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Endocannabinoids and Related Lipids in Chronic Pain

Analytical and Clinical Aspects

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To Fredric my brother who took an early departure - your hypothesis about the endocannabinoid system has not been rejected - but has been greatly inspiring me to accomplish this thesis.

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ABSTRACT

In Europe, approximately one in five adults experience chronic pain, pain that lasts more than three months. Chronic pain is a significant problem not only for those people suffering from chronic pain but also for society. The prevalence of chronic pain is higher in women and lower socioeconomic groups. Although chronic pain often originates in a specific site, it may eventually spread to several sites, transforming into chronic widespread pain (CWP), a condition evident in about 10% of the adult population. Approximately 1.2-5.4% are classified with fibromyalgia (FM). In addition to CWP, common symptoms of FM include, stiffness, fatigue, sleep disturbances, and cognitive dysfunction and common co-morbidities include depression and anxiety. Although FM/CWP has been reported to alter both central and peripheral nociceptive mechanisms, no objective biomarkers have been found that correlate with CWP/FM and no standard examinations such as blood test, X-ray or computed tomography can provide support for a diagnosis. Because there are no objective biomarkers that correlate with the pathophysiological processes associated with CWP/FM, this debilitating disease is difficult to diagnose and ultimately treat. However, there are some promising therapeutic targets for chronic pain with inter alia analgesic, anti-inflammatory, and stress modulating properties: the endocannabinoids (ECs) arachidonylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG) and their related lipids oleoylethanolamide (OEA), palmitoylethanolamide (PEA), and stearoylethanolamide (SEA).

This thesis investigates whether ECs and the related *N*-acylethanolamines (NAEs) can be used as potential biomarkers for CWP/FM. Specifically, the studies compared the peripheral and systemic levels of ECs and NAEs in 121 women with CWP/FM and in 137 healthy controls in two different cohorts. In addition, the correlation between lipid levels and common pain characteristics such as intensity, sensitivity, and duration were investigated. The EC and related lipid levels were measured using liquid chromatography in combination with tandem mass spectrometry. Multivariate data analysis was used for biomarker evaluation.

Compared to the healthy controls, the CWP/FM patients had significantly higher concentrations of OEA, PEA, and SEA in muscle and plasma ($p \leq 0.05$) and significantly higher 2-AG in plasma ($p \leq 0.01$). These results may indicate that NAEs, are mobilized differently in painful muscles compared with pain free muscles. Moreover, increased systemic levels of NAEs and 2-AG in patients might be signs of ongoing low-grade inflammation in

CWP/FM. These findings contribute to a better understanding of how peripheral and systemic factors maintain and activate chronic pain. Although the investigated lipids have statistically significant effects but biologically uncertain role in the clinical manifestations of CWP/FM. Thus plasma lipids are not a good biomarker for CWP/FM. Nevertheless, increased lipid levels indicate a metabolic asymmetry in CWP/FM, a finding that could serve as a basis for more research on pain management.

SVENSK SAMMANFATTNING

Långvarig smärta är idag en folksjukdom i Sverige där ungefär en femtedel av den vuxna befolkningen är drabbade. För ca 10 % av befolkning så är den smärtan generaliserad, d.v.s. multipla kroppsregioner är smärtande, och ca 1.2–5.4 % uppfyller också kriterierna för fibromyalgi. Vid fibromyalgi är förutom generaliserad smärta också stelhet, sömnstörningar, kognitiv dysfunktioner vanliga symptom samt ångest och depression vanliga samsjukligheter. Högre ålder, kvinnligt kön och låg socioekonomisk status är kända riskfaktorer, men kunskapen om bakomliggande biokemiska orsaker till långvarig smärta är fortfarande ofullständigt kartlagda.

Idag finns inga objektiva undersökningsmetoder (ex., röntgen, datortomografi, eller blodprov) som kan vara till hjälp vid diagnostisering av långvarig smärta. Diagnoser ställs istället med hjälp av ett antal kriterier, efter att andra kända sjukdomstillstånd har uteslutits.

Kroppens egna cannabinoidsystem upptäcktes i slutet på 1980-talet och har sedan dess utforskats intensivt. Detta system har sammankopplats med flera fysiologiska funktioner så som, regulator av smärta, inflammation och stress, vilket gör detta system till ett lovande terapeutiskt mål för långvarig smärta.

I denna avhandling har det undersökts om kroppsegna lipider med smärthämmande och anti-inflammatoriska egenskaper kan vara kopplade till långvarig smärta. Mera specifikt så har lipidkoncentrationer av endocannabinoider (ECs) och N-acetylanolaminer (NAEs) undersökts i vätska samlad från kappmuskulaturen, och i blodplasma hos individer med långvarig smärta samt hos smärtfria kontroller.

Förhöjda nivåer av flera lipider uppmättes i både muskelvätska och plasma hos patienter jämfört med kontroller. Koppling till smärta, ångest och depressions skattningar fanns delvis, men var relativt svag, vilket tyder på att det finns viktigare orsaker till dessa manifestationer än de undersökta lipiderna.

Sammanfattningsvis så tyder studierna i denna avhandling på att patienter med långvarig smärta har ökade nivåer av ECs och NAEs jämfört med friska smärtfria personer. Kopplingen till smärta var svag vilket gör att användbarheten **som ”markörer” för långvarig smärta** låg. Dock indikerar de förhöjda nivåerna på metabolisk obalans för dessa lipider hos patienter vilket kan vara användbart vid explorativ hantering av långvarig smärta.

LIST OF PAPERS

This thesis is based on the following papers, referred to by their Roman numerals in the text:

Paper I

Stensson Niclas, Ghafouri Nazdar, Träff Håkan, Anderson Chris D., Gerdle Björn, Ghafouri Bijar. Identification of lipid mediators in peripheral human tissues using an integrative in vivo microdialysis approach. *Journal of Analytical and Bioanalytical Techniques* 2016;7:306.

Paper II

Stensson Niclas, Ghafouri Björn, Ghafouri Nazdar, Gerdle Björn. High levels of endogenous lipid mediators (N-acyl ethanolamines) in women with chronic widespread pain during acute tissue trauma. *Molecular pain* 2016;12.

Paper III

Stensson Niclas, Ghafouri Bijar, Gerdle Björn, Ghafouri Nazdar. Alterations of anti-inflammatory lipids in plasma from women with chronic widespread pain - a case control study. *Lipids in Health and in Disease* 2017;16(1):112.

Paper IV

Niclas Stensson, Nazdar Ghafouri, Malin Ernberg, Kaisa Mannerkorpi, Eva Kosek, Björn Gerdle, Bijar Ghafouri. The relationship of endocannabinoidome lipid mediators with pain and psychological stress in women with fibromyalgia – a case control study (**submitted to The Journal of Pain**)

ABBREVIATIONS

AG	Arachidonoylglycerol
ACR	American college of rheumatology
AEA	Arachidonylethanolamide
BMI	Body mass index
CB	Cannabinoid
CNS	Central nervous system
CPT	Cold pain threshold
CV	Coefficient of variation
CWP	Chronic widespread pain
EC	Endocannabinoid
FIQ	Fibromyalgia impact questionnaire
FM	Fibromyalgia
HADS	Hospital anxiety and depression scale
HPT	Hot pain threshold
HPA	Hypothalamic-pituitary-adrenal axis
IASP	International association for the study of pain
IL	Interleukin
ISTD	Internal standard
LC	Liquid chromatography
MAG	Monoacylglycerol
MD	Microdialysis
MS/MS	Tandem mass spectrometry
MVDA	Multi variate data analysis
NAE	N-acylethanolamine
NRS	Numeric rating scale
OEA	Oleoylethanolamide
OPLS-DA	Orthogonal partial least squares-Discriminant analysis
PAG	Periaqueductal grey
PCA	Principal component analysis
PEA	Palmitoylethanolamide
PNS	Peripheral nervous system
PPT	Pressure pain thresholds
PPAR	Peroxisome proliferator activated receptor
QC	Quality control

RVM	Rostral ventromedial medulla
SD	Standard deviation
SEA	Stearoylethanolamide
SRM	Selected reaction monitoring
THC	Tetra hydro cannabinol
TNF- α	Tumor necrosis factor-alpha
TRPV ¹	Transient receptor potential vanilloid-1
VAS	Visual analog scale
VIP	Variable influence on projection
WDR	Wide dynamic range neuron

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How radical that the big bang occurred so that everything could be released, at least in theory thanks to **G. Lemaître and others**. How profound that the evolution could start off so that processes could spit away, at least in theory thanks to **C. Darwin and others**. How powerful of the **Homo sapiens** to survive, when other **Homos**, e.g., **erectus**, **habilis**, Neanderthals succumbed, and thanks to **C. Linné and others** the biological world is classified.

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INTRODUCTION

Pain – a brief ingress

Essential for human health and evolution, pain promotes healing of injuries and serves as a sensory detection and alarm system for escape and survival. However, pain can be harmful to health when it becomes persistent or chronic. Although pain most often has a proximate physical cause, psychological interactions and co-morbidities make it difficult to study. Moreover, pain is a subjective experience. However, it is possible to measure pain signalling (nociception) as specific nerve cells (nociceptors) and receptors along pain signalling routes have been identified and specific neurotransmitters are known to be either mediators or inhibitors of pain signalling.

Nociceptors are specialized peripheral sensory neurons – unmyelinated C-fibers and myelinated A-fibers. These neurons, also called primary afferent neurons, innervate the skin and muscles and are connected to the dorsal horn in the spinal cord [1]. Primary afferents are pseudo-unipolar neurons that enable bidirectional signalling (i.e., they send and receive signals from either nerve ending). From the dorsal horn, nociception transmits towards the brain (e.g., via the ascending spinothalamic tracts) and innervate different brain areas where the thalamus is believed to act as a relay station connecting **various cortical regions included in the so called ‘pain matrix’** [2]. There are also descending spinothalamic tracts that transmit signals from the brain back to the dorsal horn where modulation of nociceptive information occurs via distinct neurotransmitters (e.g., endogenous opioids, GABA, and substance P). Under most circumstances, transmission of nociceptive information results in pain perception; however, nociception can also be dissociable from the experience of pain. In other words, nociception can occur in the absence of the awareness of pain and pain can occur in the absence of measurably noxious stimuli [3], an interaction that emphasizes the complexity of pain. Figure 1 presents a simplified model of the nociceptive pathway.

Figure 1. Simplified illustration (Peter Lamb © 123RF.com.) of nociceptor and mechanoreceptor pathways from peripheral sites through the spinal cord and into the brain. **A δ and C fibres** comprise the primary, first-order sensory afferents coming into the gate at the dorsal horn of the spinal cord. Secondary neurons cross the cord and ascend to the thalamus as part of the spinothalamic tract. Third-order afferents (not illustrated) project to higher brain centres such as the limbic system, and the sensory cortex.

Chronic pain

Unlike acute pain, which serves as a warning and part of a protection system, chronic pain is arbitrarily defined: chronic pain persists beyond normal tissue healing time, usually defined as pain that is present for at least three months [4] (although a six-month timescale often is used in research settings). Chronic pain could be related to an initial injury or to an ongoing pathological condition, but it could also be idiopathic (i.e., no clear cause or origin can be determined).

However, multiple factors influence the emergence and maintenance of chronic pain. In addition to psychological and social factors, neurobiological and biochemical factors influence how chronic pain is perceived. The progress of peripheral and central sensitization of pain processing may influence the transition of pain from acute to chronic [5]. The International Association for the Study of Pain (IASP) defines central sensitization as follows:

Increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input and may include increased responsiveness due to dysfunction of endogenous pain control systems. (IASP taxonomy-www.iasp-pain.org)

The IASP defines peripheral sensitization as follows:

Increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive fields. (IASP taxonomy-www.iasp-pain.org)

Sensitization in the peripheral nervous system (PNS) could be the result of abnormalities in the small fibres (C and A-delta fibers) in the skin [6] and biochemical changes (metabolic, mitochondrial, and cytokine) that affect nociceptors in muscles [7]. Central sensitization mechanisms refer to alterations in the pain processing pathways in the central nervous system (CNS) **where an increased activation of the ‘pain matrix’ in the brain** [8] has been suggested. Specific changes in nociceptive pathways located in the dorsal horn due to synaptic plasticity [9] have also been proposed to drive the central sensitization processing.

Sensitization processes can also be explained as alterations in the endogenous pain modulation processes, pain modulation pathways that ascend “**bottom up**” [3]. In addition, alterations in the endogenous pain modula-

tion could be referred to as alterations in the descending “**top down**” inhibitory pain modulation pathways such as the well-studied PAG-RVM system [10]. The endogenous pain inhibitory ability has been studied in humans by conditioned pain modulation (CPM) activation (similar to diffuse noxious inhibitory controls (DNIC) in rats) and relies on the analgesic effect of a conditioning stimulus on a painful test stimulus (“**pain inhibits pain**”) [11]. Deficit CPM has been reported in several chronic pain conditions [12, 13]. However, sensitization can only be measured in settings where both input and output of a neural system are known (i.e., in animal experiments). Although phenomenon such as allodynia (pain due to a stimulus that does not normally provoke pain) and hyperalgesia (increased pain from a stimulus that normally provokes pain) can be seen as signs of sensitization, these phenomena only provide indirect information about sensitization.

Chronic widespread pain (CWP) and fibromyalgia (FM)

CWP, usually described as generalized muscle pain, is often associated with widespread hyperalgesia and/or allodynia. Compared to patients with a localized or regional chronic pain, CWP patients experience multiple pain sites and often experience high levels of anxiety and depression [14].

