessing, the statistical group analyses will be performed (section 4.5), which will be described later.

4.4.2.3 MR SPECTROSCOPY

MR spectroscopy provides information about metabolic process in the brain and is widely used in the clinic and in research (52). Even though MR spectra can be obtained from other nuclei, ^{1}H protons is the most used, due to the high sensitivity and abundance. Hence, the basic principles behind this technique is based on proton nuclear MR spectroscopy (^{1}H-NMR) (69). In the conventional MR images, we are mainly interested in the signal from ^{1}H to H_2O. For MR spectroscopy, we are not interested in the signal processing of ^{1}H to H_2O, in contrast we are only interested in the signal from ^{1}H to the other than H_2O molecules i.e. glutamate and N-acetyl-aspartate (69).

In brief, ^{1}H-NMR, which is most commonly known as MR spectroscopy, require standard radio frequency coils and a software package to analyze the brain metabolite concentrations (69). The spectra consists of a collection of peaks with different resonance frequency (chemical shift). In this Ph.D. study, MR spectroscopy were acquired using PRESS sequence from four different brain regions (for more details see Paper III) (70). The locations of those regions were placed with anatomical guidance from T1 and T2 structural scans. The collected data were then analyzed in the software package LCModel in which water scaling and eddy-current correction was conducted (Figure 4) (71). Next, the data were quality checked based on signal-to-noise ratio and full width at half maximum. Finally, statistical group analyses were performed on the selected brain metabolites.

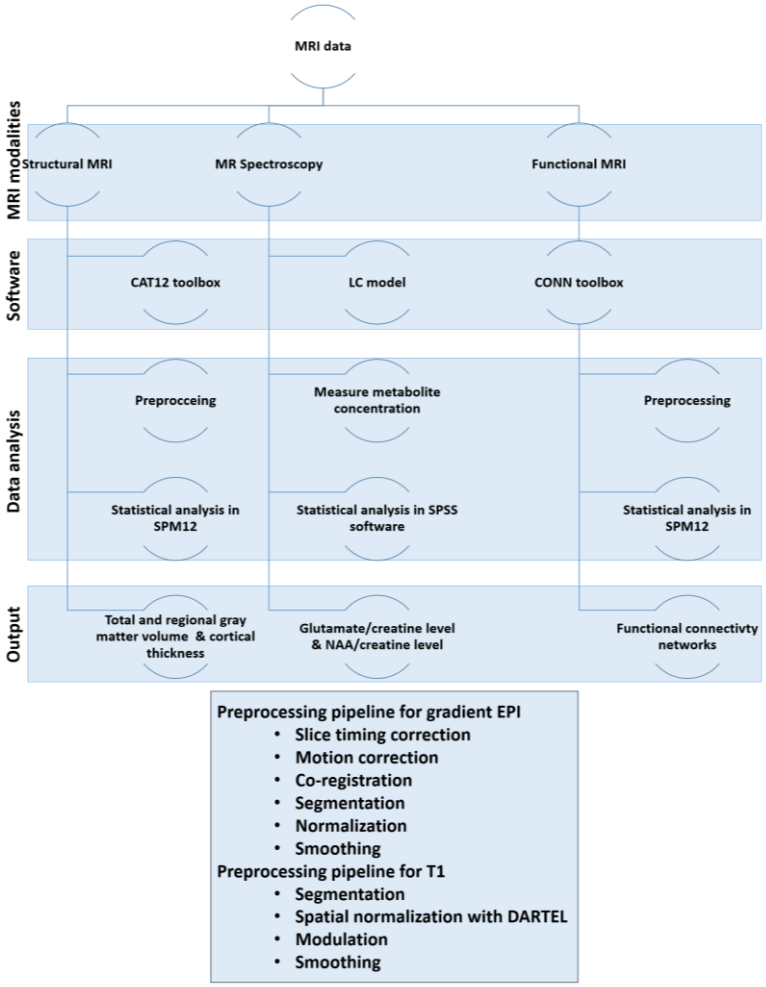

Figure 4: Overview of the MRI data analysis. Three different MRI data were acquired. Structural MRI was used to assess gray matter volume alterations using CAT12 toolbox. MR spectroscopy was used to assess brain metabolites alterations using LC model. Functional MRI was used to assess brain networks changes using CONN toolbox.

4.5 STATISTICAL ANALYSIS

Several analyses methods were used in study I and study II, depending on the study design, data construction and aims.

In study I, the MRI data were analyzed using unpaired 2-sample test for baseline data, while repeated measures analysis of variance (RM-ANOVA) was performed to assess the longitudinal changes for both healthy controls and CP patients. The clinical data

were analyzed using 2-sample t-tests, or 2-sample Wilcoxon rank sum test to detect difference between the groups. Furthermore, correlations tests were conducted using Person or Spearman's as appropriate in order to assess any association between MRI data and clinical data.
In study II, unpaired 2 sample test was performed to analyze MRI data and clinical data to detect differences between the groups (CP patients and healthy controls). Furthermore, both multiple regressions models and correlation analysis (Person or Spearman's) were performed to assess any association between MRI data and clinical data. Detailed descriptions of the statistical analysis is described in Papers I-IV.

