DEPARTMENT OF DENTAL MEDICINE
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SALIVARY BIOMARKERS IN CHRONIC MUSCLE PAIN

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The cover illustrates the protein profile signature in stimulated whole saliva of one female patient diagnosed with myalgia associated with temporomandibular disorder.

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"The knowledge of anything, since all things have causes,
is not acquired or complete unless it is known by its causes"

Abu Ali Al-Hussein Ibn Abdullah Ibn Sina (980-1037 A.D.)

Dedicated to my beloved mother and father

ABSTRACT

Background: Muscle related temporomandibular disorders (TMD myalgia), one of the most common orofacial pain conditions, is characterized by facial pain and often accompanied by jaw movement limitations. Although the underlying biological mechanisms are still unclear, a cluster of proteins and peptides is assumed may mirror the pathophysiology. These proteins and peptides may be measured in a simple non-invasive saliva sample. However, the variability in saliva sample collections and analyses should be kept to a minimum to ensure that reproducibility testing can accurately assess changes between health and disease state.

Aims: This thesis investigated whether saliva can be used to sample algogenic substances that can serve as molecular biomarkers for TMD myalgia. The specific aims of the methodological section were to compare saliva collection methods and to evaluate the daily variation of pain-related mediators. The specific aims of the clinical section were to evaluate algesic mediators and the protein profile in saliva of TMD myalgia for potential diagnostic salivary biomarkers.

Material and methods: Saliva and blood samples were collected from healthy individuals (n=69) and patients diagnosed with TMD myalgia (n=39) according to the Diagnostic Criteria for TMD. Unstimulated and stimulated whole, parotid, and sublingual saliva were analysed. The protein profiles were investigated using two-dimensional gel electrophoresis followed by identification with liquid chromatography tandem mass spectrometry. Levels of nerve growth factor (NGF), calcitonin gene-related peptide (CGRP), and brain derived neuro-tropic factor (BDNF) were determined using western blotting based technology and multiplex electro-chemiluminescence assay panel. Glutamate, serotonin, and substance P (SP) were determined using commercially available methods.

Results: The results showed that different saliva collection approaches resulted in significant differences in the protein profile as well as in the expression of NGF, BDNF, CGRP, SP, and glutamate. Stimulated whole saliva showed least variability in protein concentration (35%) and was correlated to plasma levels of glutamate ($r_s = 0.56$; $P = 0.011$). Unlike SP and glutamate, NGF and BDNF expressed a rhythmic variation in salivary expression with higher levels in the morning ($P < 0.05$). Patients with a diagnosis of TMD myalgia had significantly higher levels of salivary glutamate but lower salivary NGF and BDNF compared to controls ($P < 0.05$); in addition, the lower NGF and BDNF levels correlated to psychological dysfunction ($r_s > -0.462$; $P < 0.001$). The quantitative proteomics data revealed 20 proteins that were significantly altered in patients compared to controls. The identified proteins are involved in metabolic processes, immune response, and stress response. Dissimilarities in protein profile and clinical variables were observed between TMD myalgia and myofascial pain.

Conclusion: The thesis highlights the importance of consistency in saliva collection approaches, including the timing of the collection. It displayed significant changes in pain specific mediators and protein profile in TMD myalgia and furthermore dissimilarities between subclasses indicating different pathophysiology. After extensive validation, potential salivary biomarkers can be combined with clinical features to better understand and diagnose TMD myalgia.

نبذه مختصره عن الأطروحة

الفكرة السابقة: يعتبر الألم العضلي، المرتبط بإضطرابات مفصل الفك الصدغي، من أكثر حالات آلام الفم والوجه شيوعا. حيث أنه يتميز عادة بألم في الوجه والفك ويصاحبه قيوداً في حركة الفك في أغلب الحالات. و بالرغم من أن المسببات البيولوجية المصاحبة لهذا الإضطراب ليست واضحة، إلى أنه من الممكن الإفتراض أن تجمعات من المؤشرات والوسائط البروتينيه تعكس الفيزيولوجيا المرضية لهذا الإضطراب والتي من الممكن قياسها بواسطة أخذ عينة من لعاب المرضى بطريقة بسيطة وغير مؤلمة بغرض تحليلها. كما أنه من المهم الحفاظ على التباين في جمع عينات اللعاب حتى نتمكّن من تقييم التغيرات بين الحالة الصحية وحالة المرض بشكل موحد ومتكرر.

الهدف من الدراسة: إنّ الهدف العام من هذه الأطروحة هوالتحقق من إمكانية أستخدام اللعاب لإيجاد مؤشرات الألم البروتينية الموجودة فيه والتي يمكن أن تكون مؤشراً حيوياً جزيئياً لمسببات الآلام في عضلات الوجه. وقد حُدّدت الأهداف الخاصة بمنهجية هذا البحث لغرض مقارنة الطرق المختلفة المتعلقة بجمع اللعاب بالإضافة الى تقييم الإختلاف في تواجد البروتينات الوسيطة في اللعاب خلال اليوم الواحد. حُددت أهداف القسم الإكلينيكي لغرض تقييم البروتينات الوسيطة والمظهرالكلي للبروتينات الموجودة في لعاب مرضى الآم عضلات الوجه المتعلّقة بإضطرابات مفصل الفك الصدغي لتحديد المؤشرات الحيوية التشخيصية المحتملة.

المواد والطرق: تم جمع عينات اللعاب والدم بعدد 69 من الأفراد الأصحاء وعدد 39 من المرضى الذين تم تشخيصهم بألم في عضلات الوجه وفقًا لمعايير التشخيص الخاصه بإضطرابات مفصل الفك الصدغي. حيث تم تجميع اللعاب (محفز وغير محفز) من الغدد المختلفة في الفم. و تم تحليل المظهرالكلي للبروتينات باستخدام الفصل الكهربائي للهلام ثنائي الأبعاد متبوعًا بالتحديد باستخدام مطياف الكتلة السائل مترادف اللون. وقد تم تحديد مستويات بعض البروتينات والعوامل العصبية وهي : عامل النمو العصبي (NGF)، البيبتيدات المرتبطة بالجين الكالسيتونيني (CGRP) و عامل التغذية العصبية المستمد من الدماغ (BDNF) بإستخدام تقنية الصبغة الغربية ولوحة الفحص الكهروميكانيكية المتعددة. بالاضافة الي أنه تم تحديد المؤشرات البروتينية وهي: الغلوتامات (GLU) والسيروتونين (5HT) والمادة ب (SP) باستخدام الطرق المتاحة تجاريا.

نتائج الأطروحة: أظهرت النتائج أن طرق جمع اللعاب المختلفة أدت إلى اختلافات كبيرة في المظهرالكلي للبروتينات وكذلك في البروتينات التالية: (NGF)، (CGRP) ، (BDNF) ، (SP) و (GLU). أظهر اللعاب الكامل المحفز اختلاف في تركيز البروتينات بنسبة (35٪). بالإضافة الى وجود إرتباط لمستوى الغلوتامات (GLU) في البلازما وفي اللعاب الكامل المحفز. عند تحليل اللعاب الكامل، أظهر NGF وBDNF تباين إيقاعي خلال اليوم حيث سجّل أعلى مستويات له في الصباح (P <0.05) في حين لم يوجد هذا التباين عند تحليل (SP) و(GLU). سجل المرضى الذين يعانون من الآم عضلات الوجه الخاصة بإضطرابات مفصل الفك الصدغي بمستويات أعلى من الغلوتامات في اللعاب الكامل المحفز وإنخفاض مستويات (NGF وBDNF) وهذه المستويات ترتبط ارتباطا وثيقاً بالمشاكل النفسية (rs> - 0.462 p، <0.001). مقارنة بالأشخاص الأصحاء، سجّلت بيانات المرضى الذين يعانون من الآلام عضلات الوجه الخاصة بإضطرابات مفصل الفك الصدغي اختلافاً في 20 بروتيناً. الجدير بالذكرأن هذه البروتينات المكتشفه تكون متواجده طبيعيا في عمليات الأيض والمناعة والعمليات المتعلقة بالتوتر. وقد لوحظت إختلافات في خصائص البروتين الكلية والمتغيرات الاكلينيكية بين الآلام المحددة بمكان واحدة والآلآم المنتشرة في أكثر من مكان.

الخلاصة: تسلط الأطروحة الضوء على أهمية التناسق في النهج المتبع لجمع اللعاب متضمنة الوقت الأنسب لجمع تلك العينات. حيث أظهرت تغيرات ملحوظة في البروتينات الوسيطة الخاصّة بالألم والمظهرالكلي للبروتينات المتعلقة بالآم عضلات الوجه الخاصة بإضطرابات مفصل الفك الصدغي بالإضافه الي أنها أوجدت إختلافا بين فئات فرعية متعلقة بالآم عضلات الوجه الخاصة بإضطرابات مفصل الفك الصدغي والتي قد تشير إلى اختلاف في فيزيولوجيا المرض. بعد التحقق الشامل المكثف، يمكن الجمع بين المؤشرات البروتينية المحتملة الموجودة في اللعاب والخصائص الاكلينيكية لفهم وتشخيص الآم عضلات الوجه المتعلقة بإضطرابات مفصل الفك الصدغي في العيادة.

LIST OF SCIENTIFIC PAPERS

I. The proteomic profile of whole and glandular saliva in healthy pain-free subjects
H. Jasim, P. Olausson, B. Hedenberg-Magnusson, M. Ernberg, B.Ghafouri.
Scientfic Reports. 2016 Dec 15;6:39073

II. Saliva as a medium to detect and measure biomarkers related to pain
H. Jasim, A. Carlsson, B. Hedenberg-Magnusson, B. Ghafouri, M. Ernberg.
Scientfic Reports. 2018 Feb 19;8:3220

III. Daytime changes of salivary biomarkers involved in pain
H. Jasim, B. Ghafouri, A. Carlsson, B. Hedenberg-Magnusson, M. Ernberg.
Journal of Oral Rehabilitation. 2020 Apr 11 00:1–8

IV. Altered levels of salivary and plasma pain related markers in temporo-mandibular disorders
H. Jasim, B. Ghafouri, B. Gerdle, B. Hedenberg-Magnusson, M. Ernberg.
Submitted manuscript

V. Protein signature in saliva of temporomandibular disorders myalgia
H. Jasim, M. Ernberg, A. Carlsson, B. Gerdle, B. Ghafouri.
International Journal of Molecular Sciences. 2020 Apr 7;21(7)

LIST OF ABBREVIATIONS

2DE	Two-dimensional gel electrophoresis
BDNF	Brain-derived neurotropic factor
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CPI	Characteristic pain intensity
CRISP-3	Cysteine-rich secretory protein 3
CV-ANOVA	Cross validated analysis of variance
DC/TMD	Diagnostic criteria for temporomandibular disorders
ELISA	Enzyme-linked immunosorbent assay
FABP	Fatty-acid binding protein
GAD-7	Generalized anxiety disorder-7
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCPS	Graded chronic pain scale
ICHD	International classification of headache disorders
ICOP	International classification of orofacial pain
Ig	Immunoglobulin
IPG	Immobilized pH gradient
ISI	Insomnia severity index
JFLS	Jaw functional limitation scale
MVA	Multivariate analysis
NGF	Nerve growth factor
NRS	Numeric rating scale
OHIP	Oral health impact profile
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
PCA	Principal component analysis
PCS	Pain catastrophizing scale
PGK1	Phosphoglycerate Kinase 1
PHQ	Patient health questionnaire

PPT	Pressure pain threshold
PSS-10	Perceived stress scale-10
RDC/TMD	Research diagnostic criteria for temporomandibular disorders
SAA	Salivary alpha-amylase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SP	Substance P
TMD	Temporomandibular disorders
VIP	Variable influence on projection

CONTENTS

INTRODUCTION

Pain is an individual and subjective sensation that could be described in many ways. The International Association for the Study of Pain describes pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage”; however, there are no precise techniques to objectively measure pain, which makes it impossible to compare the sensation between individuals [1].

PAIN PERCEPTION

Nociception is the sensory process that provides the signals that lead to pain. This process occurs through nociceptors, specialized peripheral sensory neurons that are activated by physical or potential harmful stimuli. The nociceptors mediate the signals from the activated receptor in the periphery through afferent fibres that transmit the signals to the brain via the spinal cord. The pain perception occurs when the signals are interpreted by central areas of the brain [175].

The ability to perceive pain is extremely essential and may be the strongest drive to survival. Typically, pain is classified from a temporal perspective as acute and chronic, but it can also be categorized based on its aetiology as nociceptive, neuropathic, nociplastic, or idiopathic [175; 184].

Acute pain is an important warning signal that indicates a threat to the body and aims to protect the body by activating reflexes that lead to withdrawal or immobilization of body parts. The pain is provoked by a specific disease or damage to tissue and typically has a sudden onset and limited duration [175]. During their life, most people experience acute pain, for example, headache, back pain, burns, toothache, or post-surgical pain. Usually, acute pain resolves with the healing of its underlying cause. In some cases, the pain persists beyond the expected normal healing time or arises without any history of disease or damage (e.g., chronic pain) [182]. Unlike acute pain, chronic pain lacks a protective value or obvious function for survival. Usually, pain is regarded as chronic when it lasts or recurs for more than three months. Compared to acute pain, chronic pain is poorly understood and is more complex [182]. In chronic pain, the nervous system is not hardwired, which implies that the exact same noxious stimulus each time elicits a different nervous system response. Melzack and Wall suggest that repeated stimulation of nociceptors results in a progressive accumulation of electrical response in the CNS, winding up the CNS and eventually intensifying activity in secondary nerve fibres [124]. This phenomenon, called wind-up or central sensitization, is responsible for pain continuing long after expected recovery time for an injury.

Patients suffering from chronic pain may not show the behaviours associated with acute pain. Chronic pain can affect physiological systems such as immunological, endocrine, autonomic, and motoric functions. Other problems usually accompany the pain, such as fatigue, sleep disturbance, mood changes, and cognitive functions. Together, these factors can lead to social isolation and impaired quality of life [182]. In addition to suffering, the annual cost to society

related to chronic pain is relatively high, including health care service, loss of work, decreased productivity, and disability compensation [14; 86]. In the United States, the costs associated with chronic pain are estimated to be approximately $560-635 billion per year [59] and exceed the costs estimated for public health disease such as cardiovascular disease, cancer, and diabetes. In Sweden, socioeconomic costs and national healthcare of conditions associated with chronic pain run into €32 billion every year and represent a significant part of the gross domestic product [14]. A recent population-based survey shows that between 20-35% of the adult population suffer from chronic pain [13; 14; 29; 49]. The spread in prevalence between studies may reflect differences in definition of chronic pain, pain intensity, and selection of subjects. Nevertheless, the most common sites for pain are back, joints, head, and neck [13].

OROFACIAL PAIN

Orofacial pain involves pain perceived from the area of the fifth cranial nerve, trigeminal nerve. The trigeminal nerve consists of three branches on either side that innervate the skin of the face, oral mucosa, parts of the tongue, teeth, nasal cavity, paranasal sinuses, salivary glands, ear, and head. The trigeminal nerve is primary sensory, but it also has motor branches that innervate the muscles of mastication.

Orofacial pain usually starts as acute pain; however, if not treated, this pain develops into chronic pain. These pain conditions present a recurrent, persistent, or disabling pattern because of the particular complex anatomy of the orofacial area and difficulties in the diagnostics and management of chronic pain. These pain conditions are often associated with psychosocial co-morbidities such as anxiety, depression, and somatization. Chronic pain in the orofacial region is most commonly due temporomandibular disorders (TMD).

Temporomandibular disorders

TMD is a group of related conditions in the masticatory muscles, temporomandibular joint, and associated surrounding structures (e.g., ligaments and connective tissues). The disorders are characterized by a triad of clinical features involving muscle and/or joint pain, limited jaw movements, joint noises, and alteration in the mandibular movements (Figure 1) [50; 150].