Although CWP often starts as a local or regional pain condition [15], the cause of the spreading of the pain remains unclear. Risk factors for CWP include female, higher age, depression, and family history of pain, but there is no clear consensus [15]. Central and peripheral sensitization processes and defects in the inhibitory pain modulation pathways, as described above, could possibly explain the neurophysiological factors associated with CWP.

CWP patients who also have widespread hyperalgesia (determined using a tender point examination of standardized anatomical sites) fulfil the diagnosis of FM. Hence, FM is a subgroup of CWP. So, what is the difference between CWP and FM? Both CWP and FM are considered the most negative endpoint of a continuum of chronic musculoskeletal pain conditions. FM patients often report a somewhat worse situation compared to CWP patients [16]. Hence, in addition to CWP, stiffness, fatigue, sleep disturbances, depression, anxiety, stress, and cognitive dysfunction are prevalent [17, 18].

Although prolonged musculoskeletal pain and other symptoms that are currently associated with FM and CWP have been described since ancient times, the emergence of distinct classification criteria for CWP and FM have only emerged over the last 50 years. The term fibromyalgia (**fibro** = tissue; **myo** = muscle; algos- = pain), which originated from “**fibrositis**”, was coined in 1976 by P. K. Hench to describe the criteria published by

Moldofsky and Smythe. The Moldofsky-Smythe criteria included widespread pain, non-restorative sleep, and 11 of 14 distressing tender points, which became the basis of the American College of Rheumatology (ACR) classification criteria for FM and CWP in 1990. In the ACR 1990 classifications, Wolfe and co-workers concentrated mainly on the musculoskeletal aspects of FM: a history (>3 month) of chronic widespread rheumatic pain on the right and left side + above and below waist + axial pain and a high number of active tender points (11 out of 18) [19]. They ignored other key symptoms such as fatigue, sleep disturbance, and cognitive dysfunctions.

In 2010, the ACR proposed new diagnosis criteria for FM. This criteria replaced the tender point examination with a widespread pain index (WPI) and introduced a symptom severity score (SS), which included the symptoms fatigue, waking unrefreshed, and cognitive symptoms [20]. The ACR 2010 was criticized for being too time consuming and a modified version, the ACR 2011, included both the WPI and SS in a self-report survey – the FM Survey Questionnaire (FSQ) [21]. In 2013, Bennet and co-workers developed an alternative to the ACR 2011 with the artful name 2013 AltCr [22], which included more pain locations and more symptoms than the ACR 2011. In 2016, Wolfe et al. presented a revised version of the ACR 2010-2011 criteria, which refined the WPI scoring from 2010 and reintroduced the generalized pain criteria from ACR 1990 [23]. This thesis uses the ACR classification from 1990 for CWP and FM.

Chronic pain is a public health challenge

In 2006, a large epidemiological survey found a prevalence of chronic pain (moderate to severe) to be about 19% in the European adult population [24]. According to ACR 1990 criteria, the prevalence of CWP is approximately 10% in the general population [25, 26], with a 1.2-5.4% prevalence for the FM subgroup per the ACR 1990, 2010, and the modified 2010 criteria [27]. In 2010, *The Swedish Agency for Health Technology Assessment and Assessment of Social Services* (SBU) revealed that the Swedish population's prevalence for persistent pain to be about 40% and that about 5% of these people sought health care and about 1% sought specialist care. Clearly, chronic pain is a significant burden not only on those who suffer from the disease but also on society.

CWP and FM – diagnosis and treatments challenges

For chronic pain management in general, the biopsychosocial approach has been well-established and is today the state-of-the-art approach when assessing chronic pain conditions [28]. Before a diagnosis is determined, other possible **diagnosable conditions (“red flags”)** need to be ruled out. Furthermore, symptoms can vary widely between patients and for the same

patient over time. Treatments are often chosen based on a trial-and-error, where different drugs are tested (one-by-one) and evaluated to find the most suitable treatment. Diagnosing and treating CWP and FM is challenging because there are no objective diagnostic tests that can be used to complement the subjective assessment of these conditions. In addition, there is a large discrepancy between what patients report and what standard tests (e.g., blood panels and X-rays) reveal. That is, diagnosing CWP and FMS is not mechanism-based but relies on symptoms and semi-objective signs. If clinicians had access to biomarker testing, they would have a mechanism-based way to make a diagnosis and evaluate treatments [29], a clear advantage over subjective patient information and subjective clinical assessments. A biomarker is a characteristic, such as a chemical, that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention [30]. The need for biomarkers in pain medicine derives mainly from the current limitations of treatment methods, a substantial problem world-wide.

Pain pathway chemistry – from ions to proteins: a brief introduction

This section introduces some elemental components – e.g., ions, neurotransmitters, and receptors (proteins) – involved in peripheral and central mechanisms of nociception and links these components to some of the pharmacological interventions used in pain management.

Pro nociceptive components

In nociceptive transmission pathways, both high-voltage-activated Ca^{2+} (e.g., N-type, especially in the spinal cord) and low-voltage-activated Ca^{2+} (e.g., T-type) help modulate the release of pro-nociceptive neurotransmitters [31, 32].

If calcium channels malfunction, they can allow too much calcium to enter the neuronal cell body, a condition that may increase the perception of pain. To inhibit or block this Ca^{2+} influx and therefore to manage chronic pain, calcium blockers or calcium inhibitor drugs such as Gabapentin, Pregabalin, and Ziconotide are used.

In addition to high- and low-voltage Ca^{2+} , the functioning of voltage-gated sodium channels, especially the $\text{Nav} 1.7$ receptor, can influence nociception or pain signalling [33]. For example, Cox et al. found that gene coding for the $\text{Nav} 1.7$ receptor was missing in some members of a family from Pakistan who were unable to feel pain but who were otherwise healthy and fully functional [34]. However, surprisingly, no selective $\text{Nav} 1.7$ blocker has found to be clinically effective and selective $\text{Nav} 1.7$ blockers have proven to be only relatively weak analgesic drugs [35]. One non-selective sodium channel blocker has been used extensively as a local anaesthetic drug and for pain management, Lidocaine.

Ligand-gated ion channels are also located along the pain route, where glutamate (NMDA, AMPA, kainite, and metabotropic) receptors have been localized at all levels in the pain processing pathways (peripheral, spinal, and brain) [36]. These receptors also influence pain processing, so NMDA receptor antagonists (e.g., ketamine and methadone) can be used to manage chronic pain.

Another well-studied component that is found along the pain route is substance P, a broadly distributed neuropeptide in the CNS and PNS. Substance P and its main target the neurokinin 1 receptor (NK1) are involved in neuroinflammation [37]. However, NK1 receptor antagonists have failed to exhibit efficacy in clinical pain trials [38]. In addition, substance P is part of the nociceptive “**inflammatory soup**”, which also includes protons, ATP, bradykinin, histamines, and serotonin. These components are released (e.g., as the result of tissue damage) near the primary afferent nociceptor.

Other components affected when **the inflammatory soup “is cooked”** and important for pro-nociception are the transient receptor proteins (TRP), especially the transient receptor potential vanilloid 1 (TRPV¹), a non-selective cation channel also called the capsaicin (the burning pain compound in hot chili peppers) receptor. TRPV¹ was the first TRP channel to be cloned by Julius et al. [39]. Julius and co-workers discovered that TRPV¹ was activated by vanilloid compounds and noxious thermal stimuli and desensitized when sufficiently provoked in calcium-dependent fashion, a discovery that may underlie the paradoxical analgesic effect of capsaicin. TRP channels have been targeted for pain relief [40]. Qutenza is the brand name of a capsaicin containing patch, which has an anaesthetic effect and is used for management of peripheral neuropathic pain.

Cytokines are often categorized as pro- or anti-inflammatory with algetic or analgesic properties. Cytokines will be discussed more thoroughly in a separate section below.

Anti-nociceptive components

The most potent innate pain-relieving system in the body is the opioid system, including the opioids enkephalin, endorphin, and dynorphin and their molecular targets μ , δ , and κ receptors. Opioids decrease pain transmission to the brain by activating the descending nerve fibres from the midbrain and the medulla that control the endogenous opioid containing interneurons within the dorsal horn of the spinal cord. Many synthetic opioids are used for pain management, although these compounds are highly addictive. Addiction to opioids is a serious global problem that affects the health as well the social and economic welfare of many societies.

Other neurotransmitters such as the monoamines serotonin, norepinephrine, and dopamine are also mediators of endogenous analgesic mechanisms in the descending pain pathways [41], although some studies found serotonin to be both an inhibitory and promoter of pain perception via different physiological mechanisms [42]. Because serotonin and norepinephrine reuptake inhibitors (SNRIs) (e.g., Duloxetine) increase serotonin and norepinephrine levels, they are used to treat both depression and chronic pain.

Over the last three decades, the endocannabinoid (EC) system has been studied for its effects on pain perception. This system includes some lipid mediators and their receptor targets together with some metabolic enzymes. The EC system will be described more thoroughly below.

Lipid mediators and cytokines

Lipid mediators

The word lipid originates from the Greek word *lipos* (fat). No strict definition of a lipid exists, although all lipids are of biological origin and not soluble in water. In addition to being a source of stored energy (triglycerides) and a key component of cell membranes (mainly phospholipids), some lipids are also bioactive, mediating biological signalling. A lipid mediator is a lipophilic molecule that regulates cell-to-cell communication.

Various lipid mediators are involved in the regulation of the excitability of peripheral nociceptors [43] and in the mechanisms of inflammation [44]. Lipids belonging to the eicosanoids such as prostaglandins and leukotrienes are the most well-characterized pro-algetic/inflammatory lipid mediators. Prostaglandins and leukotrienes are derived from arachidonic acid via the enzymes cyclooxygenase¹⁻² (COX¹⁻²) for prostaglandins and arachidonate 5-lipoxygenase (ALOX5) for the leukotrienes. As with endocannabinoids (ECs), and related lipids are analgesic and anti-inflammatory mediators.

Cytokines

In the 1950s, the first cytokine, interferon, was observed by researchers studying the interference of heat with the influenza virus **A** and chick chorio-allantoic membranes [45]. Since then, more than 300 cytokines have been identified. Cytokines are small non-structural proteins evolved from the earliest forms of intracellular molecules before the appearance of receptors and signalling cascades. Cytokines are mediators of cell-to-cell communication and can be divided into functional classes. For example, some cytokines are primarily lymphocyte growth factors, some work as pro-inflammatory or anti-inflammatory molecules, some polarize the immune response to antigens [46].

Paper III investigated four of the more well-characterized pro-inflammatory cytokines – tumour necrosis factor- α (TNF- α), interleukin- 1β (IL- 1β), interleukin-6 (IL-6), and interleukin-8 (IL-8) – and one well-characterized anti-inflammatory cytokine – interleukin-10 (IL-10). These cytokines are expressed in numerous cell types including immune cells such as macrophages, monocytes, hepatocytes, T-cells, and mast cells [47]. It remains unclear if cytokine levels influence chronic pain conditions; however, two systematic reviews, one from 2012 [48] and one from 2014 [49], found that FM patients had elevated serum levels of IL-1RA, IL-6, and IL-8.

The endocannabinoid system

Before the story of the EC system is described, the history of cannabis and cannabis research needs to be mentioned to provide the key links between cannabis, cannabis research, cannabis therapy, and the endogenous cannabinoid system.

Cannabis history

Since the ancient times, *Cannabis sativa*, *C. indica*, and *C. ruderalis* have been used in folk medicine and as a source of textile fibre. In the mid 19th century, the Irish physician William Brooke O'Shaughnessy introduced the therapeutic use of cannabis to western medicine. In the 1830s, O'Shaughnessy worked for the East India Company in Calcutta as an assistant surgeon. While in India (where cannabis was widely used), he discovered the therapeutic effects of cannabis and he started to do experiments on various animals and eventually conducted a clinical trial with cannabis preparation. This trial resulted in one of the first published articles (1839) on medical uses of cannabis, which was also one of the first modern scientific studies – “**On the Preparations of the Indian Hemp, or Gunjah (Cannabis Indica), Their Effects on the Animal System in Health, and Their Utility in the Treatment of Tetanus and other Convulsive Diseases**” [50].

During the second half of the 19th and the first half of the 20th centuries, cannabis therapy was described in most pharmacopoeias in the western world as a treatment for a variety of disease conditions. During this period, hundreds of papers were published on cannabis. However, this research did not continue. Many factors were responsible for the decrease in cannabis research after this initial enthusiasm: the development of new synthetic drugs such as aspirin, barbiturates; the relatively new method of injecting morphine subcutaneous for localized anaesthesia; and the “**Marihuana Tax Act**” in the US in 1937 [51]. By 1952, the WHO concluded that there was “**no justification for medical use**” for cannabis. Two years before the WHO pronouncement, the medical products agency in Sweden removed the two cannabis containing drugs (Cannabisol and Cannatropin) from its registry, effectively making cannabis unavailable as a drug treatment.

After a relatively short non-eventful period of cannabis research, it took a chemist and language equilibrist to restart the research field. Raphael Mechoulam, after reading many old cannabis papers written in different languages (English, French, German, and Russian), discovered that interest in cannabis was sufficient to make cannabis investigation possible again. In 1964, Mechoulam and Y. Gaoni successfully isolated and described the structure of the main psychoactive ingredient in cannabis, delta-9-tetrahydrocannabinol (D9-THC) [52], the information that eventually lead to the first discovery of the endogenous cannabis system.