Given that the studies could be considered as explorative studies, it is challenging to estimate the sample size. However, based on previous neuroimaging studies with structural, functional and metabolic MRI data as the primary outcomes, 12-30 patients seems to be sufficient to show relevant brain changes (9,68,72).The sample size of study I is 23 CP patients and 14 healthy controls. For study II the sample size was 35 CP patients and 23 healthy controls. Due to drop-outs and poor data quality, Paper II, III, and IV have different sample size. A detailed explanation for different sample sizes is explained in Table 2. P values less than 0.05 was considered statistical significant.

Study	Paper	Sample size	Explanation
I	I	Baseline: 23 CP patients and 14 healthy controls were scanned and analyzed. Follow-up: 11 CP patients and 12 healthy controls were scanned and analyzed.	Drop-out patients (n=12) - Continued excessive alcohol drinking (n = 2) - Declined to participate (n = 4) - Dead (n = 3) - Moved to a new place (n = 3) Drop-out controls (n=2): -Breast cancer (n = 1) -Knee pain (n = 1)
II	II	35 patients were scanned. However only 33 patients were in included in the analysis. 23 healthy controls were scanned and analyzed.	2 patients were excluded due to poor data quality.
II	III	35 patients were scanned. However only 31 patients were included in the analysis. 23 healthy controls were scanned and analyzed.	In addition to the two patients with poor data quality, two patients were further excluded due to ethanol peaks in the metabolite spectra. In total four patients were excluded in Paper III.
II	IV	35 patients were scanned. However only 30 patients were included in the analysis. 23 healthy controls were scanned and analyzed.	In addition to the above four patients, a patient with poor fMRI data was excluded. In total five patients were excluded in Paper IV.

Table 2: Overview of the sample size in study I and study II. Abbreviations: CP: chronic pancreatitis. fMRI: functional magnetic resonance imaging.

CHAPTER 5. RESULTS

The key results from the studies are presented in this chapter. Mores detailed results are reported in Papers I-IV. An overview of the main results from Papers I-IV is illustrated in Figure 5.

5.1. AIM I

Aim I: To investigate changes in the brain structure over time using structural MRI in painful CP patients compared with controls (Paper I) and potential associations to pain related symptoms (Paper I) and to explore associations between morphological brain changes and disease characteristics and risk factors (Paper II).

Key results:

- Accelerated total gray matter volume decrease ($P = 0.010$) and pronounced gray matter volume loss in precentral gyrus ($P = 0.021$) and putamen ($P = 0.035$) in CP patients compared to healthy controls during the 7-year follow-up (Paper I).
- Increase in pain scores over 7-years was associated with a less reduction of thalamic gray matter volume ($r = 0.61$, $P = 0.046$), (Paper I).
- Reduced gray matter volume and cortical thickness in CP patients compared with healthy control ($P < 0.05$), (Paper II).
- Alcoholic etiology of CP was independently associated with a decreased total gray matter volume ($P < 0.001$), whereas no association was observed for pain or other disease characteristics (all $P > 0.05$) (Paper II).

Interpretation*:* Our findings implicate that longstanding pain in CP results in measurable macrostructural morphological alterations over time within specific areas of the brain involved in processing and integration of the sensory system (Paper I). Compared with healthy controls, patients had reduced gray matter volume and cortical thickness with no associations to pain symptoms, likely because of the effects of previous alcohol use, which might obscure brain changes caused by pain (Paper II). The longitudinal study provides further evidence, supporting the hypotheses that the central pain processing is abnormal in CP while the cross-sectional study indicates that alcoholic etiology is the most prominent contributing factor for structural brain alterations in CP patients.

5.2 AIM II

Aim II: To assess brain metabolites using MR spectroscopy in CP patients, compared with healthy controls, in pain related areas (ACC, insula and prefrontal cortex) and in a parietal control region, as well as to compare brain metabolite levels in subgroups with different disease characteristics including pain (Paper III).

Key results:

- CP patients had increased glutamate/creatine level in the ACC ($P = 0.045$) and reduced N-acetylasparate/creatine levels in the parietal region ($P = 0.027$) compared with healthy controls.
- Patients with highest score of pain had higher glutamate/creatine level in the ACC compared to patients with lowest pain scores ($P = 0.039$).
- Lower parietal N-acetylasparate/creatine level was found in patients with alcoholic etiology as compared to patients without alcoholic etiology and healthy controls ($P < 0.006$).