TMD causes a great deal of suffering in the community and is a widespread problem in clinical practices. It affects 10-15% of the adult population with an incidence rate between 2-4% and TMD myalgia seems to be the most frequent diagnosed TMD pain condition [37; 110; 135] with a frequency of 42% [50]. The reported prevalence of TMD in different age groups varies widely due to differences in study cohorts, diagnostic criteria, and examination methods. Several studies have demonstrated a rather low prevalence of TMD in childhood. However, TMD becomes more prevalent during adolescence and early adulthood and appears to peak during midlife and to decrease in the elderly [85; 110]. Women are more susceptible to TMD; according to epidemiological studies, two of every three patients with TMD signs are female [85]. Several studies have reported greater evoked pain, lower pain threshold and pain tolerance in women compared to men [51; 76]. However, women also show a greater adaptation to sustained stimuli and habituation to repeated stimuli [76]. The underlying pathophysiological

mechanisms for these sex differences are mainly unknown. Dissimilarities in biological, psychological, and social characteristics may interact in complex ways and contribute to the final pain experience.

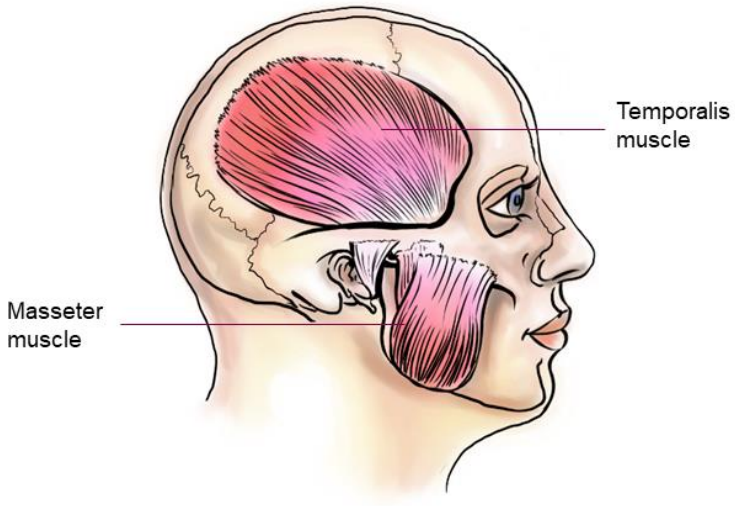

Figure 1. Anatomy of the muscles of mastication. Myalgia associated with temporomandibular disorders are characterized by an aching pain localized to the jaw muscles that is worsened upon palpation and increased with jaw function.

Aetiopathogenesis

TMD myalgia is considered a functional pain syndrome similar to fibromyalgia, interstitial cystitis, irritable bowel syndrome, and chronic fatigue syndrome. These functional disorders appear to share aetiological factors, which may explain the great co-morbidity of symptoms, to affect the patient cumulatively, to present central sensitization, and to amplify pain perception [57].

The aetiopathogenesis behind TMD myalgia remains unknown, but there is evidence for factors that predispose, initiate, and perpetuate the pain (Figure 2). These factors are biological, behavioural, and/or psychosocial [15; 65; 113]. Biological factors such as malocclusion (misalignment of the teeth), genetics, and micro-inflammation have been discussed in the literature [93]. Historically, malocclusions have gained much attention as an essential contributing factor for TMD, but recently this view has been re-evaluated [171]. Some epidemiological studies support a weak association between malocclusion and TMD myalgia, although the degree of the malocclusion does not correlate with the pain intensity [93; 121]. Genetic predispositions are also believed to affect the sensitivity to pain and increase the susceptibility for developing muscle pain [15]. TMD myalgia is often associated with specific tender areas in the muscles referred to by clinicians as ‘tender points’ or ‘trigger points’. Although no gross pathological changes have been observed in these points, there is some indication towards a micro-inflammation in these areas. Studies have reported decreased pH and elevated levels of cytokines and neuropeptides [15; 42]. In some cases, the development of tender areas and pain is related to repetitive strain injury due to behavioural factors such as teeth clenching/grinding (bruxism), lip-biting, or extensive gum chewing [15]. In addition,

evidence from animal experiments shows that repetitive lengthening contractions of the masseter muscle results in a significant increase in inflammation [84]. Repetitive strain would theoretically increase oxidative metabolism to reduce local energy supplies and consequently lead to the release of algogenic substances [15]. Indeed, patients with long-term bruxism are more likely to report complaints of orofacial pain. However, as a direct association between the severity of bruxism and development of TMD myalgia has been difficult to prove, the impact of bruxism in the development and maintenance of TMD myalgia has been questioned [15; 93].

The role of psychosocial factors in TMD myalgia has been deeply researched over the last decades. Numerous studies suggest that stress, anxiety, depression, somatic symptom, and pain catastrophizing play a significant role in the predisposition, initiation, and perpetuation of TMD symptoms [15; 52; 57; 91; 99; 119; 172]. Psychosocial stressors are thought to play a dominant role in the development of TMD myalgia. Patients suffering from TMD commonly report that their symptoms increase during stressful conditions. Since many of the proteins involved in the stress responses are the same as those associated with pain signalling, psychosocial stressors can directly and/or indirectly influence the biological processes involved in orofacial pain. Stress can activate the sympathetic nervous system, increase parafunctional activities, and affect metabolism and blood flow [15]. Psychosocial characterizations have recently been found to be important predictors of treatment outcomes. For example, high degree of depressive symptoms, pain catastrophizing, and somatic complaints are strong predictors for negative response to conventional treatments [75].

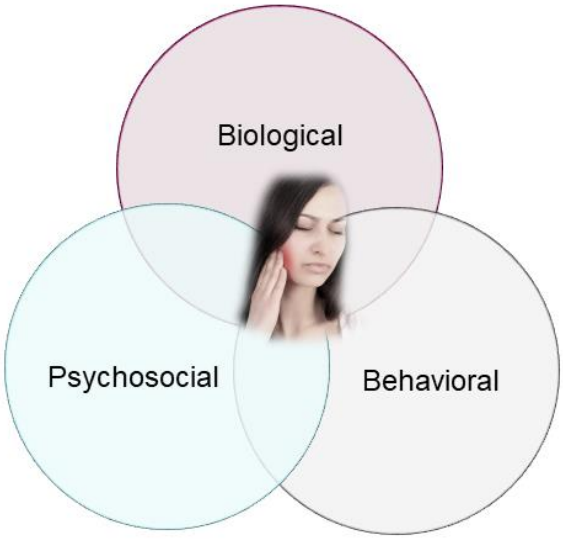

Figure 2. Myalgia associated with temporomandibular dysfunction exhibit a multifactorial aetiology including biological, behavioural, and psychosocial factors.

Classification

The Research Diagnostic Criteria for TMD (RDC/TMD) has previously been the most commonly used and recognized classification system by the scientific community for the diagnosis, evaluation, and classification of TMD. The RDC/TMD are based on a bio-

behavioural model of pain: axis I includes physical signs and symptoms and axis II includes psychological and disability factors [38; 39; 65].

In 2014, the International Research Diagnostic Criteria for Temporomandibular Dysfunction Consortium Network published an updated classification structure for TMD – Diagnostic Criteria for TMD (DC/TMD). The new criteria aimed to improve the sensibility and specificity of the previous RDC/TMD through improvement of axis and axis II, which can be used not only in research but also in clinical settings [160]. As DC/TMD is used worldwide, it has been translated into several languages. Axis I contains an effective screener for detecting any pain-related TMD and effective diagnostic criteria for discriminating between the most common pain-related TMD with a sensitivity ≥ 0.86 and a specificity ≥ 0.98. In addition, axis I also exhibits a strong inter-examiner reliability for pain-related TMD with a kappa ≥ 0.85. The axis II protocol includes both a screening and a self-assessment instrument. The screening instruments assess pain intensity, disability related to pain, psychological distress, limitations in jaw function, parafunctional behaviours, and a pain drawing to assess the location of pain. The self-assessment instruments assess in more detail limitations in jaw function, anxiety, psychological distress, and presence of comorbid pain conditions [160].

The DC/TMD includes tests for diagnosing myalgia such as pain with jaw movements and palpation of the masseter and temporalis muscles (Figure 1). Pain from the clinical examinations must resemble the patient's pain complaint from the previous month. Myalgia is further subclassified into three types: local myalgia, defined as pain localized to the site of palpation; myofascial pain, defined as pain spreading beyond the site of palpation but within the boundary of the muscle being palpated; and myofascial pain with referral, defined as pain at a site beyond the boundary of the muscle being palpated [47]. Myalgia as a class exhibited a sensitivity of 0.90 and specificity of 0.99, and the subtype myofascial pain with referral exhibited slightly reduced sensitivity (0.86) and specificity (0.98). The sensitivity and specificity for myofascial pain have yet to be established [160].

Myofascial pain has also been included in the newly published International Classification of Orofacial Pain (ICOP), a document that is aligned with the International Classification of Diseases (11^{th} revision) and the International Classification of Headache Disorders (3^{rd} edition) (ICHD-3). ICOP aims to create an instrument that will improve research as well as clinical management of orofacial pain and increase collaboration between professionals working on pain in the orofacial area. The classification committee has adopted the DC/TMD criteria for ICOP, but it only included TMD diagnosis associated with pain and modified the presentation style to that of ICHD-3 based on its frequency [1].

OBJECTIVE PAIN MEASURES

Since the nociceptive mechanisms that underlie TMD pain are still not fully understood, the clinician must rely on subjective measures such as patients' anamnesis, questionnaires, and semi-objective findings such as muscle palpation or assessment or pressure pain threshold (PPT). According to the DC/TMD, the three subclasses of myalgia differ only regarding the

presence of pain spread upon palpation as described earlier, but the pathogenesis underlying these diagnoses may not be the same. As pain is a subjective experience, semi-objective methods have limited sensitivity and correlate weakly with subjective pain scores [42]. Consequently, objective and sensitive tools are needed, a situation that has led to a growing interest in molecular biomarkers

Molecular biomarkers

Biological markers (biomarkers) are specific molecules that provide either diagnostic, prognostic, predictive, or therapeutic information and can be measured objectively in human tissues, cells, or body fluids [123]. In muscle pain, biomarkers can be separated into algesic biomarkers, tissue metabolites, and inflammatory mediators [42].

Sampling and analysing biomarkers

In TMD myalgia, clinically valuable biomarkers have to be easy to measure and correlate to pain ratings. An ideal biomarker should be measurable in samples that are easy and non-invasive to collect and handle, such as blood, urine, and saliva. However, a common limitation for all sampling methods are potential diurnal variations of the biomarker concentration. These variations need to be properly evaluated or ruled out before collection. Furthermore, potential biomarkers may be influenced by various factors such as gender, age, body mass, and general health [60; 127].

The rapid development of molecular biology and laboratory technology has led to innovative techniques that can identify biomarkers. New sensitive methods have the possibility to combine and analyse several biomarkers simultaneously, a development that saves time, sample material, and expenses. Multiplex assays such as antibody microarray, Luminex®, and Meso Scale Discovery combine the efficiencies of multiplexing with the sensitivity, precision, reproducibility, and simplicity of enzyme-linked immunosorbent assay (ELISA). Multiplex assay requires knowledge of which analytes to be measured in a specific sample. Proteomic analyses, on the other hand, usually eliminate this requirement and therefore enable the use of blind studies of all the proteins expressed in a sample (e.g., the proteome) and accurately and reliably quantify changes in protein abundance in health and disease to identify potential biomarkers. There are several proteomic methods available such as gel-based methods, including one-dimensional and two-dimensional polyacrylamide gel electrophoresis (2DE) or gel-free high throughput liquid chromatography coupled with tandem mass spectrometry (shotgun proteomic). Shotgun proteomics, 2DE, and protein microarrays can be used to obtain overviews of differences in protein abundance in a sample at a given time or under particular conditions [20].

2DE followed by mass spectrometry is considered a powerful tool for proteomics work [62] as it separates and quantifies different protein isoforms. In 2DE, proteins are separated on two dimensions. In the first dimension, the proteins are separated according to their net charge, also known as isoelectric points. In the second dimension, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins by their mass. Therefore, complex mix-

tures consisting of hundreds to thousands of proteins can be separated and the relative amount of each protein can be determined. Changes in charge and mass can also easily be detected by this approach as it is very unusual that two different proteins or protein isoforms resolves to the same place in both dimensions. Each protein spot can then be removed from the gel, digested with tryptic enzymes and identified using mass spectrometry [4].

Several proteomic studies have been performed in different painful conditions (e.g., neuropathic pain, trapezius myalgia, fibromyalgia, rheumatoid arthritis, and burning mouth syndrome) by analysing proteins in plasma, saliva, cerebrospinal fluid, synovial fluid, interstitial fluid, or biopsies [6; 7; 26; 67; 72; 94; 103; 141-143]. Saliva contains a complex mixture of proteins, peptides, and other molecules that may yield information about the pathophysiology behind TMD myalgia and can be used to identify new biomarkers for the disorder. Proteomic analysis of saliva in patients with TMD myalgia represents a new field of research as the proteomic techniques constantly improve [90; 92]. Only two studies have investigated widespread myalgia using the salivary proteome. These studies, using gel-based proteomics applied to saliva samples from patients with fibromyalgia, reported altered protein expression between patients and controls with an over-expression of transaldolase, phosphoglycerate mutase I, serotransferrin, and alpha-enolase [7; 26].

Potential biomarkers for TMD myalgia

The masticatory muscles are innervated by Aδ and C afferent fibres with free nerve endings. These trigeminal afferent nerve fibres are activated by noxious mechanical and/or chemical stimuli and express receptors for algogenic substances such as glutamate, serotonin, capsaicin, and ATP. Consistent with this role, they also contain neuropeptides [15; 60].

The most studied algogenic substance in TMD myalgia is the excitatory amino acid glutamate. Injection of pharmacological dose of glutamate in healthy masseter muscles is associated with pain symptoms and altered pain sensitivity [179]. Studies have shown that interstitial glutamate is elevated in the masseter muscles of patients with TMD myalgia compared to healthy controls [19; 31; 179]. These findings imply that glutamate may be related to ongoing pain and mechanical sensitivity in TMD myalgia [60].

Serotonin is another potential algogenic substance discussed in the pathogenesis of TMD myalgia [15]. Biopsies from human masseter muscles have revealed the presence of serotonin receptors on sensory nerves in the muscle, and patients with TMD myalgia expressed more of these receptors compared to healthy controls [24]. In addition, the interstitial concentration of serotonin in the masseter muscle of patients appears to be higher compared to the levels found in healthy controls [43; 117], and injection of the serotonin-receptor antagonist granisetron into the masseter muscle seems to alleviate myalgia associated with TMD [25].

Few studies have investigated interstitial and circulatory levels of serotonin or glutamate in TMD myalgia and the findings are inconsistent. Increased interstitial muscle levels of serotonin and glutamate are reported in patients compared to controls [19; 43], but the levels in

plasma/serum showed no differences [31; 44]. The inconsistent results may be due to methodological and diagnostic dissimilarities and the low number of subjects in certain studies.

Nerve growth factor (NGF) is a neuropeptide that facilitates neuronal regeneration and acts as a protective factor for neurons. It has been suggested that NGF plays an important role in hyperalgesia as the concentration of the molecule has been found to be increased after inflammatory injury and up-regulated in response to noxious stimuli [130]. Calcitonin gene-related peptide (CGRP), brain derived neurotropic factor (BDNF), and substance P (SP) are other examples of abundant neuropeptides in nervous tissue. These play important roles in the development of pain and hyperalgesia. CGRP and BDNF may play a role in migraine and headaches based on its increased saliva and plasma concentration during active pain periods [5; 54; 89; 196]. Some evidence suggests that salivary SP levels increase with noxious stimulation, indicating that SP may play a role in central sensitization associated with chronic pain [71; 89]. These substances have also been discussed in TMD myalgia, but their significance in clinical pain has yet to be established [50; 60].