The discovery of an endogenous signalling system

In 1988, Devane, et al. discovered the first cannabinoid receptor (CB¹) expressed in the rat CSN [53]. Later, Herkenham et al. visualized the distribution of CB¹ and suggested that CB¹ was an adenylyl cyclase inhibitor [54]. A few years later, CB¹ was cloned from rat and human cells [55, 56]. In 1993, a second cannabis receptor (CB²), mainly found in immune system cells in the PNS, was identified [57]. The discovery of the CB¹ and CB² G-protein coupled cannabinoid receptors suggested the existence of endogenous ligand(s) that could bind to these receptors and exert a physiological effect. In 1992, Mechoulam and Devane and co-workers found such a ligand in porcine brain; they named this ligand anandamide [58], using the Sanskrit word for bliss (*ananda*), a subtle reference to the country where cannabis was first studied. In 1995, another ligand was discovered [59, 60]. Since 1995, additional endogenous ligands have been found to have affinity to CB receptors, and other G-protein coupled receptors have been suggested as putative cannabinoid receptors. In addition, several enzymes are involved in synthesis and degradation of ECs; these enzymes will be more thoroughly described below. The components described above represent the EC system.

The targeted lipid mediators

Here, I present the specific lipid mediators targeted in this thesis. These lipids are either chemically classified as *N*-acylethanolamines (NAEs) or as monoacylglycerols (MAGs). In addition to the below described lipids, there are several other lipids that belong to the NAEs or the MAGs and lipids such as *N*-arachidonoyl-dopamine that are associated with the EC system, which were not included in the scope of this thesis. The targeted lipids are described with their chemical name(s) and abbreviation, and CXX:Y, where XX = number of carbons in R and Y = number of double bonds (unsaturated hydrocarbons).

N-acylethanolamines (NAEs)

Lipids with the general chemical structure

are NAEs where R is a carbon chain linked to an acyl group that is linked to the nitrogen atom of ethanolamine. NAEs are fatty acid derivatives and exist with different acyl chain length and a different number of double bonds.

Arachidonylethanolamide (AEA) (C20:4), also called anandamide, is a partial agonist at CB receptors with approximately 4-fold higher affinity for CB¹ vs. CB² [61]. AEA has also been found to have an affinity for the TRPV¹ receptor [62]. In addition, AEA can activate the two isoforms of peroxisome proliferator activating receptors (PPARs) – PPAR- γ [63] and the PPAR- α [64] receptors. Multiple functions are suggested for AEA, including modulation of pain, memory, inflammation, and energy metabolism. AEA may modulate the reward system via dopamine release [65] and be induced during physical activity [66].

Oleoylethanolamide (OEA) (C18:1) is a PPAR- α agonist [67] but is also a TRPV¹ activator [68] and a GPR¹¹⁹ activator [69]. In animal studies, OEA was primarily characterized with anorexic properties [70]. It has also been associated with analgesic properties [71] as well as the induction of visceral pain [68].

In the 1950s, it was demonstrated that lipid fractions purified from egg yolk, peanut oil, and soybean lecithin exerted anti-allergic effects in the guinea pig. Kuehl and co-workers isolated palmitoylethanolamide (PEA) (C16:0) as the agent responsible for these anti-inflammatory properties [72]. This work ultimately led to the identification of PEA in mammalian brain, liver, and skeletal muscle tissues in 1964 [73]. The PPAR- α receptor is activated by PEA, which has been suggested to be the main pathway for PEA's anti-inflammatory properties [74]. PEA also activates the GPR⁵⁵ receptor in a more potent manner than AEA [75]. PEA was also recently found to inhibit prostaglandin and hydroxyl eicosatetraenoic acid production by a macrophage cell line [76].

Although stearoylethanolamide (SEA) (C18:0) has been proposed to generate anti-inflammatory activity [77] and to activate PPAR- γ [78], no receptor target has been clearly identified. As with OEA, SEA has also been proposed to exert anorectic effects in mice [79].

Monoacylglycerols (MAGs)

Lipids with the general chemical structure

are MAGs with a glycerol linked to a fatty acid via an ester bond. MAGs can have different acyl chain length and a different number of double bonds, and they can exist in three different stereochemical isoforms, with the stereospecific numbering (*sn*): *sn*1, *sn*2, and *sn*3.

The second endogenous CB activating lipid mediator discovered was arachidonoylglycerol (AG) (C20:0), which exists as three isomers (1, 2, and 3). Although 1(3)-AG has been found to activate CB¹, the 2-AG isomer is the most potent CB¹ agonist [80, 81]. In comparison with AEA, 2-AG is substantially more abundant in the CNS [82], and AEA acts as a weak partial agonist in comparison with 2-AG (full agonist) at the human peripheral cannabinoid receptor CB² [83]. Both 2-AG and 1-AG are TRPV¹ activators [84], and 2-AG can activate both PPAR- γ and $-\alpha$ [85]. Moreover, 2-AG has also been recognized to directly activate GABA_A receptors [86]. Both 2-AG and AEA could be retrograde signalling lipids [87].

Like AEA, multiple biological functions are suggested for 2-AG, including modulation of pain, stress, inflammation [88], and systemic energy metabolism [89]. 2-AG has also been suggested to be involved in the production of exercise-induced anti-nociception in rats [90].

Receptors

The CB receptors belong to the seven-transmembrane domain family of G-protein coupled receptors. CB¹ receptors are mainly found in brain neurons and peripheral tissues, including fat (adipocytes), liver, pancreas, and skeletal muscle [91], and in other cells and tissues such as skin cells [92] and testes [93]. CB² receptors are mainly found in immune tissues (spleen, tonsils, and thymus) and cells (B lymphocytes and CD4 and CD8 lymphocytes, natural killer cells, PMNs, macrophages, microglia, and mast cells) [94]. CB² mRNA has also been localized in glutamatergic and GABAergic neurons in mice hippocampus [95].

Both CB¹ and CB² receptors primarily signal through the inhibitory G_{i/o} proteins. G protein activation stimulates CB¹ receptors to inhibit adenylyl cyclase, the activation of mitogen-activated protein kinases, the inhibition of certain voltage-gated calcium channels, and the activation of G protein-linked inwardly rectifying potassium channels. Stimulation of CB² receptors has similar consequences, with the exception of the modulation of ion channels [96].

The effects of neurotransmission through the activation of presynaptic CB¹ receptors has been linked to inhibition of the provoked release of a number of different excitatory or inhibitory neurotransmitters (i.e., GABA, glutamate, noradrenalin, serotonin, dopamine) both in the brain and in the peripheral nervous system [96].

TRPV¹ is expressed in all sensory ganglia (DRG, TG, and Vagal) and in small sensory C- and A δ fibres. TRPV¹ is also found in the CNS and in non-neuronal tissues such as keratinocytes, mast cells, hair follicles, smooth muscle, bladder, liver, kidney, spleen, and lungs cells [97]. Transduction can be initiated by a wide range of stimuli (e.g., heat, pH, touch, protons,

and different endo- and exogenous substances). Activation of TRPV¹ in nociceptive sensory neurons leads to Ca²⁺ influx, resulting in membrane depolarization and release of neuropeptides from primary afferent nerve terminals [98]. More recent studies have found that stimulation of microglial TRPV¹ in rat brain controls cortical microglia activation and indirectly enhances glutamatergic transmission in neurons [99].

The three isoforms of PPARs (**α, δ, and γ**) are nuclear receptors and ligand-activated transcriptional factors that play an essential role in energy metabolism. PPARs are expressed in multiple human tissues including liver, skeletal muscle, adipose tissue, heart tissue [100], and skin tissue [101]. PPARs are expressed in various immune cells such as monocytes, macrophages, and endothelial T- and B-cells [102, 103].

All three isoforms of PPAR have similar structural and functional features. In the classical model of PPAR activation, PPAR with the retinoid X receptor (RXR) is hetero-dimerized with the proliferation response element termed DR-1. Activation of transcription through this dimer is blocked by associated co-repressor proteins, such as nuclear receptor corepressors (NCoR), histone deacetylases (HDAC), and G-protein pathway suppressor 2 (GPS2). Formation of the PPAR activation complex leads to histone modification (e.g., through acetylation) and altered expression of genes involved in fatty acid metabolism, lipid homeostasis, and adipocyte differentiation [104]. Both PPAR-**α** and **-γ activation inhibit the transcriptional activity of nuclear factor kappa beta (NF-κB), the activator protein-1 (AP-1)** [105, 106], and inflammatory gene expression [106, 107].

There is growing evidence that other cannabinoid or cannabinoid-like receptors remain to be identified as important players of the EC system. For example, the GPCR receptors GPR55, GPR18, and GPR119 are proposed candidates. However, in a recent review (2017), the pharmacological discrepancies and the lack of selective ligands for these receptors are delaying the characterization of their relationship with the EC system, and consequently, no CB₃ receptors have been confirmed [108].

Metabolism of NAEs

Unlike other neurotransmitters (e.g., GABA and glutamate) that are stored in cell vesicles, it is traditionally accepted that ECs and NAEs are not stored in cells awaiting release but are rather synthesized on demand in response to physiological and pathological stimuli. However, some data suggest that NAEs can be stored inside the cell [109].

Several synthesis routes for NAEs have been proposed. The principal route involves a two-step enzymatic process. In the first step, catalysed by a calcium-dependent N-acyltransferase (NAT), an acyl chain is transferred from

the sn-1 position of a glycerophospholipid to the amino group of the hydroxyethyl moiety of phosphatidylethanolamine (PE). In the second step, the generated N-acylphosphatidylethanolamine (NAPE) is hydrolysed to NAE and phosphatidic acid through a reaction catalysed by a phosphodiesterase of the phospholipase D-type (NAPE-PLD). In addition to this route, there are at least four other pathways for AEA synthesis [109].

NAEs are degraded and inactivated by enzymatic hydrolysis to free fatty acids and ethanolamines. The main enzyme found to execute this degradation is fatty acid amide hydrolase (FAAH), which was first discovered to hydrolyse NAEs in rat liver [110] and further characterized and named by Cravatt et al. [111]. In addition, a FAAH isoenzyme – FAAH-2 – was discovered that prefers to degrade monounsaturated rather than polyunsaturated acyl chains [112]. FAAH is a membrane bound serine hydrolase that has been widely studied and many selective inhibitors of FAAH have been developed. NAEs can also be inactivated by N-acylethanolamine acid amide hydrolase (NAAA) which optimally operates under acidic conditions (unlike FAAH which prefers basic conditions) and is a member of the cholesterylamine hydrolase family [113], and by COX [109].

Metabolism of MAGs

The principal and most accepted biosynthetic route for 2-AG starts with the hydrolysis of membrane phospholipids (phosphatidylinositol) that is catalysed by phospholipase C (PLC) with the intermediate product 1, 2-diacylglycerol (DAG). The intermediate DAG in turn is converted to 2-AG by diacylglycerol lipase (DAGL) [109]. Like AEA, 2-AG can also be synthesized via other pathways and a second pathway is also a two-step process involving the enzymes phospholipase A1 (PLA1), PLC, and a third pathway involving the lysophosphatidic acid (LPA) phosphatase enzyme. The involvement of these latter two pathways in the production of 2-AG has not been evaluated in detail [114].

The most probable mechanism of the degradation of 2-AG (and other MAGs) is that 2-AG is metabolized by a monoacylglycerol lipase (MAGL) into fatty acids and glycerol. MAGL, a member of the serine hydrolase family and cloned by Karlsson et al. [115], is expressed in a wide range of tissues (e.g., brain skeletal muscles and adipose tissue). MAGL is mainly membrane anchored like FAAH, but it has also been found in the cytosol fraction of rat adipocyte cells [116]. Like FAAH, MAGL has been a target for drug development [117]. Other enzymes inactivate 2-AG, including FAAH, COX, α/β -hydrolase domain containing protein-6 (ABHD6), and -12 (ABHD12) [109].

Figure 2. Endocannabinoid system localization in different cell types. 2-AG, 2-arachidonyl glycerol; ABHD6, α/β -hydrolase domain-6; ABHD12, α/β -hydrolase domain-12; AEA, anandamide; CB¹, cannabinoid receptor 1; CB², cannabinoid receptor 2; DAGL- α , diacylglycerol lipase- α ; DAGL- β , diacylglycerol lipase- β ; FAP, fatty acid binding protein; FAAH, fatty acid amidehydrolase; MAGL, monoacylglycerol lipase; NAPE, N-arachidonoyl phosphatidylethanolamine; PPAR- α , peroxisome proliferator-activated receptor- α ; TRPV¹, transient receptor potential vanilloid receptor-1. Question marks refer to conflicting evidence about the cellular localization of targets. Reprinted with permission from [118].

The EC system and the targeted lipid mediators in pain modulation

This section describes a selection of evidence that associates the EC system and/or the targeted lipids with pain. Although AEA belongs to the NAEs, in the literature these lipid mediators often are divided into ECs (CB activating mediators) and NAEs (not CB activating mediators), which will be the division used here.

The EC system, the NAEs, and pain in experimental and animal studies

Components of the EC system (receptors, lipid mediators, and enzymes) are localized from PNS to CNS [119]. Injection of AEA and 2-AG and pharmacological blockade of CB receptors provided early support for the hypothesis that the EC system suppresses both acute and inflammatory pain [119]. For example, the inverse agonist SR141716A (also known as Rimona-bant) has been used to block CB¹, which has generated hyperalgesia in various pain tests (i.e., hotplate tests and formalin tests) [119]. Furthermore, studies of global-knockout mice have confirmed that CB¹ and CB² are involved in cannabinoid-induced analgesia [120, 121], which in one study was localized to be largely mediated via peripheral CB¹ in nociceptors [122]. There are also several reports on the analgesic effects as the result of inhibition of the main EC degrading enzymes FAAH and MAGL [123, 124].