Interpretation: Alterations in neurotransmitters have been demonstrated in CP patients, which is further associated to pain and alcoholic etiology. Findings from this study indicates that CP patients have neurochemical disturbance in addition to structural reorganization, which may likely be explained by nociceptive effect and neurotoxic effect. Overall imbalance of neurotransmitters in CP patients may be indicator of neuronal dysfunction.

5.3 AIM III

Aim III: To assess the resting state networks (default mode (DMN) and salience mode networks (SN)) using functional MRI in CP patients compared with healthy controls, and to explore the association between altered brain network connectivity and the clinical parameters as well as brain metabolites (Paper IV).

Key results:

- CP patients showed predominantly hyper-connectivity in both DMN and SN compared to healthy controls ($P < 0.05$).
- CP patients had significantly reduced anti-correlated functional connectivity between DMN and SN compared to healthy controls (all $P \leq 0.009$).
- The altered DMN connectivity in CP correlated to the brain metabolite glutamate/creatine level in ACC ($r = 0.363$, $P = 0.048$).

- There were no associations between altered functional connectivity and the disease characteristics such as QoL or pain symptoms.

Interpretation: The function of the DMN and SN are abnormal in CP patients compared with healthy controls. Altered DMN was further associated with the neurotransmitter glutamate/creatine level, indicating that glutamate may modulate DMN and an imbalance of glutamate may hinder deactivation of DMN. Lack of deactivation of DMN may consequently result in an abnormal anti-correlation between DMN and SN. Taken together, our findings share similarities with other chronic pain conditions, and support our understanding of altered brain circuitry associated with the CP disease and pain development.

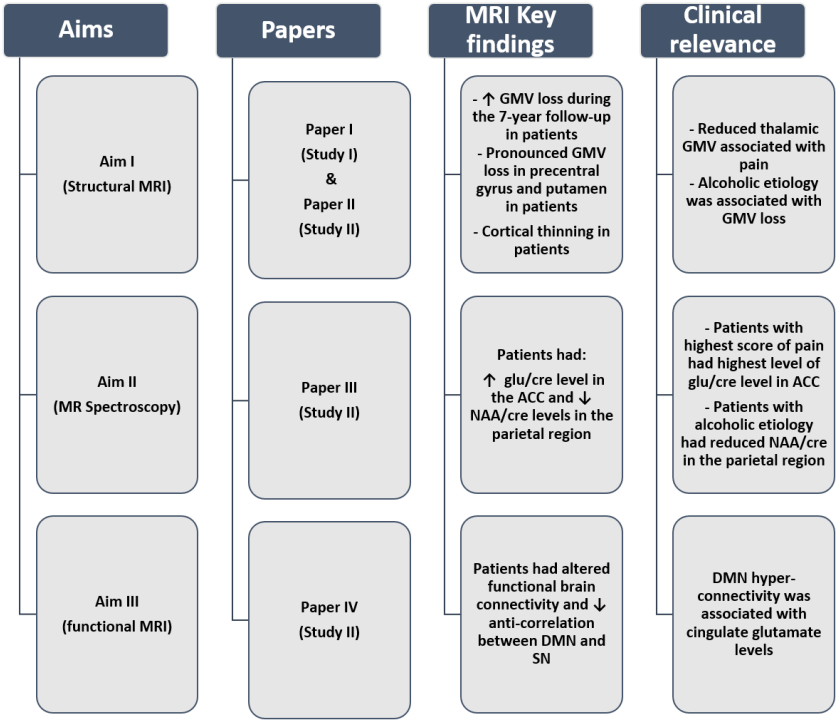

Figure 5: Overview of the main results from Paper I-IV. Abbreviations: CP: chronic pancreatitis. MR: magnetic resonance. MRI: magnetic resonance imaging. GMV: gray matter volume. Glu/cre: glutamate/creatine level. NAA/cre: N-acetyl-aspartate/creatine. ↑: increased. ↓: decreased. DMN: default mode network. SN: salience network.

CHAPTER 6. DISCUSSION

Multimodal MRI techniques were used to assess brain changes in CP patients compared with healthy controls. Two different clinical studies were conducted: a longitudinal study (study I), and a cross-sectional study (study II), to assess the brain changes with three different MRI modalities (structural MRI, MR spectroscopy, and fMRI). Those three MRI modalities provided anatomical, physiological, and chemical information of the brain. Overall, our findings provide strong evidence of structural, functional and metabolic alterations in CP patients. These alterations seem related and partly associated with both the patient reported pain, but also other disease characteristics such as alcoholic etiology. This section consists of three major parts. Firstly, the objective assessments will be discussed including structural MRI, MR-spectroscopy, and functional MRI. Secondly, the subjective assessments will be discussed. Thirdly, the methodological considerations will be discussed.