Usually, all potential biomarkers are measured in plasma, cerebrospinal fluid, or intestinal fluid; however, some studies have measured these mediators in saliva [11; 54; 89; 98; 129; 130; 196]. Although salivary glands are integrated in the neuroendocrine system, the sampling and processing techniques are limited and in need of further improvement. Clearly, measuring salivary algogenic mediators and neuropeptides could provide a valuable diagnostic and prognostic tool for chronic painful conditions and provide an objective approach to the study of pain. However, collection methods need to be evaluated and more sensitive techniques for the subsequent analysis need to be developed.

SALIVA AS A DIAGNOSTIC TOOL

Saliva performs many biological functions essential for the maintenance of oral health such as lubrication, cleansing, buffering, and digestion. However, the functions of saliva are not only restricted to the oral cavity [4; 35; 46] as saliva contains many classes of proteins and peptides that represent several significant biological functions that may mirror both oral and systemic health conditions [114]. With the advancements made in analytical technologies for saliva over the last several decades, saliva has gained increased attention also for clinical diagnostics [17; 30; 114].

The salivary glands

Healthy adults produce on average 0.3-0.7 ml of saliva per minute, producing a range of 0.5 up to 1.5 litres daily. Secretion of saliva is an active and continuous process under sympathetic and parasympathetic stimulation [194]. Saliva is not a single fluid but rather a complex mixture derived predominantly from three pairs of major salivary glands (parotid, sublingual, and submandibular gland), which contribute about 90% of the total fluid secretion, and from the minor salivary glands in the oral mucosa, which contribute about 10% of the total volume. In addition, whole saliva also contains gingival crevicular fluid, nasal and bronchial secretions, oral mucosal cells, serum filtrate, microbiota, and food debris [125; 194]. Although the parotid

glands are the largest (Figure 3), they produce only about 20% of the total saliva in the unstimulated resting state, and the minor and sublingual glands together contribute to about an additional 15%. The submandibular glands are by far the most active in the unstimulated resting state, and they are estimated to produce about 65% of the total resting volume. However, when salivary glands are stimulated, the parotid can account for more than 50% of the whole saliva volume in the mouth [83; 133]. Some of the salivary glands are purely serous (parotid gland), others are mucous (minor palatine glands), and some are mixed (submandibular, sublingual, and minor buccal glands).

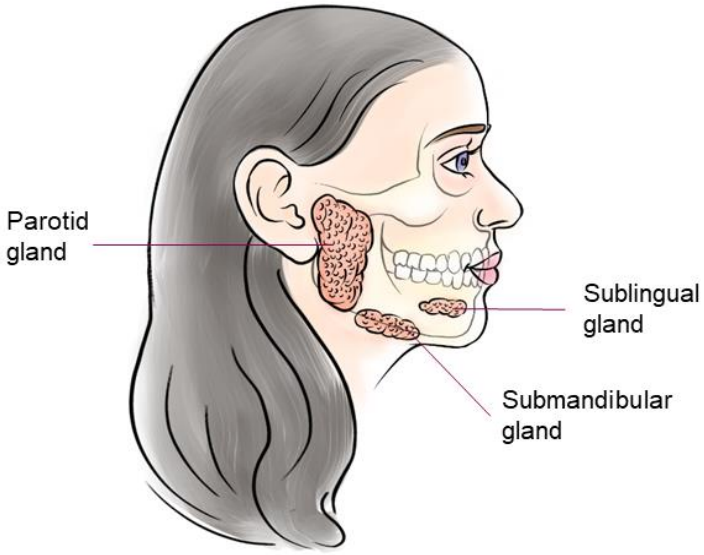

Figure 3. The major salivary glands are the parotid, the submandibular, and sublingual glands. The saliva from the parotid glands is secreted through the "Stensen´s duct", which opens into the mouth in the buccal mucosa near the parotid papilla opposite the second maxillary molar. The saliva from the submandibular and sublingual glands enters the mouth through the "Wharton duct" and several smaller sublingual ducts, which open into the floor of the mouth.

The salivary glands are composed of secretory units called acini, which are built-up of acinar cells that can be either serous or mucous. The acini cells are surrounded by myoepithelial cells, which contract the acini to secrete saliva (Figure 4). Each salivary gland is surrounded by blood vessels and is highly permeable, an arrangement that allows for the free exchange of blood-borne molecules into the acinus [194]. The saliva is composed of 99% water, 0.3% proteins, and 0.2% of inorganic substances. The formation of saliva occurs in a two-stage process. The first stage involves the secretion of an isotonic plasma-like primary saliva by the acinar cells into the luminal terminal pieces of the gland parenchyma. In the second stage, the saliva changes to a hypotonic saliva as it passes though the ducts into the oral cavity. Salivary proteins are continuously secreted by exocytosis of granules from acinar cell. Some proteins are secreted into saliva by other mechanism such as vesicular transport or transcytosis. The majority of all saliva proteins are synthesized by the salivary glands (e.g., glycoproteins, proline-rich proteins, histatins, and statherin), but a small amount of the proteins originates from the plasma [151; 194].

Mirror of bodily functions

Saliva can provide information about diseases and offers distinctive advantages over blood. Salivary diagnostic approaches have been developed to monitor cancer [77] as well as

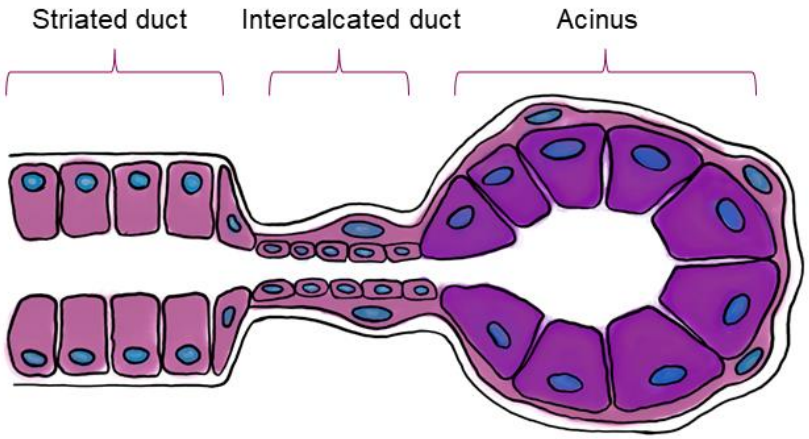

Figure 4. The salivary gland is very permeable and surrounded by blood vessels, which allows for diffusion of blood-derived molecules into the saliva. The protein content in saliva is mostly synthetized and secreted by salivary acinar cells, but a small amount is secreted from the blood.

autoimmune [81], viral [40; 120; 137], and bacterial diseases [2]. However, the full potential of saliva in medicine was recently recognized [109].

Many substances enter saliva from the blood by passing through the intercellular spaces by transcellular or paracellular diffusion [36; 73; 97; 194]. As a result, most of the abundant substances found in blood are also commonly present in saliva. Approximately 27% of the proteome in plasma and nearly 40% of the proteins that have been suggested to be candidate markers for diseases can be found in whole saliva [115]. Therefore, saliva can be regarded as functionally equivalent to plasma with respect to its ability to reflect the physiological state of the body. In addition, saliva collection provides some advantages over blood. The collection is a simple procedure and non-invasive, so it dramatically diminishes any discomfort associated with blood, cerebrospinal fluid, or interstitial collection, methods often used in pain research [53]. For the patient, non-invasive collection reduces anxiety and discomfort. Moreover, non-invasive collection simplifies procurement of repeated samples for longitudinal monitoring. In addition, saliva collection also has many advantages in terms of sampling, storage, and shipping. For the clinician, saliva is safer than venipuncture, which could expose healthcare providers to blood-borne and infectious diseases. Consequently, saliva has the potential to be used as a diagnostic and prognostic specimen in pain research [109; 163].

AIMS

This thesis investigates whether saliva can be used to sample algogenic substances that can serve as molecular biomarkers for TMD myalgia.

The methods section evaluates several saliva sampling methods and analysis techniques. The clinical section evaluates methods applied to patients. The specific objectives are as follows:

Identify the best method for saliva sampling and standardize collection procedure for studying serotonin, glutamate, SP, CGRP, BDNF, and NGF.

Evaluate if a diurnal variation in saliva exists for these biomarkers.

Compare salivary and plasma levels of the above biomarkers in patients with a diagnosis of TMD myalgia and healthy pain-free controls.

Develop a proteomic workflow for a comprehensive identification of proteins in saliva, and study the differences in the proteome expression between different types of saliva (whole and glandular saliva).

Apply the above proteomic approach to study the proteomic signature in patients with TMD myalgia and pain-free controls.

MATERIAL AND METHODS

The methods and selection of participants were approved by the Regional Ethical Review Board in Stockholm, Sweden (2014/17-31/3).

All participants were recruited through advertisement and from patients referred to the specialist clinic for orofacial pain and jaw function at the University Dental Clinic at Karolinska Institute (Huddinge, Sweden). The studies were all conducted at Department of Dental Medicine at Karolinska Institute (Huddinge, Sweden). All participants received careful information regarding the objectives and procedures of the study and signed an informed written consent form prior to participation. The study protocols followed good clinical practice and the guidelines according to the Declaration of Helsinki.

HEALTHY PARTICIPANTS

In total, 69 healthy participants were included, 47 women and 22 men. The participants in Studies I-III were matched according to age and gender. Note that samples from participants in Study I were also included in Study II. The distribution of the participants included in Studies I-V are presented in Table 1.

Inclusion criteria were age above 18 years old, good general health, and a body mass index < 30 kg/m^2. In addition, participants had to be free of fever/or cold and maintain exceptional oral hygiene on the day of collection.

PATIENTS

In total, 39 patients (32 women and 7 men) with chronic masticatory muscle pain (TMD myalgia) were included in Studies IV-V (Table 1). Note that samples from participants in Study IV were also included in Study V.

The inclusion criteria were age above 18 years old and a diagnosis of myalgia or myofascial pain with or without referral according to the DC/TMD [160].

EXCLUSION CRITERIA AND EXAMINATION

For both groups, the following exclusion criteria were used: smoking; diagnosed systemic muscular or joint diseases such as fibromyalgia and rheumatoid arthritis; whiplash-associated disorder; migraine; neurological or neuropsychiatric disorders; diseases of salivary glands such as sialadenitis and salivary gland tumours; pregnancy or lactation; obesity; regular use of medications; use of analgesics during the last 24 hours; oral complaints, such as oral dryness or mucosal lesions; participants with less than 22 teeth and extensive prosthodontics rehabilitations; and poor oral hygiene, hyposalivation, oral diseases (severe periodontal diseases and mucosal pain or ulcerations), or extensive dental abrasion.

Whether a patient should be excluded was determined using information gathered from patient questionnaires, medical histories, and dental examinations. Participants were also asked about factors influencing saliva secretion and composition such as level of physical activity. During

the clinical examination, participants were checked for attrition, decayed teeth, periodontal diseases, mucosal lesions, oral hygiene, jaw movements, and occlusal contacts.

Table 1. The distribution in number (n) of participants included in Studies I-V and their mean age (years) ± standard deviation. Note that participants in Study I are also included in Study II and participates in Study IV are also included in Study V.

Study	I	II	III	IV	V
Healthy participants					
All (n)	10	20	10	39	20
Men	5	10	5	7	6
Women	5	10	5	32	14
Age (years)					
All	23.6 ± 2.2	24.8 ± 3.1	26.3 ± 3.1	28.8 ± 6.9	28.3 ± 8.4
Men	23.7 ± 2.3	24.7 ± 3.1	26.1 ± 3.2	28.7 ± 7.9	28.7 ± 8.6
Women	23.5 ± 2.1	24.9 ± 3.3	26.4 ± 3.4	29.0 ± 6.7	28.1 ± 8.7
Patients					
All (n)	-	-	-	39	20
Men	-	-	-	7	6
Women	-	-	-	32	14
Age (years)					
All	-	-	-	28.8 ± 7.4	28.1 ± 8.8
Men	-	-	-	27.9 ± 7.2	28.5 ± 8.3
Women	-	-	-	29.0 ± 7.3	28.0 ± 9.3

SALIVA COLLECTION

Saliva was collected in all five studies. Prior to saliva collection, participants were instructed to rinse their mouth with water to remove debris and moisturize the oral mucosa. In each study, samples were collected during the same circumstances and in the same order. All participants were requested not to consume alcoholic beverages 24 h prior to collection and not to eat, drink, or brush their teeth at least one hour prior to saliva collection. They were also instructed to complete a detailed food log one day prior to collection and in Study III also during the day of collection.

To prevent degradation of sensitive proteins, all samples in Studies I-V were collected on ice or in precooled polypropylene tubes. A protease inhibitor cocktail (v/v 1:500 Sigma Aldrich, Saint Louis, MO, USA) was added to all saliva samples. Samples were then centrifuged to remove debris and the supernatant was fractionated into tubes and frozen at -70°C until analyses.

In Studies I and II, glandular and whole saliva were collected from all participants, while only whole saliva was collected in Studies III-V as described below. In Studies I and II and Studies IV and V, salivary samples were collected in the morning. In Study III, salivary samples were collected five times during the day (7:30 am, 10:30 am, 1:30 pm, 4:30 pm, and 7:30 pm).

Unstimulated parotid saliva

Parotid saliva was collected by a modified polymethylmethacrylate Carlsson-Critten collector as described by Shannon et al. [165]. The cup was placed bilaterally over the orifices of the parotid duct and secured with suction using a syringe (Figure 5C). Pure saliva from the parotid

gland was collected through a 25-cm plastic tubing placed in a precooled polypropylene tube. To reduce probable contamination, the first drops of saliva were discarded. The tubes were then weighed, and the salivary flow rate was calculated assuming a saliva density of 1.0 g/ml.

Stimulated parotid saliva

To collect stimulated parotid saliva, the Carlsson-Critten collector was used as described above. To stimulate salivary flow, aqueous 2% citric acid solution was applied bilaterally on the sides of the tongue with a cotton swab every 30 seconds. The tubes were then weighed, and salivary flow rate was calculated assuming a saliva density of 1.0 g/ml.

Unstimulated sublingual saliva

While blocking the orifices of the parotid duct with the Carlsson-Critten collector, sublingual saliva from the submandibular and sublingual gland could be collected simultaneously. Saliva was collected every second minute from the floor of the mouth with a sterile syringe into precooled tubes (Figure 5B). Similar to collection of unstimulated parotid saliva, the initial drops were discarded to neutralize salivary flow. The tubes were then weighed, and salivary flow rate was calculated assuming a saliva density of 1.0 g/ml.

Stimulated sublingual saliva

Saliva Bio Oral Swab® (Salimetrics LCC Carlsbad, CA, US) was used to collect stimulated sublingual saliva. The absorbent pad made by synthetic material was placed under the tongue for around two minutes while stimulating saliva flow with aqueous 2% citric acid solution on the sides of the tongue until the pad was fully soaked with saliva. Stimulated sublingual saliva was extracted by centrifugation (1500xg, 15 min, 4°C) of the Saliva Bio Oral Swab®.

Unstimulated whole saliva

Unstimulated whole saliva was collected while the participants were seated comfortably with eyes open and head slightly tilted forward (Figure 5A). Participants were instructed to allow saliva to accumulate on the floor of the mouth without stimulation and passively drool into precooled 5-ml polypropylene tube. Total drooling time was documented, and salivary flow calculated.