PEA's anti-nociceptive capacity has been explored in animal models of both acute and chronic pain. In one early study, orally given PEA reduced carrageenan induced hyperalgesia in a dose dependent manner [125]. Furthermore, PEA has been shown to decrease pain behaviours in mice induced by intraplantar injections of formalin when locally injected in a dose dependent manner [126] and when systemically administered [127]. Both PEA and OEA activates PPAR- α . When LoVerme et al. compared PPAR- α -null mice with wild-type (WT) mice, they found that PPAR agonists (GW7647, and Wy-14643) exert rapid and profound antinociceptive effects on acute persistent inflammatory pain and neuropathic pain [128].

In a study using TRPV¹-null and WT mice, intraperitoneal administration of OEA was shown to excite vagal sensory neurones and induce visceral pain (nociceptive pain from organs) via activation of TRPV¹ [68]. Later, OEA was tested in two animal models (formalin acidic acid) and was associated with analgesic properties. These effects were similar in mice deficient in PPAR- α [71], which suggests an alternative receptor mediating the proposed effect.

The least studied NAE in this context is SEA. In a rat testis inflammation model, NAEs including SEA were significantly accumulated [129], suggesting an inflammation modulating role for SEA. Maccarrone et al. attributed SEA with cannabimimetic activity partly similar to AEA after, for example,

a mice hotplate test [130]. SEA was reported to reduce levels of the pro-inflammatory cytokines IL-1 and IL-6 in a rat cell inflammation model [78].

EC system and NAEs in human pain studies

Compared to preclinical reports, few studies have measured how the EC system and NAEs influence in human pain, although this number is increasing. Below is a list of some of the relevant results with respect to how the EC system and NAEs influence human pain conditions. Patients with complex regional pain syndrome (n=10) have AEA plasma levels higher than healthy controls (n=10) [131]. Patients with neuromyelitis optica (n=11) have elevated plasma levels of 2-AG compared to healthy controls (n=11) [132]. Similarly, increased plasma 2-AG levels and upregulation of CB receptors gene expression have been reported in osteoarthritis pain patients (n=16) compared to healthy controls (n=14) [133]. Another study found that patients treated with total knee arthroplasty had significantly elevated levels of cerebrospinal and synovial fluid 2-AG if they experienced higher postoperative acute pain [134]. Elevated microdialysate levels of PEA and SEA sampled from the trapezius muscle have been reported in subjects with chronic neck/shoulder pain (n=11) compared to healthy controls (n=11) [135]. Finally, one study found that endometriosis (n=27) was associated with elevated plasma levels of AEA, OEA, and 2-AG compared to controls (n=29) [136].

To the best of my knowledge, with the exception of the two studies included in this thesis, only two previous studies have investigated EC and NAE plasma levels in CWP/FMS. In a 2008 gender mixed cohort study investigating elevated levels of AEA in FMS patients (n=22) and controls (n=22), Kaufmann and co-workers found no correlations between AEA and clinical variables such as pain and stress scorings [137]. In a 2016 study investigating AEA, OEA, PEA, SEA, and 2-AG in woman with CWP (n=15) and healthy controls (n=27), Hellström et al. found no statistically significant group differences or correlations between lipid levels and pain intensity or pain durations [138].

The EC system and NAEs in chronic pain management today

Almost two centuries have passed since O'Shaughnessy published one of first modern science studies about cannabis. Since then, cannabis research has revealed a great deal of information about the chemistry of the plant. Even more surprising, researchers have discovered an endogenous cannabinoid system in humans that is associated with many physiological, psychological, and pathological functions, including the modulation of pain.

Although a great deal of chronic pain research has focused on finding specific modulators of CB, PPAR, and TRP receptors or inhibitors of the lipid degradation enzymes FAAH and MAGL, traditional cannabis/marijuana,

THC extracts (e.g., Marinol and Sativex), and synthetic THC analogues remain the most commonly used drugs targeting the EC system. The use of cannabis as medication is booming **and the term “medical marijuana” or “medical cannabis” has been anchored** to the discourse. In fact, globally there is a push to legalize medical cannabis. However, the scientific community has not arrived at a consensus regarding the efficacy of medical cannabis.

Although there are hundreds of synthetic CB agonist described in the literature [139], only Nabilone (a THC analogue) has been approved (Canada, USA, Mexico, and UK), primarily as a treatment for severe nausea and vomiting associated with chemotherapy. However, Nabilone has also shown modest effectiveness in relieving FM pain [140]. In addition to Nabilone, the U.S. Food and Drug Administration (FDA) has approved the use of Dronabinol (synthetic THC) to treat patients who have failed to respond adequately to conventional treatments. In 2005, Canada approved the use of Sativex as an adjunctive treatment for MS-related neuropathy. Sativex is an oromucosal spray of a tincture of cannabis oil consisting of THC and cannabidiol in approximately equivalent amounts. In 2012, the medical products agency in Sweden approved Sativex for similar indications.

As noted above, the consensus concerning cannabis as a drug is lacking; however, the evidence of the effect of medical cannabis or mixtures of THC, or synthetic THC analogues for therapeutic use for various medical conditions (e.g., chronic pain, chemotherapy-induced nausea and vomiting, sleep disturbance, cancer, etc.) has recently (2017) been comprehensively reviewed by the National Academies of Sciences, Engineering and Medicine (NASEM) in the US. The NASEM committee concluded the following: **“There is a conclusive or substantial evidence that cannabis is effective as a treatment of chronic pain”**[141]. In addition, there is conclusive or substantial evidence that cannabis or cannabis derivatives can effectively be used to treat chemotherapy-induced nausea and vomiting and spasticity associated with multiple sclerosis [141]. However, there are short-term side effects associated with cannabis: dry mouth, short-term memory lost, and other cognitive effects. In addition, several epidemiological studies have shown a robust association between cannabis and psychosis [142].

In addition to drugs interacting with the EC system, PEA has been widely investigated as an additive during various chronic pain conditions such as FM. Currently, PEA **is marketed as a nutraceutical (Normast™, Pelvilen™, and PeaPure™) in some European countries (e.g., Italy and Spain)** and is used as a food supplement in other countries (e.g., Netherlands) [143]. A meta-analysis of twelve studies showed that PEA elicits a progressive reduction of pain intensity significantly higher than controls and the PEA effects were independent of patient age or gender and not related to the type of chronic pain [144].

AIMS

This thesis investigates whether alterations in levels of the targeted lipid mediators exist in patients with chronic pain compared to pain-free controls. In addition, this thesis evaluates how these levels are related to different manifestations and symptoms of chronic pain in order to analyse their potential as biomarkers. More specifically, we assume alterations in levels of ECs and/or NAEs in microdialysates sampled from muscles and plasma during chronic pain episodes. Furthermore, we hypothesize that an association exists between levels of the lipid mediators and clinical symptoms of chronic pain.

Paper I

Paper I investigates the suitability of a microdialysis set-up for sampling of AEA, OEA, PEA, SEA, and 2-AG from the trapezius muscle and forearm skin. The lipid levels are not only analysed in microdialysate fractions but also in MD catheter membranes. This strategy is used to gather information on the feasibility to find these compounds in the tissues in general and to estimate the degree of adsorption on the catheter membranes *in vivo*.

Paper II

Paper II, a case-control study, compares OEA, PEA, and SEA levels in microdialysates sampled from women with CWP and healthy CON during the first two hours after MD catheter insertion. This study also analyses to which extent these levels reflect an altered tissue reactivity between painful and non-painful muscle. Within this aim, this paper investigates the correlations between levels of these substances and pain characteristics (intensity and sensitivity).

Paper III

Using the same cohort investigated in Paper II, Paper III compares plasma levels of OEA, PEA, SEA, the anti-inflammatory cytokine IL-10, and the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6, and IL-8. These comparisons are used to investigate the association between levels of lipids and cytokines and their relation to pain intensity scorings.

Paper IV

Paper IV analyses plasma levels of AEA, OEA, PEA, SEA and 2-AG in women with FM and controls. In addition, this paper investigates the associations between these levels and pain characteristics, psychological aspects, and health status to evaluate their potential as biomarkers for FM.

MATERIALS AND METHODS

Table 1 presents the four papers regarding subjects, sampling, analytes, and clinical instruments included in the thesis.

Table 1. Numbers of subjects with chronic widespread pain (CWP), controls (CON), and fibromyalgia (FM) patients. MD (microdialysis) and plasma from blood samples were analysed with respect to concentrations of different analytes: arachidonylethanolamide (AEA) oleoylethanolamide (OEA), palmitoylethanolamide (PEA), stearoylethanolamide (SEA), 2-arachidonoylglycerol (2-AG) and cytokines: tumour necrosis factor- α , interleukin- 1β , interleukin-6, interleukin-8, and interleukin-10. The clinical parameters: Numeric Rating Scale (NRS), Pressure Pain Thresholds (PPT), Hot/cold Pain thresholds (HPT, CPT), Visual Analogue Scale (VAS), Hospital Anxiety and Depression Scale (HADS), and the FM Impact Questionnaire (FIQ) were assessed.

<i>Paper</i>	<i>Subjects</i>	<i>Sampling</i>	<i>Analytes</i>	<i>Clinical instruments</i>
<i>I</i>	CON (n=2)	MD	AEA, OEA, PEA ,SEA, 2-AG	No
<i>II</i>	CWP (n=17) vs CON (n=19)	MD	OEA, PEA, SEA	NRS, PPT, HPT and CPT
<i>III</i>	CWP (n=17) vs CON (n=21)	Plasma	OEA, PEA, SEA + cytokines	NRS
<i>IV</i>	FM (n=104) vs CON (n=116)	Plasma	AEA, OEA, PEA, SEA, 2-AG	VAS, PPT, HADS, FIQ

Human subjects

Paper I

This paper collected data from two volunteer controls: a 33-year-old healthy female and a 39-year-old healthy male. All procedures for sampling of microdialysate from the trapezius muscle and forearm skin were approved by Linköping University Ethics Committee (Dnr: M233-09 and 2010/164-32 and Dnr: 03250). The participants gave their informed written consent before the experiments started.

Papers II-III

Subjects with CWP

Women with CWP (n=18) were recruited to participate in this study. Inclusion criteria were female sex, age range 20-65, and widespread pain according to the ACR 1990 classification criteria. Exclusion criteria were bursitis, disorders of the spine, tendonitis, capsulitis, postoperative conditions in neck/shoulder area, prior neck trauma, neurological disease, rheumatoid arthritis or any other systemic disease, metabolic disease, malignancy, severe psychiatric illness, pregnancy, and difficulties understanding the Swedish language.

Healthy subjects

We recruited 24 healthy women (n=24). Inclusion criteria were female sex, age range 20-65, and pain-free. The exclusion criteria were the same as for the CWP group as well as any pain lasting more than seven days during the previous 12 months.

Recruitment and ethic declaration

The Nordic Ministry Council Questionnaire (NMCQ), a self-reported pain questionnaire that assesses of pain in the last 12 months, and a structured telephone interview were used for primary screening. Women with CWP were identified via FM patient organization and review of the medical reports of former patients at the multidisciplinary Pain and Rehabilitation Centre, University Hospital, Linköping. The CON subjects were recruited via advertisements in a local daily newspaper. All participants underwent a standardized and validated clinical examination of the upper extremities as described in [145] as well as a standardized clinical examination of the lower extremities. All participants signed a consent form that was in accordance with the Declaration of Helsinki. All the experimental protocols were approved by the Linköping University Ethics Committee (Dnr: M10-08., M233-09, M233-09, Dnr: 2010/ 164-32). All participants were paid for their participation.

Paper IV

Subjects with FM

The following inclusion criteria were used for subjects with FM: female sex, age range 20-65, and meeting ACR 1990 classification criteria for FM. The following exclusion criteria were used: high blood pressure ($> 160/90$ mmHg), osteoarthritis (OA) in hip or knee confirmed by radiological findings and affecting activities of daily life such as stair climbing or walking, other severe somatic or psychiatric disorders, causes of pain other than FM, high consumption of alcohol (alcohol use disorders identification test (AUDIT) score >6), participation in a rehabilitation program within the past year, resistance exercise or relaxation exercise twice a week or more, inability to understand or speak Swedish, and unable to refrain from analgesics, non-steroidal anti-inflammatory drugs (NSAID), or hypnotic drugs for 48 hours before examinations. Out of the 402 patients screened by telephone, 177 were assessed for eligibility at medical examination and 130 completed baseline examination (Gothenburg: $n=41$; Linköping: $n=42$; Stockholm: $n=47$). Blood was sampled, although not from all participants. Of the 130 patients who completed the baseline examination, 104 could be used in the analysis (i.e., $n=104$).

Healthy subjects

The following inclusion criteria was used for the healthy controls: age range 20-65 and female sex. The following exclusion criteria were used: any pain condition, high blood pressure ($> 160/90$ mmHg), OA in hip or knee, other severe somatic or psychiatric disorders, other pain conditions, high consumption of alcohol, participation in a rehabilitation program within the past year, resistance exercise or relaxation exercise twice a week or more, inability to understand or speak Swedish, and unable to refrain from analgesics, NSAIDs, or hypnotic drugs for 48 hours before examinations. Out of 182 healthy controls screened by telephone, 150 were eligible at medical examination and 137 completed the baseline examination. Of these 137 health controls, 116 provided plasma samples and therefore were used in the analysis (i.e., $n=116$).

Recruitment and ethic declaration

The subjects in paper IV were part of a randomized control multicentre trial who were recruited by advertisement in local newspapers in Gothenburg, Linköping, and Stockholm. The trial was registered with ClinicalTrials.gov (identification number: NCT01226784). The recruitment procedure has been described in detail in previous articles [146, 147]. The study was performed in accordance with the Helsinki Declaration and Good Clinical Practice. The Central Ethical Review Board in Stockholm approved the study (Dnr: 2010/1121-31/3). All participants received verbal and written

information about the study and gave their written consent. The participants were paid for their participation.