6.1 STRUCTURAL MRI

Assessment of the brain structure with MRI provided interesting insight about structural cortical reorganization in CP patients. Structural cortical reorganization was found both in the longitudinal study (study I) (73) and cross-sectional study (study II) (74). Particularly, Paper I revealed that the CP pain is related to morphological brain alterations over time involving pain related brain regions (incl. thalamus, putamen, and superior temporal gyrus), reflecting that this brain plasticity may be caused by long-term pain inputs. Importantly, CP patients showed also a trend in accelerated total gray matter volume loss compared with healthy controls. The majority of published MRI studies are cross-sectional studies (48,68,75), assessing the current brain abnormalities compared with healthy controls, however, little is known about the temporal evolution of the altered brain structure and its association to the clinical characteristics in chronic pain patients, particularly in CP patients. Knowledge from longitudinal studies may contribute with valuable information for future intervention studies.

Paper II, revealed reduced gray matter volume and cortical thinning in several brain areas. The altered brain morphology was further related to alcoholic etiology of CP and not to pain. Altered brain structure in both studies are in line with the findings from previous chronic pain studies. MRI studies with other chronic visceral pain conditions, particularly, irritable bowel syndrome (IBS) have shown reduced gray matter density in several brain areas such as prefrontal and posterior parietal region (48) as well as reduced cortical thickness (48).

Taken together, Paper I indicates that the structural brain alterations over time is associated with progression in pain, while Paper II strongly indicates that the structural reorganization is primarily associated with alcoholic etiology. Although the MRI findings overall shared similarities between the two studies, there were differences in the clinical association. Hence, there could plausibly raise doubt about the conclusion of the studies. Comparison of the two structural studies should be performed with caution given that the papers have different study design, different timeframe, and different clinical data. Paper I, studied brain changes over a 7-year period. For this purpose, pain diaries where collected both at the baseline visit and at the follow-up visit. This paper did not consider all the risk factors associated with CP. Paper II, was based on a cross-sectional study, assessing the current brain changes. Furthermore, Paper II took a major step forward into explaining the brain changes in CP patients, by taking several risk factors and patient outcomes into consideration when assessing the brain using a multivariate regression model. A limitation in the cross-sectional study was lack of pain diary prior to MRI scan. The pain in CP patients is often highly fluctuating, therefore we should have obtained data regarding the pain pattern using pain diary, to assess the true and more long-lasting pain, rather than asking the patients pain for the last 24 hours. Thus, it is not a major surprise, that the two structural MRI papers found two different clinical associations. Overall, both studies implicate that both pain and alcoholic etiology are two major contributing factors for brain plasticity in CP patients.

6.2. MR SPECTROSCOPY

The significantly higher level of glutamate/creatine level in the ACC in CP patients (76) may not be surprising, considering that glutamate is an excitatory neurotransmitter, facilitating and amplifying pain signals and is previously known to be increased in several chronic pain conditions (77,78). Most interestingly, we found that patients with highest score of pain had increased glutamate level compared with patients with lowest score of pain symptoms. This, confirms that CP patients with pain could have an imbalance of inhibitory and facilitatory descending pain modulation, resulting in greater gain of incoming nociceptive signals (19,46). However, it remains unclear whether an imbalance of inhibitory and facilitatory descending pain modulation leads to increased pain or weather increased pain leads to imbalance of inhibitory and facilitatory descending pain modulation.

Another major finding in Paper III, was the significant reduced N-acetylasparate/creatine levels in CP patients. Particularly, patients with alcoholic etiology of CP had reduced N-acetylasparatate/creatine level in parietal white matter, compared to patients without alcoholic etiology. Interestingly, the decrease did not

occur in pain related areas, but in the white matter parietal region consisting of myelin surrounded nerve fibers. N-acetylasparate/creatine is known to be a marker for the neuronal functionality and density (79), thus a decrease of N-acetylasparate/creatine implicates that previous alcohol abuse might be a significant factor contributing damage of neural structure and organization. Our findings are consistent with previous studies, demonstrating that alcohol is associated with white matter demyelination as well as degeneration (80). Taken together, we demonstrated that altered brain metabolites were both strongly associated to pain and to alcoholic etiology, however the results should be interpreted with caution, as the sample size of the subgroup analyses were relatively small.

6.3. FUNCTIONAL RESTING STATE MRI

Functional MRI was chosen to assess the resting state functional connectivity changes in CP patients in the two important brain networks: default mode network and salience network. Those networks have been shown to be altered in several chronic pain conditions i.e. fibromyalgia, migraine, and chronic lower back patients (72,81,82).