Stimulated whole saliva

Whole saliva was mechanically stimulated using sterile paraffin gum (Orion Diagnostica, Esbo, Finland) (Figure 5E). First, the participants were instructed to chew the gum until it was smooth and flexible. After about 60 seconds, the participants were asked to swallow the produced saliva and then start to chew and expectorate the secreted saliva into a precooled graded polypropylene tube until sufficient volume of saliva was collected. Total spitting time was documented and salivary flow calculated.

BLOOD COLLECTION

In Studies II-IV, venous blood samples were collected from all participants in connection with the saliva samples. The samples were collected from the cubital vein into 8.5-ml EDTA tubes. The samples were mixed gently and centrifuged within half an hour. The plasma was stored as aliquots at -70°C until analysis.

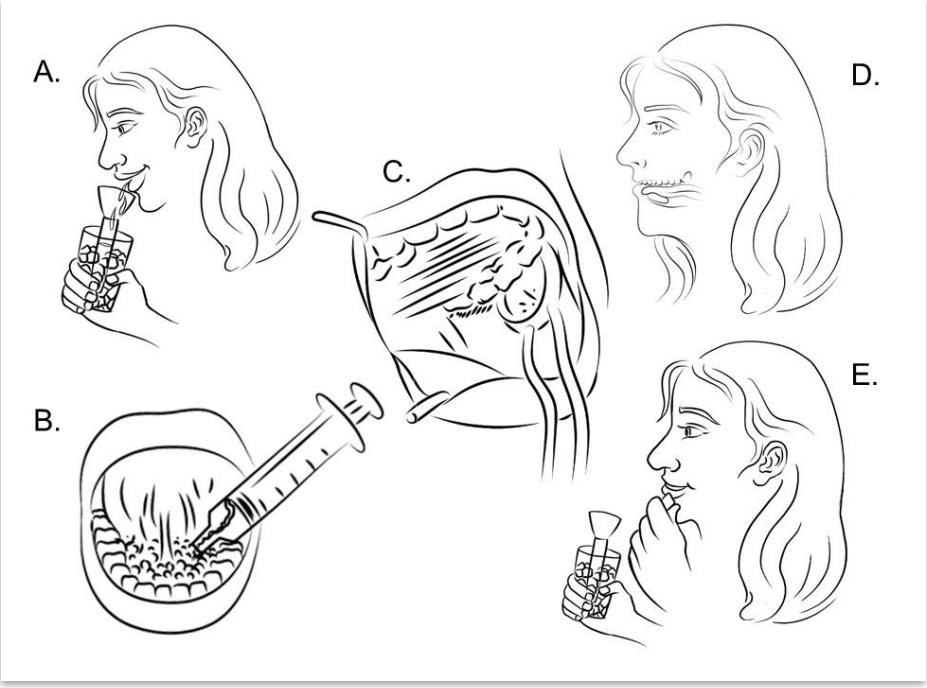

Figure 5. Overview of the different saliva collection methods used in the thesis: A. Unstimulated whole saliva; B. Unstimulated sublingual saliva; C. Unstimulated and stimulated parotid saliva; D. Stimulated sublingual saliva; and E. Stimulated whole saliva.

SUBJECTIVE MEASURES

Pain assessment

Numeric rating scale

In all studies, the participants were asked to assess their current pain intensity in the orofacial region on a numeric rating scale (NRS). The scale ranges from 0 to 10, where 0 indicates 'no pain' and 10 indicates 'worst possible pain'.

Pressure pain threshold

The PPT reflects the mechanical pain sensitivity and was assessed in Studies IV and V with an electronic algometer (Somedic Sales AB, Hörby, Sweden). The probe tip of the alogometer was one cm^2 and covered with a thin rubber pad to reduce the risk for skin irritation. Recordings were made on the most prominent part of the resting superficial masseter muscles while holding the algometer vertical to the skin surface. The pressure was increased at a standardized rate of 50 kPa/s. The digital screen on the algometer was used to monitor the force application rate. The participants were instructed to press a signal button immediately when the pressure turned

into a pain sensation. PPT was also recorded over the tip of the index finger as a reference point to register any possible systemic sensibility. Therefore, a pinch handle was attached to the algometer, which allows the fingertip to be pressed against the handle by the probe during the recording.

The participants were given a verbal and illustrated description of the procedure. PPT was then recorded three times on each location and the average threshold of the three recordings was registered.

Questionnaires

In all studies, questionnaires were used to assess psychological predispositions and conditions. The following instruments included in the DC/TMD axis II questionnaire were used to assess symptoms of depression, somatic symptoms, anxiety, psychological stress, jaw function, oral health, sleep disturbance, and pain catastrophizing: the Patient Health Questionnaire (PHQ-9 and PHQ-15), the Generalized Anxiety Disorder scale (GAD-7), the Perceived Stress Scale-10 (PSS-10), the Jaw Functional Limitation Scale (JFLS), the Oral Health Impact Profile (OHIP), the Insomnia Severity Index (ISI), and the Pain Catastrophizing scale (PCS).

The Patient Health Questionnaire

The PHQ, a validated instrument for mental health disorders, is part of a family of related measures, including the PHQ-9, which is the depression module from the PHQ, and the PHQ-15, which is the somatic symptom module from the PHQ. Studies have found good correlation between PHQ diagnoses and those of independent mental health professionals [105; 106].

The PHQ-9 includes nine symptoms of depression and assesses the level of the depression by the frequency of the symptoms within the last two weeks. Scores ranged between 0 and 27, and scores of 5, 10, 15, and 20 are considered cut-off values for mild, moderate, moderately severe, and severe depression, respectively. Studies of the reliability and validity in adults shows that the PHQ-9 has a 61% sensitivity and 94% specificity [105; 122].

The PHQ-15 includes 15 somatic symptoms or symptom clusters that account for more than 90% of the physical complaints. In determining the PHQ-15 score, each symptom is coded as 0, 1, or 2, and the total score ranges from 0 to 30. Scores of 5, 10, and 15 are considered cut-off values for mild, moderate, and severe somatic symptoms, respectively [104; 173].

Generalized Anxiety Disorder scale

The GAD-7, a frequently used diagnostic self-administered scale for assessing generalized anxiety disorder symptoms, measures anxiety based on seven items scored from 0 to 3. The whole scale score can range from 0 to 21, and scores of 5, 10, and 15 are considered cut-off values for mild, moderate, and severe anxiety, respectively [96]. The scale has been validated within a large sample of patients in a primary care setting [174] as well as within the general population [118] and is a reliable measure of anxiety. A meta-analysis shows that the GAD-7 has acceptable properties for identifying generalized anxiety disorder at cut-off scores 7-10

[149]. The scale is statistically associated with age and gender and shows high comorbidity with depressive and somatic symptoms [174].

Perceived Stress Scale

The PSS-10 is a self-reported instrument that assesses how unpredictable, uncontrollable, and overloaded participants find their lives during the previous month. The PSS-10 is rated on a five-point Likert scale (0-4). Of the ten items of the PSS-10, four are considered negative and six are considered positive. The total score is calculated after reversing the positive item scores and summing all scores and ranges from 0 to 40, where a higher score indicates greater perception of stress [45]. In some studies, high PSS scores have been correlated to high biomarker levels of stress, such as cortisol [154].

Jaw Function Limitation Scale

JFLS is designed to assess jaw function disability. The scale assesses the function of the masticatory system in three dimensions: mastication, vertical jaw mobility, and emotional and verbal expression [139; 160]. The scale consists of 20 items with each item is rated on a NRS, where 0 corresponds to no limitation and 10 to severe limitation. Calculation of a global score (0-10) as the average of the ratings for eight of the items is recommended [160]; a higher score indicates insufficient jaw function. Another suggestion is to sum all 20 items, resulting in a score that ranges 0-200 [123]. Norms have not been yet established for this scale. A recent study suggests a cut-off value of 28 or more out of 200 to define a limitation in jaw function [138]. The instrument exhibits very good psychometric properties and displays strong internal reliability for items and individual responses [139].

Oral Health Impact Profile

OHIP is a reliable and valid instrument for assessing the impact of oral health on masticatory ability and psychosocial function. All studies used a shortened (14-item and 5-item) version of the OHIP consisting of statements that have been rephrased as questions. The participants were asked to rate on a five-point Likert scale how often they experienced each problem within a period of one month. The option ‘do not know/not applicable’ was also included among the answers. Higher scores indicate a poorer oral health-related quality of life [107; 128].

Insomnia severity index

The ISI is a short screening instrument used to measure the symptoms of insomnia. The ISI consists of seven items measuring self-reported problems with sleep such as trouble falling or staying asleep, early awakenings, satisfaction with sleep pattern, distress caused by lack of sleep, and impacts on daily functioning. Each item is scored from 0 to 4, resulting in a total possible score of 28, with a score above 15 indicating clinical insomnia. ISI is a reliable and valid instrument for detecting individuals with insomnia and clinically evaluating the response to treatment [126].

Pain catastrophizing scale

Pain catastrophizing is characterized by feelings of helplessness, active reflection, and magnification of thoughts and feelings towards the painful stimulation [111]. The PCS consists of thirteen items. Each item is scored between 0 and 4, resulting in a total possible score of 52 points. Higher scores indicate higher presence of catastrophizing thoughts [176]. Previous studies have reported that a cut-off value of more than 30 points is associated with pain catastrophizing of clinical relevance. PCS exhibits good psychometric properties with high reliability and internal consistency [111; 145]. The PCS score also seems to correlate to pain intensity, pain-related disability, fear avoidance, and psychological distress [111].

BIOCHEMICAL ANALYSIS

Proteomic profiling

The protein concentration was determined using the Bio-Rad protein assay according to Bradford (Bio-Rad, Hercules, CA, USA). Saliva was desalted into 12mM ammonium bicarbonate using Amicon® Ultra centrifugal filters (Merck Millipore, Billericia, MA, USA). Proteins were then lyophilized and dissolved with 0.20 ml urea solution according to Görg et al. [70].

The denatured proteins in each sample (containing 50 ug of protein in Study I and 300 ug of protein in Study V) were separated according to isoelectric point in the first dimension by in-gel rehydration according to the manufacturer's instructions for 12 h in Study I and 10 h in Study V using low voltage (30 V) in pH 3-10 non-linear 18 cm (Study I) and 24 cm (Study IV) IPGs (GE Healthcare, Stockholm, Sweden). The proteins were then focused for up to 32 000 Vhs in Study I and up to 40 000 Vhs in Study V at a maximum voltage of 8000 V to assure a steady state. IPGs were then immediately stored at -70°C until analysed.

All the IPG gel strips were then equilibrated in SDS equilibration buffer (urea 6 M, SDS 4% (w/v), glycerol 30.5% (w/v), and Trizma-HCl 50 mM) and DTT 1% (w/v) for 15 minutes and then with iodacetamide 4.5% (w/v) for additional 15 minutes.

The second dimension (SDS-PAGE) was run horizontally in Study I and vertically in Study V. In Study I, the horizontal run was carried out by transferring the proteins to gradient gels (ExcelGel XL 245x180x0.5 mm, 12–14%T, 3%C) running at 20-40 mA, up to 1000 V for about 5 h using Multiphor (GE Healthcare). In Study V, SDS-PAGE was carried out using a vertical 2DE setup (ETTAN™ DALTsix Electrophoresis system, Amersham, Pharmacia Biotech, Uppsala, Sweden) as previously described by Bäckryd et al. [6]. Briefly, the IPGs were mounted on precast homogenous polyacrylamide gels (DALT gel 260 × 200 × 1.0 mm, 12.5 %) and run according to protocol for about 7-8 h (2.5 W per gel, 600 V, 400 mA for 30 minutes, followed by an additional 5 hours at 15 W per gel until the blue front reached the bottom of the gel) at a constant temperature of 25°C.

In addition, the staining differed between studies. In Study I, the analytical gels were stained with silver according to Shevchenko et al. [166] with a detection limit of 5ng/spot [178] using

a Stainer Shaker (Hoefer Processor Plus, Amersham Bioscience, UK). In Study V, the gels were fluorescently stained with One-Step Lumitein™ (Biotium, Hayward, CA, USA) according to the manufacturer's protocol.

The 2DE protein patterns of all the gels were visualized as digitized images using a charged coupled device camera system, Versa Doc (Bio-Rad Hercules, CA, USA), in combination with a computerized imaging 16-bit system designed for evaluation of 2DE patterns (PDQuest V 8.0.1; Bio-Rad). Protein spots were detected and matched among different samples, and the amount of protein in each individual spot was assessed as background-corrected optical density integrated over all pixels in the spot and expressed as integrated optical density.

In Study I, the protein spots were identified by comparing previously identified saliva proteins from a local database available at the laboratory [62]. However, protein spots of interest in Study V were excised from the gel and digested with trypsin (Promega Corporation, Madison, WI, USA) as previously described by Ghafouri et al. [62]. The trypsinated peptides were analysed using a nano liquid chromatography system (EASY-nLC, Thermo Scientific, Waltham, MA, USA) coupled to an LTQ Orbitrap Velos Pro MS (Thermo Scientific). Database searching was performed using software MaxQuant (version 1.5.8.3) and the findings were compared with the human Swissprot/UniProt database [27].

Capillary isoelectric focusing immunoassay

BDNF, CGRP, and NGF in Study II and III were analysed with a capillary isoelectric focusing (IEF) immunoassay. Saliva samples were thawed before the analysis and centrifuged to remove debris, and the supernatants were extracted to a new tube. The samples were then diluted with Bicince and concentrated and desalted using Amicon® Ultra centrifugal filters (Merck Millipore, Billericia, MA, USA). Total saliva protein was measured with 2D-Quant kit according to the manufacturer's instructions (GE Healthcare, Little Chalfont, UK). Plasma samples were subjected to albumin and IgG removal kit (GE Healthcare) and then concentrated and desalted using Amicon® Ultra centrifugal filters.

The saliva samples were analysed using a charge-based assay in Study II and size-based assay in Study III. The latter method was used to analyse the plasma samples. All samples were analysed using capillary isoelectric focusing with Peggy system (ProteinSimple, Santa Clara, CA, USA) per manufacturer's protocols. A protein concentration of 0.5 mg/ml was used to analyse BDNF, CGRP, and NGF. The proteins were detected using antibodies against BDNF (Mouse monoclonal, ab10505, Cambridge, UK), CGRP (Rabbit polycloncal, ab189786, Cambridge, UK), and NGF (Rabbit polyclonal, ab6199, Cambridge, UK). The signal was detected with Luminol and Peroxide and scanned with a charged coupled device camera. Higher chemiluminescence equalled to higher expression. The data generated were analysed in compass software version 2.7.1 (ProteinSimple, Santa Clara, CA, USA).

Kinetic enzymatic analysis

The concentration of glutamate in Studies II, III, and IV was determined as previously described by Gerdle et al. [61]. To remove debris, saliva and plasma samples were centrifuged at 4°C for five minutes at 12 000 × g. The supernatant was collected and transferred to a new tube and 5 µl was immediately analysed using ISCUSS analyser (CMA Microdialysis). The detection limit was 1.0 to 150 µmol/l.

Multiplex electrochemiluminescence assay panel

In Study IV, BDNF and NGF concentrations were analysed with the multiplex electrochemiluminescence assay panel from Meso Scale Discovery (MSD, Rockville, MD, USA) per manufacturer's instructions. The detection limits were 0.373 pg/ml and 0.036 pg/ml for BDNF and NGF, respectively.

Enzyme-linked immunosorbent assay

Commercially-available enzyme kits were used to quantify the levels of SP and serotonin. For detection of SP, the enzyme-linked immunosorbent assay kit (ADI-900-018) was used; for detection of serotonin, the colorimetric competitive enzyme immunoassay kit (ADI-900-175) from Enzo Life Sciences (Farmingdale, NY, USA) was used. The detection limit for SP was 8.04 pg/ml and the detection limit for serotonin was 0.293 ng/ml. All kits were used according to the manufacturer's instructions using 96 well plates.