Clinical instruments

Paper II-III assessed pain intensity using the numeric rating scale (NRS) and paper IV assessed pain intensity using the visual analogue scale (VAS). Paper II and IV assessed pain sensitivity using pressure pain thresholds (PPT). In paper II thermal sensory testing – i.e., hot pain thresholds (HPT) and cold pain thresholds (CPT) was conducted. Paper IV assessed anxiety and depression using the Hospital Anxiety and Depression Scale (HADS) and assessed general health status using the Fibromyalgia Impact Questionnaire (FIQ).

Pain Intensity

NRS and VAS

The NRS-11, a unidimensional measure of pain intensity in adults, is an 11-point numeric scale with the following endpoints: 0 = no pain and 10 = pain as bad as you can imagine or worst pain imaginable [148]. Pain ratings during the MD procedure (paper II) concerned local pain in the trapezius muscle from the most painful side (subjects with chronic pain) or the dominant side (pain-free subjects) and whole-body pain in paper III. The VAS, also a unidimensional measure of pain intensity, has been widely used in diverse adult populations, including for people with rheumatic diseases. The VAS is a 100-mm scale with the following endpoints: 0=no pain and 100=pain as bad as it could be or worst imaginable pain [148].

Pain sensitivity

PPT, HPT, and CPT

Using a handheld electronic pressure algometer (Somedic, Hörby, Sweden), paper II and IV assessed pressure pain thresholds (PPT), a measure of skin and muscle pain sensitivity. The skin contact area was 1 cm² and pressure was applied perpendicularly to the skin at 50 kPa/s. The subjects were instructed to mark the PPT by pressing a button as the sensation of “pressure” changed to “pain”. For specific descriptions of the body regions examined, see paper II and IV.

Thermal sensory testing was performed using a modular sensory analyser from Somedic (Hörby, Sweden). Thermal pain thresholds were measured using the method of limits with a baseline temperature of 32°C. HPT and CPT over right and left trapezius and tibialis anterior were determined. All tests used a thermode with a stimulating surface of 25 x 50 mm, consisting of Peltier elements and a temperature change range of 1°C/s. During the

tests, the participants sat comfortably in a quiet room with an ambient temperature of approximately 22°C. The stimulator was applied to the skin and a constant current source was connected, giving a baseline temperature of 32°C. First, the ability to perceive changes in temperature was tested (not reported). Next, cold pain and heat pain thresholds were determined. During this procedure, the participants were instructed to activate the switch when they first perceived the stimulus as painful. The lowest and highest stimulation temperatures were 10 and 50°C, respectively. The time required for the pain threshold measuring was about 15 minutes per site. QST was assessed in paper II.

Self-reported questionnaires

HADS and FIQ

HADS, a short self-assessment questionnaire (used in paper IV), measures anxiety and depression on separate 7-item scales for a total of 14 items [149]. Possible subscale scores range from 0 to 21, the lower score indicating the least depression and anxiety possible. A score of 7 or less indicates a non-case, a score of 8-10 indicates a doubtful case, and a score of 11 or more indicates a definite case.

In paper IV, FIQ was used to measure the health status. FIQ, a disease-specific self-reported questionnaire, comprises ten subscales of disabilities and symptoms ranging from 0 to 100. The total score is the mean of the ten subscales. A higher score indicates a lower health status [150].

Sampling procedures

Microdialysis sampling

Paper I and II used microdialysis (MD) sampling, a well-established technique that enables the sampling of the chemistry of interstitial fluid of tissues [151]. Introduced in its present form in 1974 by Ungerstedt and Pycock, MD was initially developed to monitor dopamine release in rat brain in response to administration of various drugs [152]. Since then, the number of tissues that have been explored by this technique includes human brain [153], human peripheral tissues, and animal and human organs [154]. Sampling of microdialysate involves perfusion of a MD membrane with an aqueous solution (perfusate) using a MD pump. The catheter contains a semi-permeable membrane and mimics a capillary blood vessel [153], allowing substances to pass by diffusion across the membrane.

MD has several advantages over other sampling methods. For example, MD estimates unbound molecule levels from a specific tissue, whereas blood sampling estimates unbound molecules from the body system as a whole.

Furthermore, as MD allows for continuous sampling, unbound molecule level changes in a tissue in response to, for example, stress or pharmacological treatments can be monitored over time. Finally, since the MD membrane works as a filter, allowing only molecules that are small enough to pass through the membrane, samples are relatively clean and need minimal preparation before analysis. However, many experimental conditions – e.g., probe membrane composition and surface area (membrane cut-off), perfusate composition, flow rate, temperature, nature of the dialyzed tissue, and physicochemical properties of the target molecules – need to be considered in the experimental design [155]. Figure 3 (panel A) illustrates a MD system containing a MD pump coupled to a MD catheter with a sample collector. Panel B illustrates how the semipermeable membrane allows components to diffuse from the tissue into the MD system.

Figure 3. Panel A illustrates a microdialysis (MD) system containing an MD pump connected to the inlet tubing where perfusate flows towards the semipermeable membrane, the location of the dialysis. The outlet tubing carries the dialysate to the sample collector. Panel B illustrates how the semipermeable membrane in the tissue allows components from the perfusate to diffuse through the membrane into the tissue and components from the tissue to diffuse through the membrane into the MD system.

In paper I and II, the subjects who participated in MD were asked to abstain from NSAIDs for seven days and/or paracetamol medication for 12 hours before MD sampling. In addition, they were asked to avoid any coffee, tea, or cigarettes or other caffeine or nicotine agents for eight hours before MD sampling.

In paper I, four catheters (20 kDa), two inserted into the left and two into the right side, were used to collect MD samples from the trapezius muscle. To study the adsorption of the compounds over time, we removed the catheters at different times. During the sampling (total time of 220 min), the participants rested in an armchair. In the forearm skin sampling, three catheters were inserted into the dermis on the left dorsal forearm and these catheters were removed at different times. The sampling lasted for a total of 140 min. In paper II, two catheters (20 kDa and 100 kDa cut-off) were used to collect MD samples from the dominant trapezius muscles. During this collection process, participants rested comfortably in an armchair for 140 min. Next, the participants performed a standardized repetitive low-force exercise for 20 min, which was followed by a 40-min recovery period, for a total of 220 min. The participants were offered a standardized light meal at the 100 min time point. MD samples were collected every 20 min. In paper II, MD samples from the first 120 min of this period were analysed (labelled as the trauma period).

Plasma sampling

In paper III, before the plasma samples were retrieved, the subjects were instructed not to drink any beverages with caffeine, not to smoke on the sampling day, and to avoid NSAID medication the week before the experiment. Venous blood was collected using EDTA-vacutainers and samples **were centrifuged for 15 minutes (1500 g, 4 C°)** within one hour after the collection. The plasma was aliquoted in Eppendorf tubes and stored at -70°C.

For the plasma samples analysed in paper IV, a uniform sampling protocol was used at three different centres. Venous blood samples were collected **with a Vacutainer (BD Vacutainer1Eclipse™ Blood Collection Needle, BD Diagnostics, Becton, Dickinson and Company, New Jersey, USA)** in a 10-mL plasma tube (BD Vacutainer1Plus Plastic K2EDTA Tubes BD Diagnostics, Becton, Dickinson and Company, New Jersey, USA). The blood samples were centrifuged for 30 min (1500 g, in r.t) immediately after collection and the plasma was aliquoted in smaller tubes and stored at -70°C.

Sample preparations

Microdialysate

The preparation of the targeted lipids from microdialysate was performed according to Ghafouri et al. [135]. On the same day as the analysis, 50 μ l of microdialysate were dried by SpeedVacc vacuum concentration system (Savant, Farmingdale, NY, USA) and dissolved in 100 μ l of methanol, vortexed, and centrifuged. The supernatants were transferred to new tubes (0.65 ml) and dried. The residues were dissolved in 20 μ l of LC mobile phase, vortexed, and transferred to glass insert vials for LC-MS/MS.

In paper I, the catheter membrane samples were prepared after withdrawal of the catheter from the tissue. The membranes were separated from the tubing, cut into two equal halves, and placed in Eppendorf tubes (Micro tube, 1.5 ml, Sarstedt), which were kept on ice during the experiment and stored at -70°C until analysis. On the day of analysis, methanol (1 ml) was added to the catheter membrane and vortexed for 15 secs before the catheter membrane was removed from the tube. The extraction solution was dried by SpeedVacc and the residue was dissolved in 100 μ l of methanol, vortexed, and centrifuged. The supernatant was transferred to a new tube and dried. The residue was dissolved in 20 μ l of LC mobile phase, vortexed, and transferred to a glass insert vial for LC-MS/MS.

Plasma

The targeted lipids were extracted from plasma using a previously described protocol [156]. This protocol was modified between paper III and paper IV. The presented protocol is valid for paper IV.

First, **300 μ L of plasma were thawed and vortexed**. Then, **30 μ L of a mixture** containing the deuterated internal standard – AEA-d4, OEA-d4, PEA-d4, and SEA-d3 (50 nM) and 2-AG-d5 (1000 nM) – were added to each plasma sample. **Acetonitrile (ACN) (1200 μ L) was added before vortexing** and centrifugation (10000 rpm, 5 min, 4°C). Supernatants were added to 4.5 mL of milliQ-H₂O containing 0.133% trifluoro acetic acid (TFA). C8 Octyl SPE columns (6 mL, 200 mg) (Biotage; Uppsala, Sweden) were activated with 1 ml of methanol and washed with 1 ml of milliQ-H₂O before the samples were added. After washing with 1.5 ml of ACN (20% with 0.1% TFA), the samples were eluted with 1.5 ml ACN (80% with 0.1% TFA) and dried by SpeedVacc and stored at -70°C until analysis. On the day of the analysis, **samples were reconstituted in 30 μ L of LC mobile phase**.

Analytical techniques

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

LC-MS/MS, an analytical chemistry technique, combines the physical separation abilities of liquid chromatography (LC) with the mass analysis abilities of mass spectrometry (MS). Using a high-performance pumping system, the LC system transfers a liquid containing the sample mixture through a column filled with an adsorbent material. The molecules in the sample mixture interact differently with the adsorbent material in the column (depending on chemical properties of the molecules and the composition of the mobile phase), causing different flow rates through the column, leading to the separation of components as they flow out the column toward the analysis device.

Composed of an ionization probe, a mass filter system, and a detector, mass spectrometer (MS) measures the mass-to-charge ratio of charged molecules. On the way from the ionization probe to the detector, ions move through a high vacuum system through one or several quadrupoles where oscillating electrical fields work as a mass selective filter. Further on, precursor ions could be either detected or fragmented to product ions that could be selectively detected (MS/MS). The precursor and product ion pair is called a selected reaction monitoring (SRM) "transition". A schematic illustration of the LC-MS/MS flow is shown in figure 4.

Figure 4. A LC-MS/MS triple quadrupole selected reaction monitoring flow diagram. The samples are introduced in the LC and molecules are separated before reaching the electrospray ionization (ESI) probe, which evaporates the liquid to charged molecules (positive or negative). In quadrupole 1 (Q1), selected masses pass through. In Q2, precursor ions are selectively fragmented by collision induced dissociation (CID) and selected product ions (and precursor ions) pass through Q3 to the electron multiplier (EM) detector.

LC-MS/MS method development

During this thesis, a LC-MS/MS method was developed. Initially, the literature was screened for LC-MS/MS methods. After reviewing several options, it was decided to use a LC mobile-phase composition based on the LC method described by Balvers et al. [156] and to use the MS/MS verified SRM transitions reported in [156, 157].

For the measurements in paper I, an instrument method was optimized for AEA, OEA, PEA, SEA, and 2-AG. A LC method was performed using isocratic elution and quantification were performed using external standard curves. This method was applied in paper II as well. In paper III, an internal deuterated standard was included in the method to correct for losses of analytes during sample preparation. In paper IV, LC was performed using gradient elution, which enhanced the resolution of the peaks. In addition, a deuterated internal standard for each analyte was included in the method to more precisely enable correction of analyte losses during preparation.

Validity of the LC-MS/MS method(s)

To ensure the quality of a measure, the validity of an analytical method should be tested. Below is a description of the validation parameters: selectivity, sensitivity, linearity, precision, limit of detection (LOD), limit of quantification (LOQ), and stability.

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample.

Sensitivity of an instrument method is related to the limit of detection (LOD). LOD of the compounds is defined as the concentration at which a signal-to-noise ratio of greater than 3:1 was achieved following direct analysis from stock solutions. The **precision** is a measure of reproducibility. The precision is determined by spiking multiple samples with known amounts of analytes and is usually presented as the coefficient of variation (CV).

The LOQ (not to be confused with LOD) could be defined as the lowest concentration of a substance that can be measured with certainty using standard tests. The U.S Food and Drug Administration (FDA) use a stricter definition for LOQ (see “**Bioanalytical method validation** – Guidance for the industry”). Unlike LOD, LOQ is matrix dependent and therefore LOQ for the measurements in MD samples and for the measurements in plasma samples needed to be determined. Moreover, both short term (24 h in 4°C) and long term (-70°C) **stability** need to be considered during validation procedures.

Measurements of Cytokines

The cytokine levels in paper II were measured using two antibody-based immunoassays. An immunoassay detects an analyte (also referred to as antigen) bound to a specific antibody. Most immunoassays use label-linked antibodies to detect a specific antigen. Labels could be enzymes, radioactive isotopes, and fluorogenic reporters, but there are also label-free immunoassays.