Based on Paper IV, we found that CP patients had hyper-connectivity within default mode network and salience network. Normally, the default mode network is highly activated during rest and deactivated while performing a task or in the presence of pain (83). In contrast to our hypothesis, we found that these patients have hyper-connectivity during rest compared to healthy controls.
Another prominent finding in the study was reduced anti-correlation between default mode network and salience network, which was not correlated to pain scores or alcoholic etiology. The reduced anti-correlation between those networks could be due to abnormal default mode network connectivity in CP patients. Abnormal anti-correlation between default mode network and salience network has previously been demonstrated in chronic pain patients (82,84) (incl. migraine patients (68), and fibromyalgia patients (85),), implicating functional damage. This abnormal anti-correlation has not only been demonstrated in chronic pain conditions, but also in several brain disorders such as in Alzheimer's disease suggesting that chronic pain share similarities with several brain disorders (84), and provides evidence that altered anti-correlation between default mode network and salience network may be related to altered attention. However, this need to be investigated further i.e. by conducting a cognitive MRI study to assess the attention in CP patients.

Moreover, we showed that increased connectivity in default mode network was associated to increased level of ACC glutamate/creatine in CP patients. Association

between default mode network and glutamate has previously been demonstrated by Hu et al 2013, suggesting that glutamate levels modulate brain activation (86). Particularly the excitatory neurotransmitter glutamate is thought to prevent deactivation of the default mode network (86). This, may explain the hyper-connectivity of default mode network in CP patients. By combining fMRI and MR spectroscopy, Paper IV has shown evidence of neurochemical characteristics that could affect the function of the default mode network, potentially providing novel insight into the function of default mode network in CP patients. Although, Paper IV failed to show an association between pain scores and brain connectivity. The results strongly implicate that the patients exhibit functional reorganization probably due to imbalance of excitatory neurotransmitter in glutamate, which primarily appears in CP patients with pain (Figure 6).

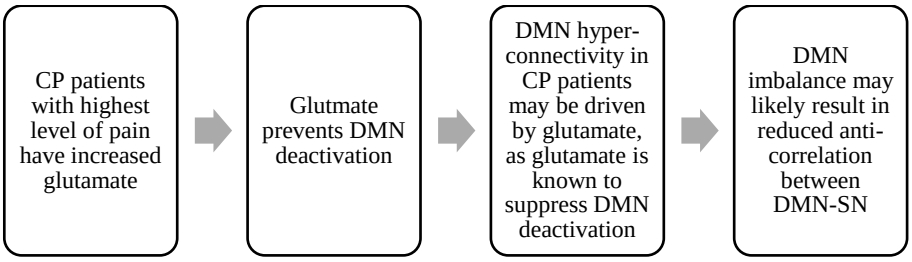

Figure 6: Potential mechanisms of glutamate involvement in default mode network. Abbreviations: CP: chronic pancreatitis. DMN: default mode network. SN: salience network.

6.4. BRAIN CHANGES AND ITS ASSOCIATION TO CHRONIC PAIN AND ALCOHOL

Previous EEG and QST studies have shown abnormal pain processing in the CP patients (5,27). None of those studies have directly considered disease characteristics in the data analysis, such as etiology, use of opioids, diabetes, and disease duration. Addressing these disease characteristics is crucial, as each factor is known to potentially affect the brain (70).

Overall, the four papers implicate that the brain changes are associated to both pain and alcoholic etiology (Figure 7). Differentiating of whether the brain changes are associated with chronic pain or alcoholic etiology can be challenging, as they are likely a complex combination of both. Thus, structural, functional and metabolites brain changes may be both due to a combination of alcoholic etiology, which is the most prevalent cause for development of CP, and long-lasting pain which is the most severe clinical manifestation of CP.

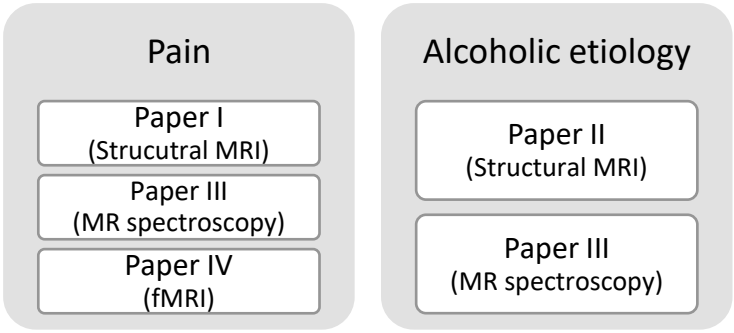

Figure 7: Paper I, III and IV provide structural, metabolic and functional brain alterations related to pain in chronic pancreatitis patients, while Paper II and III provide evidence of structural and metabolic brain alteration in relation to alcoholic etiology. Overall, Paper I-IV, implicate that brain alterations may be caused by a combination of both nociceptive and neurotoxic effect.