STATISTICAL ANALYSIS

Univariate statistics

The Shapiro-Wilks test was used to test for normality. For continuous variables with normal distribution, independent t-test was used to study differences between two independent groups or repeated measures analysis of variance (ANOVA) for repeated observations with Bonferroni as post-hoc test. Only substances that were detected in more than half of the samples were included in the statistical analysis.

For categorical variables or variables that were non-normal distributed, the Mann-Whitney U-test was applied to study differences between two groups or Friedman's ANOVA for repeated observations. When significant, post-hoc analysis with Wilcoxon matched pair-test was applied with Bonferroni correction.

The Pearson's correlation test was used to test for significant correlations for normally distributed data. Otherwise, correlations between variables were tested for statistical significance using the Spearman correlation test adjusted for multiple comparisons according to Bonferroni.

Descriptive data are presented as mean and standard deviation (SD) or median and interquartile range (IQR). For all analyses, the significance level was set at $P < 0.05$. Statistical analyses were performed using Statistica version 13 (StatSoft, Tulsa, OK, USA).

Multivariate statistics

Multivariate analysis (MVA) is the statistical analysis of several variables simultaneously. In proteomics, each sample can generate hundreds to thousands of potentially equally interacting proteins. MVA has the ability to find interactions between several supposedly independent variables and to determine the contribution of each variable to the measured gains. Multivariate techniques provide a powerful tool to test for significance compared to common univariate techniques [6; 41; 189]. MVA was performed in Study I and Study V using SIMCA-P+ v.15.0 (UMETRICS, Umeå, Sweden).

In the first step, when little is known about the data, a simple overview of the information in the data is needed. This overview can be obtained using principal component analysis (PCA). PCA produces a summary and has the ability to uncover time trends and sudden changes in the data, displaying how the observations are related and if there are any outliers or deviating groups of observations in the data. PCA requires the definition of a few latent variables – i.e., principal components – that describe the principal structure in the data. The principal components are uncorrelated to one another, but they simplify and summarize the data and facilitate the discovery of important patterns in the data. The PCA analysis produces a score plot and a loading plot: the score plot describes the relationship between the participants and the loading plot describes the relationships between variables.

In the second step, orthogonal projections to latent structures discriminant analysis (OPLS-DA) were used to investigate the multivariate correlations between the proteins and to identify the variables responsible for discriminating between patients and controls. Therefore, the outcome variable (Y) nominally represented patient or control, and the predictor variable (X) numerically represented each protein spot measured by integrated optical density. The variable's influence on projection (VIP) indicates the relevance of each X variable pooled over all dimensions and the Y variables the group of variables that best explains Y. Variables with VIP $\geq$ 1.0 and with a 95% confidence interval are usually considered significant in MVA. However, in Study I and Study V, VIP > 1.2 and 1.5, respectively, were considered significant. The sign of the corresponding loading was used to determine whether the relationship was positive or negative.

The R^2 value describes the goodness of fit – the fraction of sum of squares of all the variables explained by a principal component. R^2 ranges between 0 and 1, with higher value indicating higher predictive accuracy. The Q^2 describes the goodness of prediction – the fraction of the total variation of the variables that can be predicted by a principal component using cross validation methods. Q^2 ranges between 0 and 1, with the higher the value indicating better predictability of the model. If R^2 is considerably higher than Q^2, the strength of the model is poor; therefore, R^2 should not exceed Q^2 by too many units. That is, the difference between R^2 and Q^2 should not exceed 0.3. To validate the obtained model, cross validated analysis of variance (CV-ANOVA) was used. The OPLS-DA model was considered significant if the CV-ANOVA showed $P < 0.05$.

RESULTS

METHODOLOGICAL STUDIES

These studies aimed to analyse and compare different saliva collection methods by analysing the general protein profile as well as specific proteins participating in pathways of pain and the daily variation of these proteins.

Descriptive data

Features of the participants in Studies I-III are presented in Table 2. Participants exhibited normal body mass index and jaw function. They reported no signs of pain, depression, somatic symptoms, anxiety, or perceived stress. Salivary flow differed significantly between stimulation and rest but were within the normal reference value and were similar in all studies.

Table 2. Overview of the participants in the methodological studies (Studies I-III). Questionnaire scores are presented as mean ± standard deviation or as median (interquartile range).

Variable	Study I	Study II	Study III
Body Mass Index (kg/m^2)	23.0 ± 3.5	23.0 ± 3.0	21.6 ± 3.0
Pain duration (years)	0 (0)	0 (0)	0 (0)
Number of teeth	29.5 (4)	30 (3.5)	31 (3)
PHQ-9 Score (0-36)	0.5 (1)	0.5 (2)	1.5 (4)
PHQ-15 Score (0-30)	2 (2)	1 (1.5)	4 (3)
GAD-7 Score (0-28)	0 (1)	0 (1)	1 (4)
PSS-10 Score (0-40)	5.5 (4)	6 (4)	7.5 (6)
JFLS Score (0-10)	0 (0)	0 (0)	0 (0)
Unstimulated salivary flow (mL/min)	0.18 ± 0.075	0.19 ± 0.074	0.26 ± 0.169
Stimulated salivary flow (mL/min)	2.18 ± 1.164	2.01 ± 0.827	1.96 ± 0.945

PHQ = The Patient Health Questionnaire; GAD = Generalized Anxiety Disorder; PSS = perceived stress scale; JFLS = Jaw Functional Limitation Scale.

Proteomic profile of different saliva collection methods

The protein concentration, number of specific proteins, and protein pattern showed great differences between the sampling methods. The inter subject coefficient of variation (CV) of the protein concentration varied between 35% and 92% between the six collection methods. Least intersubject variability was observed for stimulated whole saliva (CV 35%), whereas unstimulated parotid saliva expressed the highest variability (CV 92%) followed by unstimulated whole saliva (CV 62%). No significant differences were found in total protein concentration between these methods ($F = 1.899$; $P = 0.130$).

Between 94 and 464 protein spots could be detected in each gel. The smallest number of protein spots were detected in saliva originated from the parotid gland, while sublingual and stimulated whole saliva had the highest number of specific protein spots.

Differences in the protein pattern were typically detected in the area for isoelectric point between 3 and 5 and molecular weight between 10 and 20 kDa. There were also differences in the typical salivary proteins such as salivary Alpha Amylase (SAA), Cystain N, Cystain S, and

prolactin-inducible protein between collection methods. SAA expression was similar among all methods, while Cystain N, Cystain S, and prolactin-inducible protein varied considerably.

Statistical analysis of the comparative proteomic data revealed that several of the 25 protein spots with VIP value above 1.2 were considered significant and distinguished the different saliva collection methods. These proteins are extracellular proteins involved in response to stimulus.

Pain biomarkers between different saliva collection methods

Variations of NGF, CGRP, and BDNF were found in saliva. NGF and BDNF were detected in five different isoforms, while CGRP expressed eight different isoforms in saliva. The isoform pattern showed significant variations in expression between the different collection methods. In addition, when analysing the total expression/level of NGF, BDNF, CGRP, SP, and glutamate, there were significant variations between the different saliva collection approaches (Figure 6).

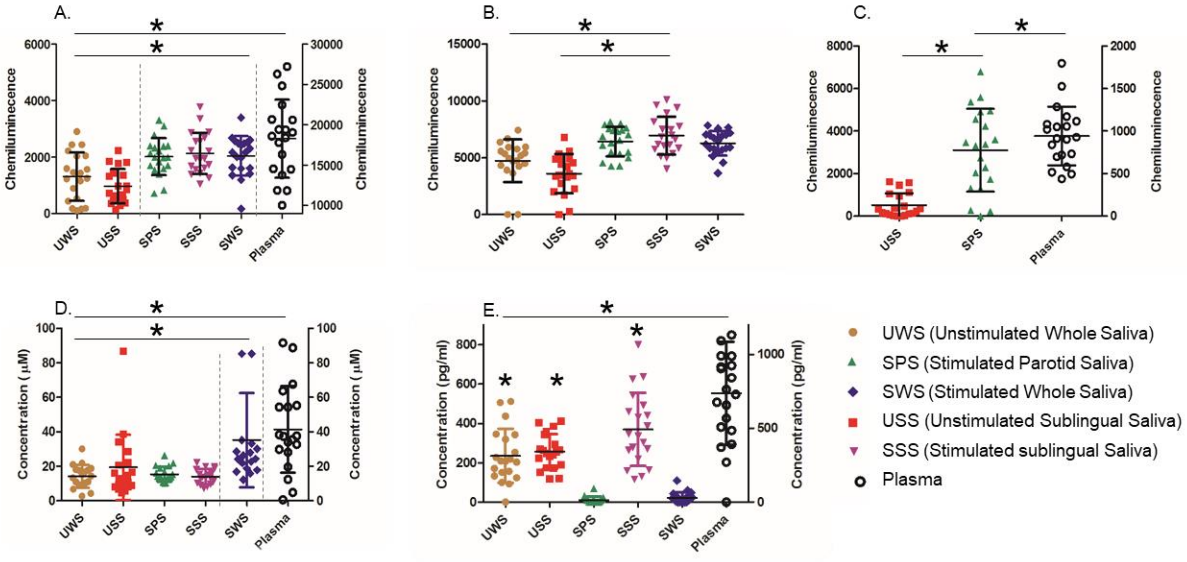

Figure 6. Salivary and plasma A.) nerve growth factor (NGF), B.) calcitonin gene related peptide (CGRP), C.) brain derived neurotrophic factor (BDNF), D.) Glutamate, and E.) Substance P (SP) expression in 20 healthy individuals. Large discrepancies were observed between different saliva collection methods. * indicates significant differences, $P < 0.05$.

NGF levels in unstimulated whole saliva (1,313 ± 860) and sublingual saliva (966 ± 609) showed lower expression compared to the other collection approaches. All stimulated samples showed significantly higher expression of NGF compared to the unstimulated samples ($X^2 = 35.2$; $P < 0.001$). A similar tendency (i.e., elevated levels in stimulated samples compared to unstimulated samples) was also observed for CGRP ($X^2 = 4.6$; $P < 0.001$). However, post-hoc analysis was only significant for stimulated sublingual saliva ($Z = 3.7$; $P < 0.001$). NGF could be detected in plasma and showed significantly higher expression compared to the saliva ($P < 0.001$). Plasma CGRP was not possible to detect adequately with the current analytical protocol.

BDNF could only be detected adequately in unstimulated sublingual and stimulated parotid saliva. The expression was significantly higher in stimulated parotid saliva ($Z = 3.7$; $P < 0.01$) compared to unstimulated sublingual saliva ($Z = 3.7$; $P < 0.001$) and plasma ($Z = 3.4$; $P < 0.001$).

Similar to NGF, CGRP, and BDNF, glutamate and SP showed large variations between the different saliva collection methods (glutamate: $X^2 = 30.3$; $P < 0.001$: SP $X^2=54.6$; $P < 0.001$). Additional post-hoc analysis revealed significantly higher levels of glutamate in stimulated whole saliva compared to all the other saliva types ($Z = 3.8$; $P < 0.001$). The glutamate level in stimulated whole saliva (34.2 ± 26.1 µg/L) were similar to the plasma level (39.4 ± 26.1 µg/L) and moderately correlated ($r_s = 0.56$; $P = 0.011$) after adjustment for multiple comparison.

SP was significantly more concentrated in sublingually derived saliva compared to saliva high in parotid content. Plasma SP was significantly higher compared to all evaluated saliva collection methods ($X^2 = 69.0$; $P < 0.001$) (Figure 6).

Daily variation of pain biomarkers

To study the variations of NGF, BDNF, glutamate, and SP across the day, unstimulated and stimulated whole saliva were collected repeatedly from early morning to late evening. Plasma samples were also collected in association with the first and last saliva sample.

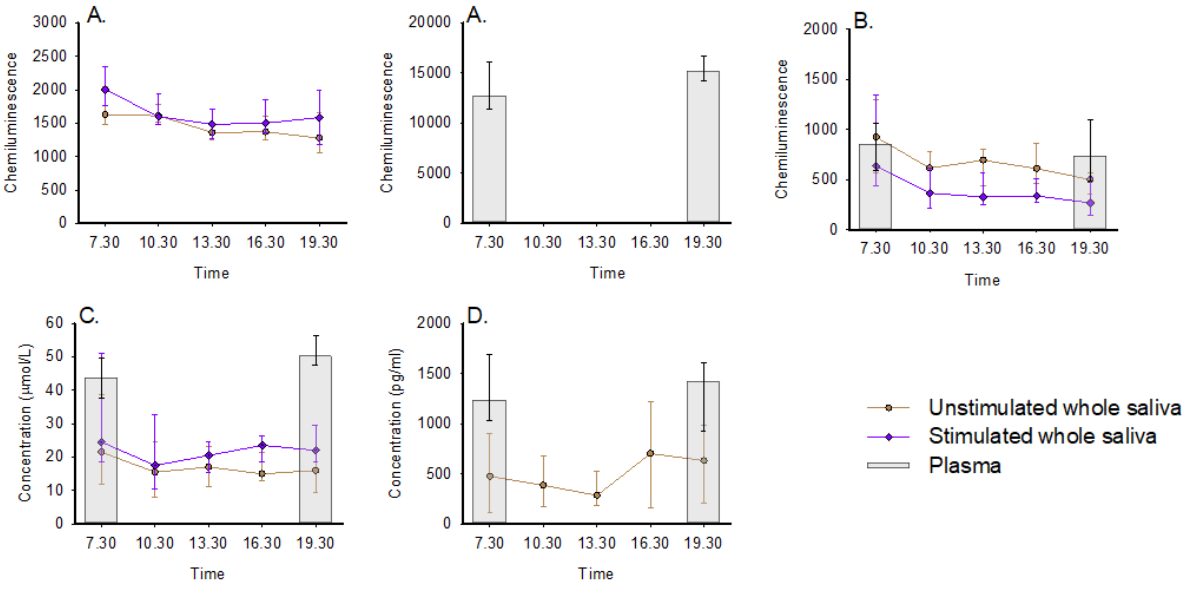

Figure 7. Salivary and plasma. A) nerve growth factor (NGF), B) brain derived neurotrophic factor (BDNF), C) Glutamate, and D) Substance P (SP) expression in ten healthy individuals throughout the day.

The NGF ($X^2 = 14.3$; $P = 0.006$) and BDNF ($X^2 = 17.04$; $P = 0.019$) expression in unstimulated and stimulated whole saliva showed significant differences across the day. The post-hoc test confirmed that NGF and BDNF were significantly higher in the morning sample and the expression decreased during the day (Figure 7A-B). Plasma NGF showed the opposite relation, with significantly lower expression in the morning sample compared to the evening sample

(P = 0.028). Glutamate concentration did not express any significant changes throughout the day (X^2 = 7.289; P = 0.121). SP expression in unstimulated whole saliva and plasma did not change significantly over time. In stimulated whole saliva, SP could not be analysed adequately in a minimum of 50% of all samples and according to the criteria.

CLINICAL STUDIES

These studies aimed to investigate potential salivary biomarkers in TMD myalgia by analysing the general protein profile in patients as well as specific pain-related markers.

Descriptive data

Descriptive data of all patients and healthy controls in Study IV and Study V are presented in Table 3. Patients and controls shared similar background factors such as country of birth, occupation, education level, and level of physical activity. Patients showed significantly higher signs of psychological distress and decreased jaw movement compared to controls. The patients also showed on average mild depressive symptoms and insomnia, moderate levels of somatic symptoms and perceived stress, and almost no signs of pain catastrophizing of clinical relevance. The pain duration was 6.7 ± 6.3 years and the CPI was 60 (20). The average current intensity (NRS) was 4 (2). Patients with TMD myalgia could be divided into subgroups based on the main diagnoses according to DC/TMD criteria. In Study IV, 14 patients were diagnosed with myalgia, 16 with myofascial pain, and nine with myofascial pain with referral. In Study V, ten patients were diagnosed with myalgia, eight with myofascial pain, and two with myofascial pain with referral. Therefore, the myofascial pain diagnosis in Study V was combined into one group.