The Luminex Assay

TNF- α and IL-1 β were analysed with the commercial High Sensitivity Human Cytokine Magnetic Bead Panel Immunoassay, MILLIPLEX[®] MAP for Luminex[®] xMAP1 Technology kit. The kit comprises all components (microplate, magnetic beads, antibodies, standards, and buffers). This immunoassay identifies analytes in plasma bound to specific monoclonal antibodies linked to magnetic beads coated on a microplate. After washing, biotinylated antibodies that bind to the analytes are added. After a second washing, the biotinylated antibodies are labelled with streptavidin-phycoerythrin; phycoerythrin is the detectable fluorophore. A Luminex 100 instrument (Biosource, Nivelles, Belgium) was used to measure Luminex bead-based fluorescence. Standard curves were fitted using five parameter logistic regression.

The Proximity Extension Assay

IL-6, IL-8, and IL-10 were analysed using multiplex proximity extension assay (PEA) at Olink Bioscience in Uppsala, Sweden. PEA is based on pairs of antibodies that are linked to oligonucleotides having slight affinity to one another (PEA probes). Upon target binding, the probes are arranged in close proximity, and the two oligonucleotides are extended by a DNA polymerase to form a new sequence that acts as a unique surrogate marker for the specific antigen. This sequence is typically quantified by quantitative real-time PCR (qPCR), where the number of PCR templates formed is proportional to the initial concentration of antigen in the sample. Figure 5 describes the flow of the PEA assay.

Figure 5. Design of the PEA assay.

(A) Pairs of specific antibodies are equipped with oligonucleotides (PEA probes) and mixed with an antigen-containing sample. (B) Upon sample incubation, all proximity probe pairs bind their specific antigens, which brings the probe oligonucleotides in close proximity to hybridize. The oligonucleotides have unique annealing sites that allow pair-wise binding of matching probes. Addition of a DNA polymerase leads to an extension and joining of the two oligonucleotides and formation of a PCR template. (C) Universal primers are used to pre-amplify the DNA templates in parallel. (D) Uracil-DNA glycosylase partly digests the DNA templates and removes all unbound primers. (E) Finally, each individual DNA sequence is detected and quantified using specific primers in microfluidic qPCR. The unit is signal-to-background (dCq). Reproduced from [158] with permission.

Statistics

Traditional statistics

Traditional uni- and bivariate statistics were applied in paper II-IV. Student's *t*-test of independent samples was used for group comparisons. When needed, *p*-values were corrected for inequality of variance using Leven's test for equal variance. In paper II, when several measurements of the same dependent variable were taken at different time points, One-way ANOVA with repeated measures was used for pairwise comparisons. Pearson's correlation analyses were used to investigate associations between variables. The IBM SPSS version 22.0 (IBM Corporation, Route 100 Somers, New York, USA) and the GraphPad Prism programme (GraphPad Software Inc, San Diego, CA, USA) were used for the traditional statistical analyses.

Multivariate data analysis

Multivariate data analysis (MVDA) was used to complement traditional statistics. Principal component analysis (PCA) and Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA) were used in paper II-IV. PCA and OPLS-DA are multivariate projection methods where the measured variables are modelled as linear combinations of a small set of latent

variables. The software SIMCA 14 (Umetrics) was used for the MVDA analyses.

In MVDA, pre-treatment of data before analysis is often needed, where scaling and mean centring of data are common transformations. Variables could have substantially different numerical ranges and by scaling and mean centering data is normalized to a unit scale and centered around zero, which makes variables comparable.

In PCA, a multivariate correlation analysis method, after scaling and mean centring of data the first principal component (PC 1) could be computed, which is the vector that represents the maximum variance direction in the data. The second principal component (PC 2) is orientated orthogonal to PC 1 such that it reflects the second largest source of variation in the data. Two PCs define a plane, which is a window into to the K-dimensional (K = number of variables) variable space. By projecting and plotting variables onto this space, it is possible to visualize the structure of a dataset. The main plots generated from PCA analysis are the score and the loading plot. The score plot illustrates the relationship between the observations and the loading plot describes the relationships between variables. PCA enables identification of multivariate outliers and overviewing multivariate correlations.

This thesis used another tool for analysing multivariate data, OPLS. OPLS, a regression method, is modified to make it easier to interpret versions of Partial Least Square (PLS). In its simplest form, PLS is a method that relates two data matrices (X and Y) to each other by a linear multivariate model. In OPLS-DA, the outcome (Y) is nominal (e.g., patient or healthy control), so it can be used for the multivariate regression analysis (i.e., prediction) of group memberships. OPLS-DA also generates score and loading plots. An OPLS-DA score plot illustrates how well observations from groups (e.g., patient and control) are separated, and a loading plot describes how each variable (X) in a model is related to the nominal Y .

The validation parameters R^2 and Q^2 diagnostic were used to evaluate model quality in PCA and OPLS models. R^2 describes the goodness of fit – the fraction of sum of squares of all the variables explained by a principal component [159] ($R^2 = 1$ explains 100% of the data). Q^2 , calculated by cross-validation, estimates the predictive ability of a model. R^2 should not be considerably higher than Q^2 .

Moreover, OPLS models use the CV-ANOVA and the variable influence on projection (VIP) parameters to validate estimations and to estimate the relative importance of variables in a model, respectively. The CV-ANOVA diagnostic corresponds to a hypothesis test of the null hypothesis of equal cross-validated predictive residuals of the two compared models [57], measures the significance of the observed group separation, and returns a

statistically significant p-value [55]. In SIMCA, the computed influence on Y of every term (X) in a model is called VIP. VIP is the sum over all model dimensions of the variable influence of the contributions. One can compare the VIP of one term to the VIP of others. Terms with large VIP, larger than 1, are the most relevant for explaining Y.

RESULTS AND DISCUSSION

Paper I

This paper evaluated the suitability of a MD set-up for sampling of AEA, OEA, PEA, SEA, and 2-AG in muscle and skin. As MD recovery of lipophilic compounds is low due to their adsorption to catheter membranes, this paper focused on identifying endogenous lipids in dialysate samples and determining to what extent the lipids adsorb to the catheter membrane at specific time points.

The main finding was that OEA, PEA, and SEA could be detected in all dialysate and membrane samples collected from human trapezius muscle and from forearm skin tissue. Although PEA and SEA had previously been reported in dialysate collected from trapezius [135], this was the first study to report about OEA in dialysate from human muscle and to report OEA, PEA, and SEA levels in microdialysate sampled from human skin tissue. 2-AG was not detected in dialysate, but it was substantially present in the membrane samples. This result indicated that 2-AG is present in the extracellular compartment of both human muscle and skin. A trend of decreasing levels of OEA, PEA, and SEA in microdialysate over time could be observed in both muscle and skin.

This was an exploratory *in vivo* study concerning feasibility aspects of a specific MD sampling set-up. The aim was to investigate the feasibility of this set-up in human muscle and skin tissue; however, only two subjects were studied (one per tissue), which limits the interpretation of the results.

The integrative approach of analysing both the microdialysate samples and the catheter membranes for the targeted compounds provided information on the feasibility to find these compounds in the tissues in general and could fill an information gap and enhance an adequate interpretation of microdialysate data outcomes.

Papers II-III

Papers II-III use the same cohort. Each paper will be discussed separately and then the results from the two papers are combined and discussed. For this purpose, additional statistical analyses have been performed and some new results are presented that were not included in either of the two published papers.

Paper II

This study investigated the acute consequences (i.e., first two hours) of MD probe insertion with respect to PEA, SEA, and OEA levels in dialysate and to what extent differences exist between healthy subjects and CWP patients. Within this aim, correlations between levels of these substances and pain characteristics (intensity and sensitivity) were investigated.

Pain intensity rating from CWP was significantly higher compared to CON for all time points ($p < 0.001$). CWP had significantly lower pressure pain thresholds ($p < 0.001$) for both sides of the trapezius and tibialis muscles. Significant differences in the thermal pain thresholds were found in three anatomical areas in CWP compared to CON ($p < 0.05$) (for detailed presentation see the paper).

Levels of OEA and SEA were significantly higher in CWP ($p < 0.05$) at all five time points during the first two hours after the MD probe insertion (Figure 6). PEA was higher for all time points, but only significantly higher for one time point.

Figure 6. Mean concentrations (nM) with error bars (SEM) for oleoylethanolamide (OEA) and stearoylethanolamide (SEA) in microdialysate sampled from the trapezius muscle of women with chronic widespread pain CWP and healthy female controls (CON) during the acute tissue trauma phase (20-120 min) immediately after the insertion of the MD probe. * implies statistical significance.

A significant positive bivariate correlation existed between SEA and pain intensity in CWP ($r = 0.55$, $p < 0.05$). However, if removing the possible outlier (the right upper corner), the significance is lost, which obviously weakens this relationship (Figure 7).

Figure 7. Scatter plot of pain intensity (NRS) and stearoylethanolamide (SEA) with a fitted linear regression line. (Pearson correlation: $r = 0.55$, $p < 0.05$).

When regressing group membership using OPLS-DA, pain intensity and pressure pain thresholds were the most important (significant) regressors, followed by OEA and SEA. Thermal pain thresholds were not associated with age or BMI (i.e., age and BMI were not significant).

The rate of change of the lipid concentrations between time points was not significantly different between the groups or within the groups, which indicates that painful and non-painful muscle respond similarly to catheter insertion in this context. However, some differences could be observed between groups in the change speed plots (attached as a supplementary file in paper II). Moreover, when comparing the relative differences (quota: CWP/CON) and including the time point following the trauma phase (data retrieved from another paper [160]), the SEA levels was substantially different between trauma and after trauma. Hence, an altered tissue reactivity in response to MD probe insertion cannot be ruled out. However, the higher levels of NAEs in CWP compared to CON is probably also a result of habitually increased levels.

The two-hour trauma period was derived from earlier studies on metabolites such as pyruvate and lactate; these studies concluded that these metabolites concentrated in dialysate stabilize approximately one hour after probe insertion [161-163]. However, no studies have provided evidence for the original substrate used as the rationale for this idea (obtained from PubMed). This study is limited because it includes only measurements of NAE levels from the first two hours after MD probe insertion. Since no clear stabilization of the lipid levels could be elucidated (Figure 6), this indicates that such stabilization could occur after the two-hour trauma period. Previous to this study, PEA and SEA were analysed at two-time points (140 and 180 min after catheter insertion, before and after a repetitive low intensive exercise). The MD sampling of that study and the sampling in paper II were part of the same experiment. However, if all the time points (11

points in total) had been analysed collectively in the same analytical batch, we would have had a more reliable view of the effect of MD probe insertion on NAEs level with respect to healthy muscles and chronic pain muscles.

Paper III

This paper investigated systemic levels of OEA, PEA, and SEA, the pro inflammatory cytokines TNF- α , IL-1 β , IL-6, and IL-8, and the anti-inflammatory cytokine IL-10 related to these lipids. Significantly higher levels of OEA and PEA were found in CWP ($p < 0.01$), but no significant group differences were found in levels of cytokines (Table 2). These results were confirmed by OPLS-DA regression of group membership, where PEA, OEA, and SEA were important regressors, but not the cytokines. No significant correlations existed between cytokines and NAEs.

Table 2. Mean concentrations and standard deviations of lipids and cytokines in subjects with chronic wide spread pain (CWP) and healthy controls (CON). OEA, PEA, and SEA in nM. The cytokines TNF- α and IL-1 β in pg/mL and IL-6, IL-8, and IL-10 in dCq. * implies statistical significance.

Analyte (unit)	CWP	CON	<i>p-value</i>
OEA (nM)	11.1 (3.0)	7.6 (3.7)	0.003*
PEA (nM)	18.1 (9.7)	10.5 (6.2)	0.006*
SEA (nM)	38.6 (28.7)	27.2 (20.7)	0.164
TNF-α (pg/mL)	5.6 (1.7)	5.8 (3.6)	0.777
IL-1β (pg/mL)	0.6 (0.5)	2.1 (6.0)	0.295
IL-6 (dCq)	2.7 (1.7)	1.9 (1.5)	0.216
IL-8 (dCq)	29.8 (25.8)	19.0 (5.9)	0.120
IL-10 (dCq)	3.2 (1.0)	3.0 (0.6)	0.424

Concerning the associations between the different substances and pain ratings, no significant correlation existed, except for TNF- α levels, which correlated negatively with pain intensity in CWP ($r = -0.50$, $p < 0.05$) (Figure 8). This result is not in line with other reports. For example, Wang et al. found elevated levels of circulating TNF- α in FM, but no correlation between these levels and pain intensity [164], and Christidis et al. did not find any difference between plasma levels of TNF- α between FM and controls and or any correlation with pain intensity [165]. In addition, more recently Ohgidani et al. reported that protein levels and the expression of TNF- α at mRNA level significantly increased ATP-stimulated induced microglia-like cells in patients with FM compared healthy controls. TNF- α expression correlated with clinical parameters of subjective pain and other mental manifestations of FM [166]; however, this result was from ATP-stimulated cells, so it cannot be compared with circulating levels of TNF- α .

Figure 8. Scatter plot of pain intensity (NRS) and tumour necrosis factor- α (TNF- α) with a fitted linear regression line illustrating the negative direction of the association (Pearson correlation: $r = -0.50$; $p < 0.05$).

Significant positive inter-correlations existed between the three lipids in CON (OEA vs. PEA, $r = 0.78$, $p < 0.01$; OEA vs. SEA, $r = 0.61$, $p < 0.01$; PEA vs. SEA) ($r = 0.92$, $p < 0.01$), but in CWP similar correlations only existed between PEA and SEA ($r = 0.93$, $p < 0.01$) – i.e., no correlation existed between OEA and PEA ($r = -0.05$) or OEA and SEA ($r = -0.27$), results that strengthen the supposition that there was an altered NAE metabolism between the two groups.