Lack of associations between pain and altered brain morphology in Paper II may differ likely due to the fact that pain in CP patients is often highly fluctuating and that the reported pain at the time of MRI scan does not exactly reflect the long-lasting pain input to the brain. Furthermore, as a results of external inputs, chronic pain patients have an altered internal milieu, and/or altered endogenous processing (47). Examples of external inputs in CP patients could be risk factors associated with the disease (87,88) i.e. alcohol abuse for a long period, nicotine consumption, hyperlipidemia, etc. In addition to risk factors, external inputs could also be extensive use of opioids, psychological problems, social factors, and depression, and diabetes (Figure 8). Both in Paper I and II, diabetes were taken into consideration in the MRI analysis, as previous studies have shown that diabetes may alter brain structure, function (89) and metabolites (90). Based on multiple regression model, Paper II showed that diabetes had no association to gray matter volume loss in CP patients.

Alcohol abuse remains the most prevalent risk factor for the development of CP and may likely play an essential role in the brain alterations in CP patients. For instance, if the patients have had many years of previous alcohol abuse and only experienced pain the last few months, then previous alcohol abuse may obscure the brain changes related to pain. Thus, not only nociceptive inputs play an essential role in the structural and functional reorganization, but previous neurotoxic inputs play equally an effect on the brain, both anatomically and chemically, referring to Paper II and Paper III (Figure 7). To identify the unique brain changes related to alcoholic etiology (and other potential factors influencing the brain), future studies should ideally recruit a

new control group, for instance subjects with extensive alcohol use but without a CP diagnosis. To study the brain changes related to pain, future studies need to recruit CP patients who had never experienced abdominal pain, which is practically very difficult. Furthermore, given that, it is complicated to detect a clear association between pain and brain alterations, future CP studies should include biological (pain (56), medication), psychological (anxiety, depression (91), sleep) and social (alcohol (92), trauma (47)) data when assessing the structural brain, as those factors a known to affect the brain structure (Figure 8).

6.5. SUBJECTIVE ASSESSMENT OF PAIN

In study I, pain diary and BPI questionnaire were assessed in CP patients, showing no significant differences between baseline and follow-up visit. No significant difference between those visits, does not necessarily mean that pain pattern has been stable during the 7-years, due to the fact that we did not measure the pain between baseline visit and follow-up visit. Another major limitation in this study was that we did not collect clinical data during the study period, such as medication, lifestyle changes i.e. alcohol consumption, psychological status i.e. depression (Figure 8) which are known also to affect the brain structure. Lack of controlling for those confounders between the two visits, generates a "black box" of factors that could contribute to alterations of the brain. Therefore, it can be fundamentally challenging to assess whether the altered brain morphology solely is associated to the effect of long-lasting pain. Future longitudinal studies are strongly recommended to repeatedly and systematically obtain physiological, psychological and social data during the entire study period.

In study II, BPI and QoL data were collected for all patients to subjectively assess the pain and QoL in CP patients. To date, there are no ideal objective pain assessment tools to assess pain given that pain is a subjective response, however it would have been optimal to have included QST, in order to assess nociceptive activity (93). QST provides physiological information of the underlying pain mechanisms involving different modalities such as thermal, mechanical, chemical and electrical compounds. Association of such QST data with MRI findings, could potentially been more informative than only correlating MRI findings with questionnaires.

Based on other chronic pain conditions, it is known that the longer the pain persists and the stronger it is, the more difficult is it to treat the pain, as the ongoing and long-lasting pain is an important driver for development of central sensitization (41). Therefore, the first step in treating pain could be examination and profiling the sensory

system in CP patients, enabling the clinicians to provide a more precise and effective (personalized) treatment based on the central pain mechanisms (41).