Table 3. Overview of the patients and controls included in the clinical studies (IV-V). Questionnaire scores are presented as mean ± standard deviation or as median (interquartile range). Statistical parameters are only reported when the distributions between patients and controls differed significantly, $P < 0.05$ (T-test or Mann–Whitney U-test).

Variable	Study IV		Study V		Statistics
	Patients	Controls	Patients	Controls	
Body Mass Index (kg/m^2)	23.7 ± 3.9	22.7 ± 3.3	24.4 ± 3.8	22.7 ± 3.0	NS
Number of teeth	28 (3)	30 (3)	28 (2)	30 (3)	NS
Pain-free opening (mm)	40.6 ± 9.9	56.5 ± 6.2	41.4 ± 10.8	56.4 ± 5.9	P < 0.001
Maximum unassisted opening (mm)	52.5 ± 6.4	57.9 ± 6.2	54.0 ± 6.0	57.7 ± 6.1	P < 0.001
Pain duration (Years)	6.7 ± 6.3	0	6.3 ± 6.3	0	P < 0.001
Current pain intensity (NRS)	4 (2)	0 (0)	6 (2.5)	0 (0)	P < 0.001
CPI	60 (20)	0 (0)	65 (27)	0 (0)	P < 0.001
PHQ-9 Score (0-36)	6 (7)	1 (4)	6.5 (7)	1 (3.5)	P < 0.001
PHQ-15 Score (0-30)	10 (7)	3 (4)	11.5 (9)	2.5 (4)	P < 0.001
GAD-7 Score (0-28)	4 (5)	1 (3)	3.5 (8.5)	1 (2.5)	P < 0.01
PSS-10 Score (0-40)	17 (11)	10 (9)	15.5 (10)	10 (8)	P < 0.01
JFLS Score (0-10)	1.15 (1.8)	0 (0)	1.65 (2.0)	0 (0)	P < 0.001
PCS Score	14 (17)	3 (10)	15 (18)	5 (10)	P < 0.01
ISI Score	10 (9)	5 (5.5)	10 (15)	5 (5)	P < 0.01
Salivary flow (mL/min)	1.61 ± 0.56	1.98 ± 0.93	1.57 ± 0.50	1.74 ± 1.05	P < 0.05
PPT reference (kPa)	356 ± 121	439 ±119	382 ± 127	437 ±130	NS
PPT masseter muscle (kPa)	180 ± 56	268 ± 71	179 ± 63	272 ± 81	P < 0.001

NRS = Numeric Rating Scale; CPI = Characteristic Pain Intensity; PHQ = The Patient Health Questionnaire; GAD = Generalized Anxiety Disorder; PSS = perceived stress scale; JFLS = Jaw Functional Limitation Scale; PCS = Pain Catastrophizing Scale; ISI = Insomnia Severity Index; PPT = Pressure Pain Threshold.

Proteomic profile of TMD myalgia

For comparative proteomic profiling, 20 patient samples and 20 control samples were selected. The patients were well matched with respect to age, gender, and demographic variables to reduce bias from these factors during the discovery stage. The total protein concentration was measured prior to 2DE analysis, and equal amount of protein from each sample was used for the proteomic analysis.

Statistical analysis of the comparative proteomics data revealed that 20 proteins (VIP > 1.5) were at least two-fold higher or lower expressed in patients compared to controls. Among these proteins, twelve showed significantly higher levels, whereas the remaining eight showed significantly lower levels in TMD myalgia compared to controls (Table 4). These identified proteins are involved in metabolic processes (n = 11), immune response (n = 6), and response to stress (n = 7).

Table 4. Identified salivary proteins that were altered in patients with temporomandibular disorder myalgia compared to healthy controls. Proteins with a variable of importance (VIP) above 1.5 in the orthogonal partial least squares discriminant analysis model are shown. The *P*-value is according to the Mann-Whitney data analysis. Arrows ↑ and ↓ indicate up and down regulated proteins in patients compared to controls.

Spot no.	Protein	UniProt ID	VIP	*P*-value	Pat vs. Con
211	Immunoglobulin J chain	P01591	2.02608	0.005	↓
9502	Phosphoglycerate kinase 1	P00558	1.95887	0.056	↑
7202	Glyceraldehyde-3-phosphate dehydrogenase	P04406	1.94448	0.04	↑
2102	Fatty acid-binding protein	Q01469	1.82302	0.042	↓
6202	Immunoglobulin kappa light chain	P0DOX7	1.79123	0.04	↑
4801	Alpha-amylase 1/Alpha-amylase 2B	P04745/P19961	1.78309	0.213	↑
2501	Alpha-amylase 1/Alpha-amylase 2B	P04745/P19961	1.75617	0.007	↓
9205	Cysteine-rich secretory protein 3	P54108	1.7448	0.053	↑
1602	Zinc-alpha-2-glycoprotein	P25311	1.73792	0.026	↑
9404	Chitinase-3-like protein 2	Q15782	1.71259	0.033	↑
5401	Alpha-amylase 1/Alpha-amylase 2B	P04745/P19961	1.68841	0.06	↑
5501	Alpha-amylase 1/Alpha-amylase 2B	P04745/P19961	1.67398	0.027	↑
209	Interleukin-1 receptor antagonist protein	P18510	1.6386	0.025	↑
3601	Alpha-amylase 1/Alpha-amylase 2B	P04745/P19961	1.63012	0.168	↓
8001	Protein S100-A8	P05109	1.62158	0.004	↓
1202	Albumin (N terminal fragment)	P02768	1.57152	0.285	↑
212	Immunoglobulin J chain	P01591	1.5693	0.009	↓
5603	Alpha-amylase 1/Alpha-amylase 2B	P04745/P19961	1.52998	0.172	↑
12	Thioredoxin	P10599	1.52274	0.176	↓
210	Immunoglobulin J chain	P01591	1.51922	0.028	↓

There were no correlations observed between the significantly altered proteins (Figure 2) and any of the following clinical parameters in TMD myalgia or subclasses: mouth opening, pain duration, current pain intensity, CPI, GCPS, JFLS, PHQ-9, PHQ-15, GAD-7, PSS, PCS, ISI, and PPT masseter.

The alerted proteins were analysed together with the clinical parameters to identify any differences between patients diagnosed with myalgia and patients diagnosed with myofascial pain. A significant OPLS-model was found (model characteristics $R^2 = 0.7$, $Q^2 = 0.4$, CV-ANOVA = 0.01), where the enzyme phosphoglycerate kinase 1 was the most important protein (VIP > 2) for separation between the two subclasses (Table 5).

Table 5. Differences between patients diagnosed with myalgia (n=10) and patients diagnosed with myofascial pain with/without referral (n = 10) according to the diagnostic criteria for temporomandibular disorders. OPLS-model characteristics: $R^2 = 0.7$, $Q^2 = 0.4$, CV-ANOVA = 0.01. Variables with variable of importance (VIP) above 1.0 in the orthogonal partial least squares discriminant analysis model are shown. The *P*-value is according to the Mann-Whitney data analysis.

Variable	Myalgia	Myofascial pain	VIP	*P*-value
Phosphoglycerate kinase 1	1,282 ± 519	323 ± 441	2.09004	0.001
PPT masseter muscle (kPa)	227 ± 59	141 ± 31	1.94463	0.001
Level of physical activity*	≥ 3 times/week	1-2 times/week	1.63919	0.039
PHQ-9 SCORE (0-36)	4 (6)	8 (8)	1.57094	0.121
Alpha-amylase 1/Alpha-amylase 2B	2,927 ± 1,885	1,102 ± 1,624	1.5689	0.017
Current pain intensity (NRS)	3.5 (3)	6 (1)	1.51112	0.023
CPI	53 (20)	73 (17)	1.47441	0.023
GCPS (Grade 0-IV)	2 (2)	2.5 (1)	1.43058	0.131
Alpha-amylase 1/Alpha-amylase 2B	1,785 ± 1,498	845 ± 827	1.38901	0.140
Chitinase-3-like protein 2	1,679 ± 1,177	675 ± 636	1.26495	0.026
Glyceraldehyde-3-phosphate dehydrogenase	5,862 ± 4,225	2,568 ± 4,822	1.25951	0.011
PHQ-15 Score (0-30)	8 (11)	12 (5)	1.21037	0.273
PPT reference (kPa)	419 ± 151	353 ± 103	1.21008	0.450
ISI Score	9 (13)	12 (14)	1.18766	0.488
Headache duration (years)	3.0 ± 3.9	7.7 ± 4.4	1.11962	0.037
PSS Score (0-40)	13 (11)	18.5 (7)	1.07296	0.121

PPT = Pressure Pain Threshold; PHQ = The Patient Health Questionnaire; NRS = Numeric Rating Scale; CPI = Characteristic Pain Intensity; GCPS = Graded Chronic Pain Scale; ISI = Insomnia Severity Index; PSS = Perceived Stress Scale; *Median level of physical activity/week.

Pain biomarkers in TMD myalgia

There were significantly different levels of salivary NGF, BDNF, and glutamate in patients with a diagnosis of TMD myalgia compared to pain-free healthy controls. Serotonin and SP did not show any significant differences between patients and controls ($P > 0.05$).

Patients expressed significantly lower levels of salivary NGF ($P = 0.032$) and BDNF ($P = 0.028$) compared to pain-free controls. A similar pattern with lower levels of NGF in patients compared to controls was found in plasma, but the difference was not statistically significant ($P = 0.618$). Plasma BDNF showed significant higher levels in patients compared to controls ($P = 0.022$).

There were no significant correlations between salivary NGF or BDNF and psychological variables in TMD myalgia. There was a reverse correlation between NGF and somatic

symptoms (r_s = -0.462; n = 78; $P < 0.001$) in the study sample. Among the healthy controls, a moderate correlation could be observed between salivary BDNF and perceived stress (r_s = -0.608; n = 38; $P < 0.001$), anxiety (r_s = -0.605; n= 38; $P < 0.0005$), and somatic symptoms (r_s = -0.593; n = 38; $P < 0.001$). No correlations were found between NGF or BDNF expression and pain measures.

Salivary ($P < 0.05$) and plasma ($P < 0.05$) levels of glutamate showed significant differences between patients and controls with the patient group expressing higher levels of glutamate both in saliva and in plasma compared to controls. There were no signs of correlations between glutamate and psychological variables or pain measures in any group.

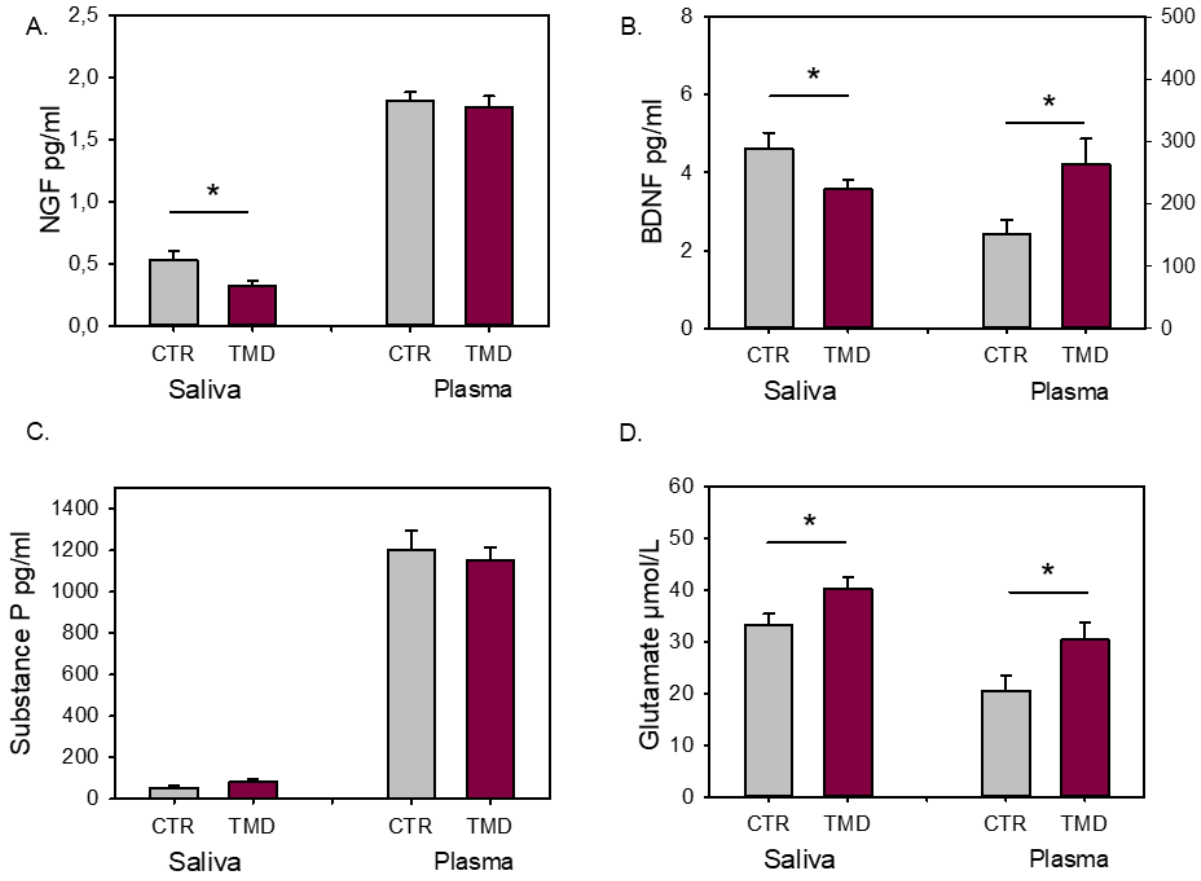

Figure 8. Salivary and plasma levels of A) nerve growth factor (NGF), B) brain derived neurotrophic factor (BDNF), C) substance P (SP), and D) glutamate in 39 patients diagnosed with temporomandibular disorder (TMD) myalgia according to the diagnostic criteria for TMD and 39 healthy pain-free controls (CTR). Patients expressed significantly lower levels of salivary NGF in comparison to controls ($P = 0.032$). Plasma NGF was not statistically significant between groups ($P = 0.618$). Salivary BDNF was lower in patients than in controls ($P = 0.028$), while plasma BDNF was higher in patients compared controls ($P = 0.022$). There were no significant differences for SP. Salivary ($P = 0.026$) and plasma ($P = 0.043$) levels of glutamate were significantly higher in the patients.

DISCUSSION

METHODOLOGICAL STUDIES

Saliva is usually neglected as a body fluid with diagnostic or prognostic value even though sampling in general is well accepted by the patients. This neglect is due to lack of standardized collection procedure and reliable analytical protocols. The methodological section of the thesis aimed to compare different saliva collection methods on specific pain-related mediators as well as the total protein profile and further to study the periodicity of pain-related markers. The main findings were that different saliva collection approaches resulted in clear differences in the protein profile as well as in the expression of several pain-related mediators. Furthermore, some neuropeptides expressed a rhythmic variation in their salivary expression (NGF and BDNF), but other neurotransmitters showed no variation throughout the day (SP and glutamate).