Papers II-III: a comparison of the lipid levels in microdialysate and plasma

The association between the systemic levels and the locally sampled MD levels of OEA, PEA, and SEA was investigated using bivariate correlation analysis of the separate groups (CWP and CON). Correlations between the lipids in plasma and in microdialysate were analysed at each time point (20, 40, 60, 80, and 120 minutes after catheter insertion) to reveal the fluctuation between systemic and locally sampled levels. The blood samples were collected at one time point (200 min after MD catheter insertion). No significant correlation existed between the lipid levels in plasma and in microdialysate in the two groups except for OEA at two time points (40 min; $r = 0.64$; $P = 0.008$ and 120 min; $r = 0.52$; $P = 0.04$) in CON (Figure 9). A possible trend of higher association between the lipid levels in plasma and in microdialysate and higher fluctuation in correlation between different time points could be seen in CON (Figure 9). In addition to the other results (change speed and CWP/CON quota analysis) presented in paper II, this trend possibly gives further support to the idea that there might be a different tissue reactivity between painful and non-painful muscles.

Correlation between [OEA]plasma and [OEA]microdialysate

Correlation between [PEA]plasma and [PEA]microdialysate

Correlation between [SEA]plasma and [SEA]microdialysate

Figure 9. Association between levels of oleoylethanolamide, palmitoylethanolamide, stearoylethanolamide (OEA, PEA, and SEA) in plasma and in microdialysate in patients with chronic widespread pain (CWP) and in healthy controls CON during the first two hours after catheter insertion expressed with Pearson correlations coefficient r . * indicates a significant correlation between plasma and microdialysate levels.

Paper IV

In paper IV, levels of OEA, PEA, and SEA and levels of AEA and 2-AG were analysed in plasma from patients with FM and healthy controls. The group difference of the lipid levels was tested and the association between lipids and pain ratings, psychological (depression and anxiety) ratings, and general health status was investigated using bivariate and MVDA statistics. As expected, the patients had significantly higher pain intensity (VAS), depression and anxiety (HADS), and FIQ ratings and significantly lower pain sensitivity (PPT) (Table 3).

Table 3. Means (standard deviations) for pain intensity (visual analogue scale; VAS), pressure pain thresholds (PPT; kPa), the two subscales of the Hospital Anxiety and Depression Scale (HADS) and the Fibromyalgia Impact Questionnaire (FIQ) in fibromyalgia (FM) and in healthy controls (CONTROL). * indicates statistical significance.

<i>Parameter</i>	<i>FM</i>	<i>CONTROL</i>	<i>p-value</i>
VAS (1-100)	51.2 (21.6)	2.7 (6.6)	0.000*
PPT (kPa)	187.6 (84.7)	358.4 (105.6)	0.000*
HAD-depression	7.3 (3.6)	1.8 (2.4)	0.000*
HAD-anxiety	8.7 (4.1)	3.4 (3.2)	0.000*
FIQ	63.3 (15.3)	7.4 (9.2)	0.000*

The lipid levels of 2-AG, OEA, PEA, and SEA were significantly higher in FM (Table 4).

Table 4. Means (standard deviations) and concentrations in nM and standard deviations of the lipids AEA, OEA, PEA, SEA, 2-AG in subjects with fibromyalgia (FM) and healthy controls (CONTROL). * implies statistical significance.

<i>Analyte (nM)</i>	<i>FM</i>	<i>CONTROL</i>	<i>p-value</i>
AEA	0.31 (0.15)	0.31 (0.15)	0.986
OEA	6.23 (2.28)	5.44 (1.83)	0.006*
PEA	9.49 (2.39)	8.62 (2.52)	0.010*
SEA	2.62 (1.02)	2.08 (0.93)	0.000*
2-AG	14.5 (9.20)	11.1 (5.14)	0.001*

Significantly higher BMI ($p < 0.001$) and a tendency to higher age ($p=0.064$) was also found in FM patients. After controlling for age and BMI (using multiple linear regression), significant differences remained for OEA ($p=0.029$) and SEA ($p<0.001$) with a non-significant tendency for 2-AG ($p=0.085$).

When regressing group membership using OPLS-DA modelling, the clinical parameters pain ratings, depression, anxiety, and general health status were substantially stronger group separating regressors than the lipids, indicating that the clinical measures are better diagnostic tools than the biochemical measures. The multivariate correlation analysis indicates that 2-AG levels were negatively associated with pain intensity, FIQ, and anxiety (HADS) and positively correlated with duration of FM. After analysing the bivariate correlation, only the duration of FM and 2-AG correlated significantly ($r = 0.29$; $p = 0.005$) (Figure 10). However, if removing the possible outlier in the right upper corner, the significance is lost, which makes this association more of a tendency.

Figure 10. Scatter plot of years of fibromyalgia (FM) duration and 2-arachidonoylglycerol (2-AG) with a fitted linear regression line, illustrating the positive direction of the association (Pearson correlation: $r = 0.29$; $p = 0.005$).

AEA levels did not differ between groups; however, AEA correlated significantly stronger with OEA and PEA in FM than in controls ($p = 0.05$ and 0.01 , respectively), which indicates differences in the mobilization of AEA between groups to some extent. AEA correlated positively ($r = 0.20$; $p = 0.05$) with depression scorings (HAD-D) (Figure 11).

Figure 11. Scatter plot of levels of arachidonylethanolamide (AEA) and depression scorings (HADS-D) with a fitted linear regression line illustrating the positive direction of the association ($r = 0.20$; $p = 0.05$).

Methodological considerations and limitations

Selection of participants

Because both cohorts were defined according to the ACR 1990 criteria, it is possible to compare the groups. In papers II and III, 16 of 18 patients were classified with FM. Two patients did not meet the tender point criteria ($\geq 11/18$ tender points) and were classified as CWP patients. Paper IV was a sub study of a trial examining the effect of physical exercise. As the participants in this study were recruited via newspaper advertisement, it may have resulted in recruitment of participants who were motivated to exercise, which potentially could bias the results. The participants included in these studies were paid to participate, which also risks biasing the results. Such compensations are and have been the source of substantial debate, in particular about whether, or the extent to which, payment induces individuals to participate [167]. However, since the participants often need to leave work to participate and since experiments could entail a certain measure of pain and discomfort, it would also be ethically dubious to not pay the participants. In addition, not providing pay would potentially only displace the risk of volunteer bias to other groups in the general population, and it would probably be a far more challenging task to perform an experiment or study. In paper IV, BMI differed significantly between groups and patients were also older (non-significantly). Future studies should include patients and controls with similar BMI and with a narrower age span.

Sampling – collection and preparations

MD

Sampling of hydrophobic substances using MD is more challenging than the sampling of hydrophilic substances. In addition to the adsorption of lipophilic compounds onto the MD catheter membranes, the liquid used for perfusion was hydrophilic, which possibly makes it more difficult for lipophilic compounds to cross the membrane. A relatively high flow-rate and the addition of cyclodextrins to the perfusion fluid seem to increase the recovery of lipophilic substances [168]. We used a relatively high flow-rate but did not include cyclodextrins, which might limited the possibility for lipids to cross the catheter membrane. However, Zoerner et al. did add cyclodextrins, but did not succeed with the sampling of 2-AG from adipose tissue [169], indicating other important factors are at play when recovering lipophilic compounds. The measured concentrations of the NAEs in the microdialysate do not reflect the interstitial tissue concentrations since the recovery is considerably lower than 100%. The recovery of PEA in 20-kDa catheters has been measured *in vitro* and was 29-35% (depending on the concentration). The recovery *in vivo* could not be calculated since no internal reference was used.

Plasma

The *stability* of ECs and NAEs in blood, plasma/serum, and in sample extracts has been evaluated by several groups. Concerning whole blood, it is well known that NAEs are synthesized *ex vivo* in blood [170, 171]. Therefore, plasma should be harvested from whole blood as soon as possible after sample collection. More importantly, the time between blood withdrawal and plasma/serum harvesting should be similar. The short term stability (**24 h in 4 °C and in R.T**) of NAEs in plasma is good, although alteration of 2-AG has been observed [170], which suggests that plasma should be divided into aliquots without delay, snap-frozen, and kept at -80°C until analysis. Substantial deviations of ECs and NAEs due to freeze/thaw cycles before analysis have been reported [156, 171] and should be avoided. The two plasma sampling protocols used in this thesis have approximately the same design, with the exception of the centrifugation parameters. In paper III, plasma was harvested after 15 min centrifugation in 4°C, and in paper IV plasma was collected after 30 min centrifugation in R.T., which possibly could affect the recovery of the lipids. However, since no comparisons of lipids between cohorts were performed, this difference was not important. Comparing plasma contra serum used for extraction of ECs and NAEs, Lam et al. found slightly higher levels of serum AEA than plasma AEA [172]. However, in the same study, PEA levels were substantially higher in plasma than in serum, which indicates that the choice of plasma or serum could be important for specific lipids, although not uniform for all ECs and NAEs.

Analytical methods

LC-MS/MS

There are a variety of MS instruments (e.g., MS, MS/MS, MS-time of flight (TOF)), coupled to various separation devices (e.g., LC, gas chromatography (GC), and a variety of capillary electrophoresis, CE). In addition, there are many ionization techniques (e.g., ESI, matrix-assisted laser desorption/ionization (MALDI), and atmospheric pressure chemical ionization, APCI). When applying targeted quantitative measurements of small molecules (< 1000 Da), LC- or GC-MS/MS are optimal instruments. These instruments have been frequently used to target the molecules studied in this thesis [157].

The advantages of LC over GC include less time-consuming sample preparation, mainly due to the fact that pre-derivatization of non-volatile analytes (such as ECs and NAEs) is usually not needed in LC, and shorter retention times, which makes it possible to analyse many samples in a relatively short time [157]. Modified LCs or ultra-pressure LCs (UPLC) have also been used for EC analysis [173] as these instruments can operate at very high back pressure with the advantage of high chromatographic resolution, high sensitivity, and shorter analysis times. In addition, the ionization techniques ESI and APCI have frequently been used for ECs and NAEs [157]. The selectivity and sensitivity of SRM based MS/MS methods (triple-quadrupole) are generally higher compared to MS or MS-TOF methods, since it allows for the simultaneously measuring of a precursor ion with a selected fragment (product ion) of a targeted analyte, reducing the risk of including unwanted molecules with similar masses.

In the method used in paper IV, each analyte was quantified using the area of its deuterated internal standard. Figure 12 presents the chromatograms of all the analytes with their corresponding ISTD from a plasma sample.

Figure 12. Chromatograms of the analytes AEA, OEA, PEA, SEA, and 2-AG with their corresponding deuterated internal standards AEA-d4, OEA-d4, PEA-d4, SEA-d3, and 2-AG-d5 received from a plasma sample from a fibromyalgia patient. Peaks are shown with their retention time.

For the specific instrumental parameters, see the different papers. The validity of the LC-MS/MS methods used in this thesis is described by the validation parameters below.

The extraction **recovery** of the analytes and their corresponding internal standards were calculated by comparing the peak area of standards and internal standards with the peak area in spiked samples before and after the sample preparation. The recovery was ~ 40% for 2-AG/2-AG-5, ~30% for AEA/AEA-d4, ~20% for OEA/OEA-d4 and PEA/PEA-d4, and ~10% for SEA/SEA-d3. In paper III, only AEA-d4 was used as internal standard therefore adjustments of OEA, PEA, and SEA concentrations were performed. In paper IV, no adjustments of the concentration were needed since recoveries were approximately the same for the analytes and their corresponding internal standards.

The linearity in plasma was calculated from five point standard curves spiked in plasma in duplicate (Figure 13) (standard curve for AEA). Quantification was performed using similar five point standard curves prepared in LC mobile phase in duplicate. The slopes of standard curves in plasma compared to standard curves in mobile phase were estimated to be parallel for the various lipids, so no corrections for different slopes were needed. Linear regression was used, and data were $1/X^2$ weighted since this resulted in higher accuracy than other weightings (e.g., equal, $1/x$ weighting), which has been applied by others [156]. The precision was calculated from pooled plasma samples ($n = 5$) and from spiked quality control (QC)-samples ($n=5$) (standard in mobile phase) and was between 15-22%, which is comparable with precisions reported from Balvers et al. [156]. The linearity, range, and precision for the method used in paper IV are presented in Table 5. For linearity and ranges of the methods used in papers I-III, see the papers.

Figure 13. Five point standard curve for AEA with the concentrations 1, 2.5, 5.0, 10.0, and 25 nM, $R^2 = 0.89$.

Table 5. Linearity, range, and precision defined as coefficient of variation (CV) values of the method used in paper IV. Quality control (QC) samples at “low” (n=5) and “high” (n=5) levels within the linear range were used for calculation of the precision.

Analyte	Linearity	Range	Precision			
	R ²	nM	QC-low (nM)	CV (%)	QC-high (nM)	CV (%)
AEA	0.89	1–25	1.8	22	8.3	21
OEA	0.97	10–500	63.1	15	212.9	16
PEA	0.95	10–500	101.6	15	355.6	19
SEA	0.97	10–500	33.1	20	126.6	17
2-AG	0.92	50–1250	162.7	15	301.8	22

LOD was ~ 0.1 fmol for OEA, PEA, and SEA, ~ 0.5 fmol for AEA, and ~ 50 fmol for 2-AG, which is comparable with other methods in the literature using similar instrumentation [156, 170] and similar LOD definition.