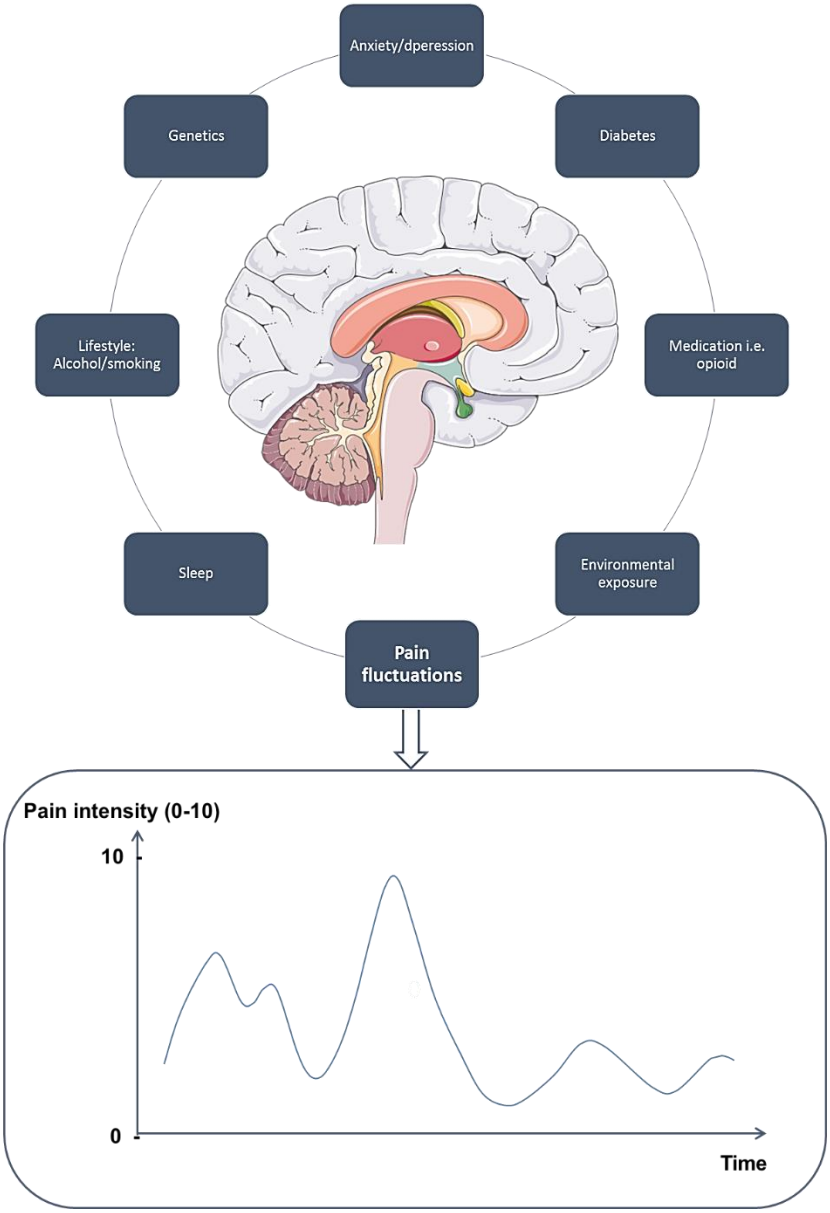

Figure 8: Several factors may affect the brain structure. The confounding factors could be biological (pain fluctuations), psychological (depression, anxiety) and social (lifestyle – alcohol/nicotine use, trauma etc.).

6.6. LIMITATIONS

For study I, the sample size was relatively small and moreover there was a large drop-out rate (52% of the patients and 14% of the healthy controls). The drop-out reasons are described in Paper I. Due to the fact that half of the patients discontinued, this may potentially have affected the MRI results, as these patients could have been those where regional brain atrophy and cortical thinning progressed the most, resulting in selection bias. Another limitation in this study was, that our sample of CP patients was clinical heterogeneous, meaning that the patients had different pain pattern (intermittent pain, constant pain, and constant pain with exacerbation). This indicates that the pain progression is individual and that there is a high chance of inter-subject variability in the general response to CP pain. For study II, lack of comprehensive data of the patient's previous alcohol abuse was a limitation, as only categorical data were obtained (whether the patients had alcoholic etiology or not). Thus, to assess the effect of alcoholic etiology, more data is needed such as years of alcohol abuse, amount of alcohol intake, etc. Also, Study II, was a cross-sectional study, hence, it was not possible to determine the true causal relationship between cerebral abnormalities and the disease characteristics.

Both study I and study II share some common limitations. Firstly, CP patients used medication i.e. opioids. Secondly, the pain was only assessed through clinical questionnaires. Future studies should include QST to better assess the underlying changes in the sensory system in CP patients. In regards to clinical data, futures studies should collect the clinical data more systematically. For instance in the longitudinal study, only baseline and follow-up data were collected, which resulted in generation of "black box", not knowing what that patients is going through during the 7 years. Also, both study I and study II, did not include exocrine insufficiency data. Those data would be valuable when assessing the brain in CP patients.

CHAPTER 7. CONCLUSION

This Ph.D. thesis had an overall objective, divided into three aims.
In conclusion, we have demonstrated that, CP patients after a 7-year follow-up period had accelerated gray matter volume loss and cortical thinning, particularly in the pain related areas. The altered brain structure was associated with progression in pain score. Reduced gray matter volume and cortical thinning in CP patients were further confirmed by the cross-sectional study. Although, the latter study found alterations in the pain related brain regions, no associations were found to the pain. Interestingly, we demonstrated that alcoholic etiology is the most prominent risk factor associated with altered brain morphology in CP patients. Taken all together, the structural MRI technique showed long-term structural reorganization with involvement of both nociceptive effect and previous neurotoxic effect on the brain (aim I).
Demonstration of potentially both alcoholic neurotoxic effects and long-lasting nociceptive effects on the brain in CP patients was further confirmed by the MR spectroscopy study. This technique detected reduced parietal N-acetylasparate/creatine in CP patients with alcoholic etiology, and increased ACC glutamate/creatine level in CP patients with highest level of pain compared with CP patients with lowest level of pain (aim II). Finally, functional reorganization was demonstrated using the resting state fMRI technique. Particularly, this technique showed hyper-connectivity within the default mode network and salience network, as well as reduced anti-correlation between those two networks. Most, importantly, we found an association between altered glutamate level (MR spectroscopy) and altered default mode network (fMRI), suggesting that glutamate may be involved in modulation of brain function such as the default mode network. Altogether, the fMRI study implicates that the CP patients have disrupted communication between the networks, similar to other chronic painful disorders, which may be potentially influenced by altered glutamate level (aim III).