The variable nature of saliva secretion and composition suggests that different approaches may have to be adopted when studying its possible role as a diagnostic and/or prognostic tool. To date, there is extensive literature on the diagnostic possibilities of saliva, but there are still no standardized techniques for saliva sample collection. Different studies apply different sampling methods, and countless studies fail to describe the sample collection procedure such that the procedure can be reproduced [4; 53; 80]. Additionally, the description of the enrolled participants is often insufficient, and a proper clinical examination is often lacking [192]. Taken together, these failures make it difficult to compare results from different studies. A majority of the studies focus exclusively on whole saliva [4; 62; 69; 79; 89; 101; 129; 146; 181; 196] because it can be easily obtained by simply spitting into a test tube, chewing on a sponge, or passively dribbling from the mouth. Only a few studies have focused on ductal saliva such as parotid, sublingual, or submandibular saliva obtained separately from the major glands in the oral cavity [34; 78; 185; 192]. As far as we know, these are the first attempts to structurally compare whole and glandular saliva in a carefully characterized and clinically examined cohort.

Some studies have compared the proteome of different saliva types [34; 185; 192]. Using similar approaches, Waltz et al. describe differences in the protein expression in whole, parotid, and sublingual-submandibular saliva using samples from four participants [185]. They reported less specific protein spots in glandular saliva compared to our findings. The loss of proteins can be because of the additional saliva filtration these authors applied to remove insoluble material and bacterial contamination. In another small poorly described cohort, Wu et al. found significantly diverse protein profiles resolved in parotid and submandibular/sublingual saliva [192]. The salivary proteome depends on the contribution from each salivary gland [17]. Samples collected by the same method display closer correlation than those collected by different methods. Close correlation was observed between the protein pattern of unstimulated and stimulated parotid saliva as well as unstimulated sublingual and whole saliva.

When investigating specific pain-related peptides, significant differences could also be observed between different collection methods and between times of sample collection. When

quantifying NGF, BDNF, and CGRP, automatized western-blot was applied based on charge and size. This technique has the benefit of identifying different isoforms based on protein charge. NGF, BDNF, and CGRP were consequently found in different isoforms in saliva and showed great variation between different collection approaches. A few studies of healthy subjects have been performed on these mediators in saliva. Nam et al., measuring NGF in three sources of saliva, found similar levels to our study [130]. We could also demonstrate a significant association between increased salivary flow by stimulation and increased NGF expression. Although some previous studies have investigated salivary concentration of NGF, no studies have investigated the daytime periodicity of the neuropeptide in saliva. We were able to show daily fluctuation of salivary NGF similar to those described in plasma by Bersani et al. The elevated NGF levels in the morning suggest an increased NGF production during the night similar to what has been observed for other endocrine molecules [10].

Similar to NGF, BDNF expression increases by saliva stimulation (Study III). It is noteworthy that charge-based methods could only detect BDNF in glandular saliva. Nevertheless, when a size-based approach was applied, BDNF could also be detected in whole saliva and similar to NGF expressed a daily variation throughout the day. Other studies have confirmed a circadian variation of BDNF secretion with significant decrease throughout the day [8; 23; 148; 181]. In addition, the amplitude of the fluctuation varies across the menstrual cycle [148].

The levels of SP and glutamate in the methodological studies were analysed using commercially available methods. SP levels were significantly more pronounced in saliva high in sublingual/submandibular secretion, such as unstimulated whole saliva and sublingual saliva. This finding may be explained by SP containing sensory fibres in the submandibular glands [68]. However, SP and glutamate levels did not show any variation throughout day. Interestingly, glutamate levels in stimulated whole saliva showed association to circulatory levels in plasma.

When searching for the best saliva collection methods to study proteins and peptides related to pain, there are strengths and limitations with all sampling methods that need to be properly addressed before choosing a method. Parotid saliva has the advantage of being highly viscous and pure, which makes it easy to process during analysis. The collection, however, is time-consuming and requires special devices [108] and blockage from the other unrequired glands. Because of the slow flow-rate in the unstimulated state, it was not possible to acquire enough material for the analysis in Study II. Therefore, parotid saliva is not suitable when larger sample volumes are needed and not practical in large cross-sectional or clinical studies involving individuals with impaired health states.

The two glands on the floor of the mouth (i.e., submandibular and sublingual glands) are anatomically closely situated and can sometimes share the same ducts [78]. Therefore, it is difficult to separate the saliva from these glands safely and without accidently traumatizing the duct and cause bleeding. This is why saliva was collected from both glands simultaneously in the methodological studies even though they show different protein expressions [78]. Furthermore, the use of absorbent materials to collect saliva is common in saliva research but

could be problematic when the expected volume of saliva is small and can negatively affect the validity of the assays when the saliva is filtered through a cotton pad [17]. Contrary to glandular saliva, whole saliva is easy to control and access and express high levels of proteins and pain-related markers. The collection of unstimulated whole saliva by simply allowing saliva to accumulate in the mouth and then allowing it to dribble out through a funnel or straw into a tube is subject to considerable variation. For example, to varying extents the participants may try to speed up the collection by moving their tongue and cheeks or by mental exertion. These factors could partly explain the large variability in saliva secretion and protein concentration (CV 62 %). Compared to unstimulated saliva, whole stimulated saliva is also subject to some concerns because of the higher possibility of contamination by debris and bacteria in the oral cavity while chewing and spitting. In our studies, all participants were instructed to not eat/drink prior to collection and all exhibited good oral health, which diminishes the risk of contamination. Furthermore, all samples were collected in chilled tubes, treated with a protease inhibitor, and immediately centrifuged to reduce the risk for bacterial overgrowth and protein degradation. Whole stimulated saliva had the highest volume obtained, least variability in protein expression (CV 35%), low levels of glycoproteins, and high levels in all analytes except BDNF and SP.

A strength of the study methodology was that saliva samples were collected in the same structured manner, at the same time, and with the same surroundings conditions. To reduce the effect of external factors on salivary flow and protein expression, the inclusion criteria for the participants enrolled in the studies were very rigid. The health of the participants was ensured via their anamnesis and careful oral and dental examination. According to some findings, age may affect protein expression and saliva quality [28; 56; 127], but the participants were within a limited age interval to reduce the influence of such a confounding factor.

However, some limitations need to be considered. One study has shown the possible influence of sex hormones on BDNF secretion [148]. However, since it is not clear how the menstrual cycle affects the saliva compositions, we did not control for the phase of the menstrual cycle. Another limitation is that the strict criteria in combination with the limited age interval reduced the external validity of the findings.

Based on these results, it can be concluded that there are great variations between different collection methods both on the protein profile and specific pain-related peptides. It could be suggested that stimulated whole saliva may be a preferable collection method based on its simplicity, low variability, and significantly higher expression of several biomarkers. The samples should be collected at the same time of day to minimize the influence of the daily variation. The methodological studies stress the importance of consistency in the saliva collection approach rather than the method or collection time itself.

CLINICAL STUDIES

Saliva contains a complex mixture of proteins, peptides, and other substance that may yield information about the pathophysiology behind TMD myalgia and be used to identify new disease-specific biomarkers. The clinical section of the thesis aimed to evaluate specific pain-related mediators as well as the total protein profile in saliva of TMD myalgia to identify potential diagnostic or prognostic salivary biomarkers and to investigate the relationship between these proteins and clinical features. The main findings were significantly altered levels of salivary NGF, BDNF, and glutamate as well as altered levels of 20 proteins involved in metabolic processes, responses to stress, and immune response in TMD myalgia. Patients showed elevated plasma levels of BDNF and glutamate. However, there were no correlations between any of these proteins and pain measures.

Previous studies of conditions associated with chronic pain have suggested the involvement of serotonin, glutamate, NGF, SP, and BDNF [9; 54; 88; 101; 134; 146; 170]. Increased local and circulatory levels of NGF have been reported in chronic migraine [89], burning mouth syndrome [11], interstitial cystitis [95], and rheumatoid arthritis [156; 164]. Only two studies have explored salivary NGF in chronic pain. Both showed higher levels of salivary and circulatory NGF in chronic pain, a finding that contrasts with our study [11; 89]. However, the lower levels of salivary NGF in TMD myalgia and comparable plasma levels in both of our groups are in line with a recently published article on fibromyalgia [88]. Reduced levels of NGF have previously been reported in patients with depressive disorders [22], and a possible explanation for the lower levels of NGF in TMD myalgia may be related to the psychological maladjustments of these patients [88]. In addition, patients with TMD in general exhibit greater psychological distress compared to pain-free individuals [42; 52; 91; 172]. This well-established finding is also confirmed in our study cohort where patients reported significantly higher levels of depressive and somatic symptoms, anxiety, stress, pain catastrophizing, and sleep disturbances compared to pain-free controls. In addition, we found a moderate correlation between salivary NGF and somatic symptoms after adjustment for multiple comparison. This finding may indicate that the altered salivary NGF levels in patients are not caused by the pain itself but reflect a psychological maladjustment. Similar to salivary NGF, salivary BDNF was significantly lower in TMD myalgia. There was also an inverse correlation between salivary BDNF and perceived stress, anxiety, and somatic symptoms in the controls. One may speculate that salivary BDNF, like salivary NGF, may reflect psychological maladjustment. However, further studies are needed to confirm such an interpretation. Circulatory BDNF, on the other hand, was higher in patients compared to controls. This is in line with several studies of different chronic pain conditions [9; 54; 88; 170]. Some studies have found decreased BDNF levels in individuals with depressive symptoms or insomnia [18; 64]. The patients in Study IV reported a higher degree of insomnia and depressive symptoms compared to the controls. However, we found no relationship between circulatory BDNF levels and psychological factors. Therefore, the increase of circulatory BDNF might be interpreted as a general reaction to nociception [88].

Salivary SP decreased in chronic pain patients compared to pain-free controls [146] and similar findings were also observed for burning mouth syndrome [11]; however, studies on salivary levels of SP in chronic pain are inconsistent and show diverse findings. Jang et al. reported increased plasma and salivary SP levels in migraine and observed an increase in SP with higher pain intensity [89]. On the contrary, a recent study by Källman et al. reported no differences in SP levels in chronic neuropathic pain compared to pain-free controls [98]. Our findings support the latter study [98]. The plasma levels of SP in Study IV were in line with previous reported concentrations [90; 98], whereas saliva showed lower concentrations of SP compared to earlier studies [89; 146]. These differences are most probably due to different collection approaches, since salivary SP concentrations seem to decrease with increased flow rate [89]. Therefore, the low levels of SP in whole stimulated saliva are most probably due to the collection approach as this saliva contains 20% of the levels detected in resting whole saliva [90]. This collection approach may also have affected the traceability in our saliva samples, resulting in measurable levels in only half of the samples.

Salivary serotonin was excluded because it was difficult to detect in sufficient amount. As serotonin occurs in platelet-rich plasma to a higher extent than saliva, measurable levels were detected in most of the plasma samples. The levels were higher in healthy controls compared to patients but within normal reference values [12; 32]. The non-significant differences in TMD myalgia and healthy controls agree with earlier studies in serum and plasma [32; 44].

Glutamate has previously been studied in the interstitial fluid of the masticatory muscles and in plasma of patients with TMD myalgia, but to date not in saliva [19; 31; 60; 117; 167]. Interstitial muscle levels of glutamate were shown to be elevated in TMD myalgia compared to pain-free individuals [19; 31], but there were no significant differences in circulatory levels [19]. Based on the close vicinity of the salivary glands to the masticatory muscles, one may hypothesize that glutamate and other local biomarkers may diffuse into the saliva and therefore saliva levels reflect muscle levels. The concentration of the biomarker may also increase with salivary flow due to the reduced modification in the ducts. The patients in Study IV displayed significantly higher levels of salivary as well as circulatory glutamate compared to healthy individuals, suggesting that elevated salivary glutamate may be an indicator of TMD myalgia. This finding is also in line with a recent study showing that patients with chronic migraine had significantly higher levels of salivary glutamate compared to episodic migraine and healthy controls [129].

By applying proteomic approaches to saliva samples, we were able to show alteration in several proteins associated with metabolic processes, stress, and immunity in TMD myalgia, and interestingly also in subclasses of TMD myalgia (myalgia and myofascial pain). The most important proteins for separation between patients and controls and further between subclasses of TMD myalgia were PGK1, GAPDH, CHI3L2, and SAA. PGK1 expression has previously been described in various malignancies [177] and high expression of PGK1 has been observed in synovial tissue and blood of patients with rheumatoid arthritis [195]. Over-expression of salivary PGK1 in TMD myalgia has to date never been described. Therefore, the significance

of the enzyme in myalgia remains unclear and needs further investigation. GAPDH, similar to PGK1, is also overexpressed in various malignancies and positively correlates with tumour progression [183; 188]. Glycolytic enzymes such as PGK1 and GAPDH are usually found in the cytoplasm of the cell and released in the general circulation during pathological states that are associated with cell damage or apoptosis [183]. In this context, it may be assumed that conditions of oxidative stress, which lately has been discussed in relation to myalgia, may increase the need of PGK1 and GAPDH and lead to the observed elevated levels in saliva [48; 159].

The levels of SAA were significantly altered in TMD myalgia compared to controls and further significantly increased in patients with myalgia compared to myofascial pain. The interest in this digestive and antimicrobial enzyme has gradually increased because of its role as a potential marker for sympatho-adrenal medullary activity [131; 132; 187]. Studies have shown that SAA levels increase in individuals during physical and psychological stress [21; 66; 132; 136; 190], so the enzyme has been suggested as a marker for psychological and physical stress [16; 131; 180]. The patients in our study sample reported significantly higher levels of perceived stress, but no significant correlation could be observed between SAA and perceived stress level. Therefore, the validity of SAA as a stress marker remains debatable. Nonetheless, orofacial pain can act as a potential stressor and affect psychological as well as physiological systems [91]. Recent studies have proposed SAA as a biomarker for an objective assessment of pain intensity [3; 169]. One study reported a significant decrease in SAA level after pain reduction in chronic back pain and similar findings were also found in odontogenic pain [3; 169]. On the other hand, several studies have failed to observe a significant correlation between ongoing pain and SAA levels [16; 55; 186].

Inflammatory proteins and peptides associated with myalgia have been debated [60] and some attempts have been made to study cytokines and other inflammatory markers in TMD myalgia [116]. The proteomic analysis revealed significant alterations in several proteins related to inflammation and immunity such as immunoglobulin j chain (JCHAIN), immunoglobulin kappa light chain (P0DOX7), and interleukin-1 receptor antagonist protein (IL-1RA). The JCHAIN is expressed by mucosal and glandular plasma cells and regulates polymer formation of immunoglobulin (Ig)A and IgM. P0DOX7 is also a subunit of an antibody. The secretary protein IL1RA was elevated in patients. This protein is a member of the IL-1 cytokine family and is secreted by various types of cells to inhibit the pro-inflammatory effect of IL1α and IL1β. It also modulates different IL-1-related immune and inflammatory responses [144]. Significantly altered expression of some more specific immunity-related proteins was also observed. Cysteine-rich secretory protein 3 (CRISP 3), for example, has recently been suggested as a potential biomarker for prostate cancer [63] and was found up-regulated in TMD myalgia, while the fatty-acid binding protein (FABP) was significantly lower in patients compared to controls. This protein family has recently been discussed as a novel marker for diagnosis of diseases associated with oxidative stress, such as heart diseases, renal failure, Sjögren´s Syndrome, and Alzheimer's disease [58; 140; 157; 168; 193]. FABPs are intra-cellular lipid-binding proteins that exhibit a variety of expressions depending on the specific

organ or cell type. To date, at least nine different isoforms have been identified. FABP1 has been suggested as a marker for renal failure, FABP3 for myocardial infarction, and FABP5 has lately been debated as a diagnostic marker for Sjögren´s Syndrome [140; 157; 168; 193]. FABP5 has also been shown to be highly expressed in nociceptive dorsal root ganglia neurons and FABP inhibitors exert analgesic properties on a peripheral and supraspinal level. These findings indicate that peripheral FABP inhibitors may be used therapeutically to reduce pain and inflammation [147]. Another interesting observation was the significantly lower level of S100-A8, also known as MRP8. S100A8 is not only increased locally in sites of inflammation in rheumatoid arthritis but also in the general circulation. Moreover, the concentration of the protein seems to be strongly associated with disease activity [153].