The LOQ was defined as the lowest calibration point, where an accuracy ≥ 80 % compared to the actual concentration was required and was estimated to ~1 fmol for OEA and SEA, ~5 fmol for PEA in the MD analysis, ~10 fmol for AEA, ~100 fmol for OEA, PEA, and SEA, and ~300 fmol for 2-AG for the plasma analysis. However, due to the **lack of “blank” sample materials**, it was difficult to determine an accurate LOQ for plasma, a limitation noted by others [156].

Concerning contamination, traces of PEA and SEA from chloroform solutions during sample preparations has been reported [174], and polypropylene tubes have been shown to release additional amounts of PEA and SEA during evaporation in the presences of chloroform [170]. Although we did not use chloroform during sample preparation, background levels of SEA, PEA, and OEA were detected in mobile phase used for the LC. The origin of these background levels was not thoroughly investigated in this thesis; however, one recently published study (2017) reports a widespread **PEA contamination in standard laboratory glassware and especially in 5.75” glass Pasteur pipettes** [175], which could be one source of contamination in our work as well.

Although 2-AG was detected in several of the plasma samples analysed in paper III, its levels could not be measured adequately. This lack of accuracy was probably due to the limits of the isocratic LC method in separating background noise from analytes and the **method’s** lack of ISTD.

Levels of NAEs in papers III and IV differ substantially, especially for SEA. This difference is probably due to the lack of specific ISTDs in paper III, but different batches of standards could also contribute to this quite large difference. Concerning levels of ECs and NAEs in general, the comparability between different studies are not valid. Fanelli et al. investigated the possibility to establish reference intervals for these compounds and they concluded the following:

... until certified material for calibrations and quality controls are available, the establishment of shared reference intervals is difficult to achieve. In these concerns, an effort toward the harmonization of pre-analytical and analytical procedures would help in improving the agreement and in moving the EC field out from basic to clinical research. [170]

Clinical instruments

In addition to the two pain intensity scales NRS and VAS (both used in this thesis), the Verbal Rating Scales (VRS) and the Faces Pain Scale-Revised (FPS-R) are valid instruments for assessing pain intensity [176]. In chronic pain clinical trials, NRS was demonstrated to be more accurate than the VRS [177].

Pain sensitivity was assessed with an algometer, a valid device for measuring pain sensitivity for pressure [178]. In paper IV, because the PPTs were assessed by three different examiners, some error could potentially be introduced which could bias the results. Using a thermode and a modular sensory analyser for measuring thermal pain thresholds has been demonstrated to have a high reliability for both CPT and HPT and to be a valid method [179].

Anxiety and depression scores using HADS has been found to perform well in assessing the symptom severity of anxiety disorders and depression in both somatic, psychiatric, and primary care patients and in the general population [149]. The FIQ instrument is a validated FM specific tool to assess the current health status in clinical and research settings and it is well correlated to disability status [180].

Statistics

In this thesis, both traditional (uni- and bivariate) and multivariate statistics were used. The OPLS-DA models in papers II-III confirmed the traditional statistical analyses and were useful for determining the relative importance of the investigated variables when group membership was regressed. In paper IV, the PCA model of the FM group confirmed the bivariate correlation (e.g., the correlation between NAEs and the lack of correlation between 2-AG and the NAEs) in some respects, but it also implicated

other possible associations. Multiple comparison increases the risk of measuring just sampling error. A possible limitation concerning the traditional statistical group comparisons is that we chose not to correct for multiple comparisons, for example, using Bonferroni correction. Such a correction assumes that the hypothesis tests are statistically independent, which was not true for the lipids.

Expanded discussion and interpretation of papers II-IV

The case control trials (papers II-IV) investigate whether lipid mediators with anti-nociceptive and anti-inflammatory characteristics are altered in women with CWP or FM compared to healthy controls. Both the studies investigating the systemic levels (papers III and IV) found significantly increased levels of the PPAR- α activating OEA and PEA in patients. In addition, women with FM also had significantly increased levels of the CB activating 2-AG and SEA.

If the increased levels of OEA and PEA in plasma reflects different pathological mechanisms of CWP conditions (at least partly), how are we to interpret the high circulating levels of these lipids? We also measured PEA and OEA in muscles tissue and its expected that muscles contribute to the systemic levels; furthermore, when comparing MD levels from muscle with plasma levels, a substantial increase in the correlation (significant correlation) existed for OEA in the control group 40 min (and 120 min) after catheter insertion (Figure 9), results that further support this proposition. This result might also indicate that healthy muscles respond more intensely to the insertion of the MD catheter (with respect to NAE levels) than painful muscles compared to their habitual levels. Hence, chronic pain muscles could be debilitated with respect to their ability to mobilize NAEs, which could reflect a NAE metabolic dysfunction in CWP muscles. Moreover, since no association was evident between muscle levels and systemic levels in CWP, although relatively associated in CON, it is possible that the sources of systemic NAE levels differ between CWP and CON.

If PPAR activating NAEs are increased in CWP, the hypothesis of associations between NAEs and cytokines are reasonable as NAEs have been related to specific cytokines (via PPAR receptors) [78, 107, 181-183] *in vitro* and in animal studies; however, no such associations were found in paper III. Although PPAR- α and the targeted cytokines are expressed in same cell type (e.g., PPAR- α [102] as well as TNF- α , IL-1 β [184], and IL-6 [185]) are expressed human macrophages, it is likely that circulating levels of NAEs and cytokines do not reflect their association through PPAR- α . Perhaps the association between NAEs and cytokines could be studied better using MD in muscles. Carson et al. reported an increase in IL-6 and IL-8 levels two to

six hours following insertion of a MD catheter in muscle [186]. To measure NAEs simultaneously during such a period might be an approach to study their association.

What do higher circulating levels of 2-AG mean?

In contrast to the NAEs, which were measured in both muscles and plasma in patients and controls, 2-AG was measured only in plasma. Hence we can only speculate about its origin, which could be various tissues and organs, circulation cells, and brain [187]. If 2-AG reflects the pathology of FM (or different aspect), it could be related to different signs of its manifestations. In paper I, we found 2-AG in MD membranes used in muscles, and since CB¹ are highly expressed in A-delta and C primary afferents innervating skeletal muscle [188], muscles are a possible source. As calcium mobilizes 2-AG in neurons [189], overexpression of 2-AG could be a result of high calcium levels in muscle neurons, which potentially affects nociception. Second, since CB² receptors are widely expressed in human leukocytes [190] and since CB² activation is associated with a reduction in pro-inflammatory cytokine release [191-193], high levels could reflect a spill-over in a immunological homeostasis process. However, significant positive correlation between circulating 2-AG and IL-6 has also been reported [194]. Third, CB¹ receptors are widely expressed in brain and associated with stress regulating activity [195], and the EC system has been proposed to regulate basal HPA activity [196]. Since ECs pass the blood-brain-barrier, high levels of 2-AG in circulation could also reflect the abundance of stress regulating activity.

Furthermore, if the lipid mediators are limited to the most well-recognized targets, these results imply that there are four distinct main signalling routes that could be affected in the investigated chronic pain conditions: 1) the PPAR- α route; 2-3) the CB routes; 4) the TRPV¹ route (Figure 14).

Figure 14. Signalling routes possibly affected in the chronic pain patients and potential effects of such activity. 2-AG via CB receptors could modulate neuronal calcium, stress activity, energy metabolism, and the immune system. OEA, PEA (via PPAR- α), and SEA could modulate the immune system and energy metabolism. Both 2-AG and OEA could via TRPV¹ have the potential to modulate the neuro immune system.

Confounders

Age has been reported to positively influence 2-AG and PEA in females [170], and age of the patients was higher in both cohorts investigated in this thesis and significantly higher in paper II. After correction for age in paper IV, significant group differences remained for OEA and SEA. However, since age also increases the risk of developing FM [197], such corrections could be disputed.

BMI and circulating 2-AG seems to be positively correlated [187], which was found in paper IV as well. Activating of CB¹ increases food intake, and the hunger inhibiting hormone leptin decreases hypothalamic EC levels [198]. Several studies have found that BMI is associated with FM symptoms [199, 200], which might be linked to disturbances in leptin production [201]. In addition, leptin has been associated with pain in FM [202]. Hence if higher BMI is linked to the pathogenesis of FM, it might be questionable to control for BMI when comparing the levels of lipids between the two groups.

Diet influences lipid levels. Circulating levels of AEA were significantly increased and this was followed by a fall in AEA concentration after food consumption [203]. 2-AG levels were significantly higher at food presentation

and during and after consumption [204, 205]. OEA, PEA, and SEA have also been reported to be influenced by diet [206, 207]. Circulating OEA increased in humans after consuming a diet enriched in monounsaturated fat [208]. Diet was not controlled for in this thesis.

Physical exercise affects circulating NAEs and ECs in rats and humans and ECs have been associated with exercise-induced reward [90, 209, 210]. NAEs increased significantly in healthy trained male cyclists after 60 min of exercise and continued to increase after a 15-min recovery [211]. Moreover, PEA and SEA decreased in trapezius muscle of CWP patients after 20 min of low intense exercise [160]. In addition to the suggested rewarding well-being effects, Heyman and Di Marzo et al. have proposed that NAEs could produce metabolic effects in skeletal muscles during exercise including such as enhanced glucose uptake and mitochondrial biogenesis [212]. Moreover, exercise-induced anti-nociception has been suggested to be mediated by the EC system [90, 213]. Because we did not document how much exercise the participants engaged in before biological sampling, exercise confounding effects could not be ruled out.

In 2015, Hanlon et al. reported that circulating concentrations of 2-AG is significantly circadian with an average low point in the early morning (04:00) and an average high point between 12:00 and 15:00 [214], which makes it important to collect samples during the same phase of the day. The blood sampling in paper IV was performed both in the mornings and afternoons, so this the timing of sampling could be a confounder.

Moreover, a single nucleotide polymorphism in the FAAH gene (385C->A mutation) encodes a FAAH protein more susceptible to degradation, and is associated with a ~30% reduction of FAAH activity [215], which has been reported to affect circulating levels of NAEs [216]. Although only about 4% of Europeans encode for this mutation [217], it is a possible confounder.

Perhaps, although far-fetched, acetaminophen (paracetamol) is metabolized by FAAH to the AEA analogue arachidonoylphenolamide (AM404) [218]. The long-term effects on NAEs level due to paracetamol consumption is not known. Therefore, it is possible that chronic pain patients consume more paracetamol than non-chronic pain individuals, which possibly could affect the levels of NAEs. The long-term consumption of paracetamol was not controlled for and could possibly be a confounder.

CONCLUSIONS

The main conclusions of thesis are as follows.

1. OEA, PEA, and SEA can be measured in microdialysate collected from trapezius muscles and forearm skin. AEA and 2-AG were not robustly detected in the microdialysate, but 2-AG was detected in all MD membranes, which indicates its presence in the tissues. The consideration of data conserved in the membrane could enhance an adequate interpretation of microdialysate data outcomes and be relevant in situations such as choice of membranes.
2. Chronic painful muscles might be altered in their ability to mobilize NAEs, although the association between increased lipid levels in CWP muscles and pain characteristics was low or absent.
3. High levels and altered relative composition of NAEs in CWP might indicate imbalanced metabolism. The link between NAEs and cytokines was not reflected in plasma levels.
4. Increased levels of OEA, PEA, SEA, and 2-AG might indicate that these lipids play important roles in the complex pathophysiology of FM. However, the investigated lipids could not sufficiently explain the manifestations of FM or be used as biomarkers.

FUTURE PROSPECTIVE

According to papers II-IV, the investigated lipids ability to work as biomarkers for chronic pain conditions in the clinic is low because we found a low association with clinical manifestations, the source of systemic levels have multiple confounding problematics, and there are no shared reference intervals. However, to measure these lipids before and after a standardized provocation test (e.g., CPM activation test) could reduce several confounding issues and could potentially be a measure of function, which might is a better way to study the pathology of chronic pain conditions than using single point measures.

In the future, it's possible that the targeted lipid mediators are included in a panel of biomarkers, together with other molecules e.g. cytokines, neuropeptides, and other proteins, which in combination with increased knowledge in pain genetics could help clinicians to diagnose and prescribe drugs in an optimal way for chronic pain conditions.

There are few available pharmacological therapeutics that interact with signalling routes, which are highlighted (possibly affected in the pain patients) in this thesis. These therapies include medical cannabis, synthetic THC, and extracted phyto-cannabinoids, micronized PEA, and a capsaicin patch used for its anaesthetic effect. Optimised drugs operating at these signalling routes and with smaller side effects may be available in the near future. A novel selective FAAH-1 inhibitor (ASP8477) was recently reported to have an analgesic effect like pregabalin (with no dizziness and motor coordination deficits like pregabalin) in a mouse model of neuropathic pain [219]. Moreover, a compound (OMDM198) with dual effect as both FAAH inhibitor and as TRPV1 antagonist has shown to have potent analgesic effect in a rat pain model of osteoarthritis [220].

Obviously, it is important to continue to search for and reveal possible biochemical alterations that could explain the mechanisms of chronic pain. This in turn could lead to a more mechanism-specific chronic pain diagnosis and better treatments. Moreover, some data suggest that chronic pain could be related to life style. In addition to the non-modifiable risk factors of chronic pain such as age, female sex, socioeconomic background, and genetics, there are modifiable life style risk factors such as lack of physical exercise, obesity, and poor nutrition [221]. However, there is a lack of human trials that examine the relation between life style risk factors, chronic pain, and biochemical measures, including genetics. If aiming to reveal the complex aetiology of various chronic pain conditions, the long and hard way of longitudinal observational studies could be one way forward. If sampling biochemical measures and life style factors over several decades, a

large amount of data will be produced, which preferably could be analysed using MVDA to capture the system wide aspect. **Let's do it!**

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