To date, CNS abnormalities associated with pain has been receiving increased attention. However, this Ph.D. thesis, provides evidence showing that the pain (nociceptive effect) is not the only modulator for CNS abnormalities in CP patients, but a combination of both pain and previous alcohol use (neurotoxic effect) result in complex CNS abnormalities. This has been demonstrated using a multimodal MRI approach including structural, functional and metabolic assessment of the brain.
Structural, functional and neurochemical abnormalities of the brain may be reversed upon successful resolution of pain (94,95) and in combination with cessation of alcohol.

CHAPTER 8. CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

The results from this Ph.D. project has improved our understanding of CNS abnormalities in CP patients, demonstrating that the CNS changes is not solely driven by long-lasting nociceptive inputs, but it is most likely a complex combination of ongoing nociceptive and neurotoxic effects (i.e. alcohol) affecting the brain. Therefore, future studies should aim to distinguish between those effects.

To date, changes in the pancreatic morphology has received a lot of attention, resulting in invasive treatments (incl. endoscopic pancreatic duct clearance, pancreatic duct stenting and surgery). The rationale for such treatments is that decreasing the ductal pressure or resecting an inflammatory pancreas mass will lead to pain reduction (31). However, the pain has often a neuropathic origin involving central sensitization, structural and function reorganization and neurochemical alterations, hence invasive treatment would not always be efficient. In this case, new treatments with less side-effects targeting the CNS are highly warranted.

One such option is a treatment with transcutaneous vagal nerve stimulation (t-VNS). T-VNS has been shown to have an analgesic effect as well as anti-inflammatory effect, even though the exact mechanisms by which t-VNS modulates pain is unclear. T-VNS treatment is FDA approved for the acute treatment of migraine patients (96). Based on this rationale, a randomized, double blind, sham-controlled, cross-over, controlled study has recently been conducted by our research group (97). The protocol is published in BMJ Open, and registered at Clinicaltrials.gov with following number: NCT03357029. This study was conducted to investigate the analgesic effect a two-week t-VNS treatment in CP patients, as well as to explore the mechanisms of the anti-nociceptive effect using advanced neuroimaging techniques, QST, clinical questionnaires, pain diary, collection of blood samples and by measuring cardiac vagal tone. Among others the main efficacy parameters to be assessed are clinical pain relief and induced brain alterations using MRI. The data has not been analyzed yet.

Moreover, future studies should also assess microstructural white matter changes using diffusion tensor imaging (DTI). Although, the DTI was collected in both study I and study II, DTI data was not analyzed due to technical issues and due to limited time. Based on structural MRI, functional MRI, MRI spectroscopy and DTI, future

studies should attempt to conduct multivariate pattern analysis combining information from all of the four techniques to understand the complex CP disease effects across the brain.

Given that there are no objective assessments for pain, monitoring progress with structural, functional and metabolic scans, may help measure changes in patients with pain, in addition to QST. The latter enables to characterize the pain profile and identifying relevant pain mechanisms in CP patients with pain. In the same way, structural scans may contribute with information on cortical thickness and volume loss (73,74,98). Metabolic scans enables to detect alterations in several neurotransmitters as a results of treatment (99,100). Functional scan facilitates to define some objective outcomes such as sustained central sensitization or responses to specific analgesia (53,54). Future clinical trials are highly recommended to include MRI techniques to assess the effect of a treatment on the brain.

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APPENDIX: PAPER I-IV

I. **Muthulingam J**, Olesen SS, Hansen TM, Seminowicz DA, Burrowes S, Drewes AM, et al. Progression of Structural Brain Changes in Patients With Chronic Pancreatitis and Its Association to Chronic Pain: A 7-Year Longitudinal Follow-up Study. Pancreas. 2018; 47 (10):1267–76.

II. **Muthulingam JA**, Hansen TM, Olesen SS, Drewes AM, Frokjaer JB. Altered brain morphology in chronic pancreatitis patients and its association with pain and other disease characteristics. Eur J Gastroenterol Hepatol. 2019 Jun 31(9):1092-1098.

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IV. **Muthulingam, JA**. Hansen, T.M, Drewes, A.M., Olesen, S.S., & Frøkjær, J.B (2019). Disrupted functional connectivity of default mode and salience networks in chronic pancreatitis patients. *Clinical Neurophysiolgy* (Under review).

ISSN (online): 2246-1302
ISBN (online): 978-87-7210-508-6

AALBORG UNIVERSITY PRESS