The clinical studies have some limitations that need to be considered. The studies were performed in adults between 18 and 45 years old representing the peak of TMD prevalence; other age groups were not considered because of the possibility of age variability [152]. Sub-categories of myofascial pain could not be properly addressed in Study V due to the limited number of patients in each subclass. Moreover, female participants represented a majority of the study population to reflect the distribution in the clinic where women to a higher extent seek care for TMD myalgia [85]. Consequently, sex differences in proteins and mediators could not be properly addressed in any of the clinical studies. The female sex hormones can alter pain levels and therefore another limitation is that the female participants were not screened for menstrual cycle phase. However, it is most likely the women included were in different phases of their menstrual cycle, which would remove such a confounding factor.

In these studies, there are also some strengths that should be raised. Patients and controls enrolled in the studies were matched and they were all properly examined by the author to exclude systemic or oral conditions that may affect the salivary composition and/or biomarker levels. The author was calibrated to a reference standard researcher according to the most recent DC/TMD criteria to ensure accurate diagnosis of TMD myalgia. Patients with pain conditions other than TMD were excluded from the study. In both studies, the diurnal variation of salivary proteins were considered and a majority of the samples were collected between 9 and 11 am. The saliva collection was also standardized and followed a specific protocol to decrease the inter- and intra-individual variability.

To conclude, the clinical studies displayed significant changes in salivary pain-specific mediators as well as proteins related to metabolic processes, stress, and immunity in TMD myalgia compared to healthy controls. Significant differences in some proteins and clinical parameters could be observed between subclasses of TMD myalgia, indicating that they display different aetiopathogenesis. Patients showed significantly higher levels of salivary and plasma glutamate as well as plasma BDNF compared to healthy pain-free individuals, but lower levels of salivary NGF and BDNF, which further correlated to psychological variables. Future larger studies are needed to validate these potential biomarkers in a subset of TMD patients and evaluate any potential clinical correlation between the candidate biomarkers and clinical features.

IMPLICATIONS AND FURTHER PROSPECT

Saliva has considerable potential to mirror local and systemic health conditions. Nearly 40% of all the proteins that have been suggested to be candidate biomarkers for different diseases can be found in saliva. These comparisons together with the growing advancement in technology have made the analysis of saliva more attractive in pain research, whereas other sampling techniques such as microdialysis, biopsies, and cerebrospinal fluid present complications [17; 60; 115]. However, saliva is not a homogenous and stable body fluid as it is constantly changing. For example, the composition of saliva is affected by sampling methodology, circadian rhythms, environment, age, gender, oral hygiene, physical activity, medications, psychological status, and general health [17; 33; 87; 112; 127; 151; 152]. This characteristic of saliva limits its ability to be used as a diagnostic medium is the inter- and intra-individual variability, which was also obvious in this thesis, making the comparison between studies and patients challenging. For saliva-based diagnostics and techniques to be useful, there is a need for proper evaluation and standardization. Most studies using saliva as a diagnostic or prognostic medium do not provide proper description of participant preparation, time of sampling, sampling procedure, or the subsequent management of the sample [4; 53]. Since many studies also apply different collection approaches and sometimes do not describe their methods adequately, it is difficult to compare findings across studies. To our knowledge, the methodological studies included in this thesis are the first to properly address these aspects by evaluating different saliva collection approaches in healthy individuals within a limited age interval.

Significant differences in the protein profile signature and the relative amount of specific pain-related molecules were observed between saliva collection approaches. These results support previous studies in saliva [34; 185; 192]; however, there are substantial differences. That is, we investigated up to six saliva collection methods and carefully considered factors known to affect saliva composition. In addition, using new sensitive methods, we observed for the first time several isoforms for NGF, BDNF, and CGRP and showed that plasma glutamate can be reflected by the concentration in simulated whole saliva. Another relevant observation that emerged from the thesis was the daily variation in salivary NGF and BDNF, a finding that resembled previous studies of plasma [8; 10; 23; 181]. Our findings have led us to conclude that independent of the method chosen several conditions should be standardized. The differences between saliva collection approaches for specific molecules as well as for the protein profile made it clear that the collection method is a key factor for successful detection of peptides and proteins. What kind of saliva collection protocol is recommended in pain research? As previously described, whole saliva is a mixture of the saliva from the major and minor salivary glands as well as other fluids present in the oral cavity [17]. Consequently, gland-specific saliva should be used for gland-specific pathologies, and whole saliva should be used for local and systemic diseases. A drawback against the collection of whole saliva is the contamination of exogenous compounds such as microbiota, nasal secretions, blood contamination, and food debris. However, a majority of saliva research studies used whole instead of glandular saliva [17]. The findings in this thesis suggest that the collection of gland-

specific saliva is somehow invasive, requires special device, time-consuming, and results in small volumes. As in other studies, we also witnessed greater variability and less protein spots in the gland-specific saliva [34; 69; 185]. Based on findings in this thesis and in the literature, whole saliva shows compelling advantages. However, the study of whole saliva can be done using different collection procedures. Collection of unstimulated whole saliva by passive drooling seems to be the most frequently used and is regarded by researchers as a gold standard [3; 4; 7; 17; 26; 62; 82; 103; 129; 162; 181]. However, in clinical practice, this method is subject to some concerns. It is difficult to maintain the individual steady for several minutes without affecting and stimulating the saliva secretion, which most probably is the reason for the great inter-individual variability. However, unstimulated whole saliva is preferable for substances such as SP, whose concentration and therefore detection is considerably affected by the flow rate. Given the simplicity of the method, high volume, and low variability in stimulation, this thesis used stimulated whole saliva to study proteins and peptides in chronic pain. To improve reproducibly, the sample should be collected in the morning two to three hours after awaking to reduce the influence of the circadian rhythm and the participants should rinse their mouth with water prior to collection.

The collection protocol as described was applied and stimulated whole saliva samples were collected in the morning from patients with a diagnosis of TMD myalgia to detect diagnostic-specific biomarkers. Saliva can potentially be used as a specimen for diagnosis in TMD because it can exchange substances with blood. A thin layer of epithelial cells separating the salivary ducts from the systemic circulation enables the transfer of substances from the saliva by means of active carriage, diffusion through cell membrane, or passive diffusion. Nevertheless, there are also disease-specific biomarkers that are only present in saliva but not in blood, including some biomarkers for oral cancer [30].

This first study of the saliva proteome in TMD myalgia reveals that proteins related to metabolism, stress, and immunity were altered in patients and supports some of the pathological aspects discussed in the literature [42; 50; 65; 75; 113]. The present technique combining 2DE analysis with LC-MS/MS allows for an explorative approach, not focusing on predetermined proteins, to understand the mechanisms and discover novel biomarkers. Using this technique, we observed significant differences between myogenic TMD diagnoses in biological as well as clinical parameters, suggesting distinctive pathological mechanisms. Patients diagnosed with TMD myofascial pain with referral expressed decreased levels in the glycolytic enzymes PGK-1 and GAPDH and the digestive enzyme SAA compared to patients with myalgia. These findings suggest that pathological mechanism associated to oxidative and psychological stress are more pronounced in TMD myalgia or may indicate a depletion in myofascial pain with referral following prolonged or excessive secretion as described for cortisol under conditions of chronic stress [74]. Interestingly, patients with myofascial pain with referral reported higher pain intensity, depressive and somatic symptoms, perceived stress, and co-morbid headache. In addition, these patients had significantly lower pressure pain threshold of the masseter muscle than patients with myalgia. This finding in combination with the presence of pain spreading in myofascial pain correspond to central sensitization, whereas

changes in the properties of neurons in the CNS leads to increased pain hypersensitivity [191]. Thus, patients with myofascial pain seem to have an altered nociceptive function that is unlike what patients with myalgia experience. The results from the thesis support that myofascial pain should be regarded as nociplastic pain condition and treated as such. The term nociplastic pain was recently introduced by the International Association for the Study of Pain Taxonomy to describe pain states that arises from altered nociception even though no clear evidence exists of tissue damage causing the activation of nociceptors or evidence for disease or lesion to the CNS triggering the chronic pain. Nociplastic, pain in contrast to nociceptive pain, usually responds better to centrally-targeted therapies [102; 184].

When investigating specific pain-related proteins in saliva, patients surprisingly showed lower levels of NGF and BDNF, a finding that also correlated to psychological dysfunction. These findings are supported by meta-analysis showing that circulatory NGF and BDNF are indicative biomarkers for depressive disorders [22; 100]. One may speculate that salivary NGF and BDNF mirror psychological maladjustment usually associated with TMD myalgia. Although patients with myofascial pain reported higher pain intensity and scored higher in psychological malfunction, there were no statistically significant differences between subclasses.

Glutamate was increased in patients, and these findings were in line with other studies showing that glutamate levels positively correlate with perceived pain sensitivity [19; 60; 158; 161; 167]. Increased glutamate levels contribute to the nociceptive process by lowering neural threshold and/or increasing the pain response creating central sensitization [42; 161]. This could explain the increased salivary and plasma levels in TMD myalgia compared to pain-free controls. Elevated glutamate levels have also been reported in other pain conditions such as migraine, headache, and chronic widespread pain; consequently, glutamate is suggested as a potential biomarker for pain [60; 61; 129; 155; 161]. However, our studies are the first to support stimulated whole saliva as an alternative diagnostic medium to measure glutamate and to show that glutamate in saliva, as with interstitial fluid, is increased in TMD myalgia [19; 42; 60].

The greatest challenges for saliva diagnostics it to positively translating the identified biomarkers and results from the laboratory bench to clinical practice. Candidate biomarkers need to be verified and validated on a larger study group with appropriate clinical classification system. Proteome analysis provided significant insight, but 2DE shows only a certain part of the proteome. Therefore, there is a need for complement analysis of proteins and peptides not detectable by this technique. Furthermore, there is a need to validate potential biomarkers and their clinical value in larger patient cohorts. We may be able to combine these potential biomarkers with other clinical features to better understand and diagnose TMD myalgia as well as subclasses and evaluate therapeutic outcomes.

POPULÄRVETENSKAPLIG SAMMANFATTNING

En stor del av den vuxna befolkningen i världen lever idag med långvarig muskelsmärta, hos drygt varannan person är smärtan lokaliserad till ansiktet och käkar. Detta innebär att drygt 10-15% av den vuxna befolkningen är drabbad. Att leva med långvarig käkmuskelsmärta påverkar många aspekter av livet och har därför stor betydelse för livskvalitén hos den drabbade individen, men även för de närstående. Symptomen är vanligtvis lokal smärta, trötthet och ömhet i käkmusklerna ofta i kombination med nedsatt käkfunktion och huvudvärk. Förutom det individuella lidandet är långvarig käkmuskelsmärta ett stort problem för samhället med ökad sjukfrånvaro och sjukvårdskostnader som följd.

Individer som lider av långvarig käkmuskelsmärta har ofta träffat många olika vårdgivare. De har vanligtvis genomgått ett flertal olika undersökningar inom hälso- och sjukvården, och prövat en mängd olika behandlingar, men ofta utan framgång. Långvarig käkmuskelsmärta anses idag av många kliniker som en diagnostisk utmaning som kräver stora vårdresurser och i flera fall specialistkompetens. Processen är ofta lång innan den drabbade individen erhåller korrekt diagnos och behandling, och resan från de första symptomen till adekvat omhändertagande tar ofta många år, vilket tyvärr ökar risken för en smärtöverkänslighet i nervsystemet s.k. central sensitisering. Den långa processen är ofta även förknippad med psykisk ohälsa.

De bakomliggande sjukdomsmekanismerna vid långvarig käkmuskelsmärta är till stor del okända, varför diagnostiken bygger på uppgifter från patientens sjukdomshistoria och den kliniska undersökningen. Liksom för andra långvariga smärttillstånd är forskarna dock överens om att de innefattar flera faktorer som tillsammans bidrar till, underhåller och förvärrar tillståndet. Psykisk ohälsa i kombination med stressrelaterad tandgnissling och tandpressning är viktiga bidragande faktorer. Dessutom har biologiska och genetiska faktorer uppmärksammas på senare tid som andra viktiga faktorer. Nya forskningsrön har visat att flera substanser som har samband med smärta och inflammation, till exempel signalsubstanserna glutamat, serotonin och nervtillväxtfaktor, är förhöja i den smärtande käkmuskeln, vilket antyder att de kan bidra till sjukdomsutvecklingen. I olika studier har dessa substanser analyserats i muskel, blod och ryggmärgsvätska; mätmetoder som ofta är förenade med smärta och obehag. Munhålans saliv, som är en mycket informationsrik kroppsvätska, förbises ofta som ett prov av diagnostiskt värde, troligen på grund av avsaknad av standardiserade rutiner för provtagning. Saliven är en komplex vätska som främst består av sekret från tre stora parade körtlar och ett hundratal små körtlar samt vätska från tandköttsfickan och annat sekret. Saliven är ett filtrat av blodplasma och många substanser når därför salivkörtlarna från blodbanan. Analyser har visat att drygt en tredjedel av blodets proteiner kan återfinnas i saliven. Därför finns det goda skäl för att använda saliv som ett diagnostiskt redskap i forskningen kring långvarig käkmuskelsmärta.

En metod som kan användas för att urskilja markörer för ett tillstånd är att studera alla de proteiner som finns i ett prov, t.ex. ett salivprov, från en sjuk patient och jämföra det mot en frisk kontrollperson. Proteomik, det vill säga läran om vilka proteiner som finns i kroppen, är

en mycket ung och växande vetenskap. Det så kallade proteomet, det vill säga de proteiner som uttrycks av en cell, kroppsvävnad eller organism vid en specifik tidpunkt, är en komplex funktion av de speciella förhållanden som cellen/vävnaden/organismen utsätts för. Proteinerna är inte bara av stor vikt för att upprätthålla en god hälsa utan förändringar i uttryck och koncentrationen av dessa kan även avslöja sjukdom. Ökad förståelse för vilka proteiner som uttrycks hos patienter med långvarig käkmuskelsmärta i jämförelse med friska individer kan ge oss värdefull kunskap om de biologiska mekanismerna bakom långvarig käkmuskelsmärta. Vi kan genom omfattande analys av hela proteomet även urskilja karaktäristiska för patienterna, och på detta sätt förbättra diagnostiken. Det kan på sikt leda till effektivare behandlingar som riktar sig direkt mot sjukdomsmekanismerna. Det övergripande målet med avhandlingen var att öka kunskapen avseende orsaksmekanismerna vid långvarig käkmuskelsmärta för att därigenom förbättra diagnostiken och ge ett mer rationellt omhändertagande. Men för att kunna använda saliven som diagnostiskt redskap behövs först omfattande arbete med att skapa standardiserade rutiner för salivprovtagning. Därför syftade avhandlingens första del till att studera skillnader mellan olika salivprovtagningsmetoder i avseende att hitta den bästa provtagningsmetoden för att studera proteiner och andra substanser hos individer med långvarig käkmuskelsmärta. Dessutom behöver man undersöka om det finns dygnsvariationer av dessa substanser i saliven och bestämma den optimala provtagningstiden.

I denna avhandling samlades först sex olika typer av saliv in från friska individer, både saliv från de olika salivkörtlarna och helsaliv, det vill säga den blandade saliven som finns i munhålan. För att studera sammansättningen samlades proverna in både i vila och genom att stimulera salivutsöndringen med citronsyra eller mekaniskt genom tuggning. Prover togs även upprepade gånger under dagen från ett antal friska individer för att följa dygnsvariationen.

Resultaten från analyserna visade att det finns mycket stora skillnader mellan de olika salivtyperna, både avseende hela proteomet och vissa specifika smärtrelaterade substanser. Tuggstimulerad helsaliv uppvisade bättre stabilitet och fler proteiner jämfört med de övriga salivtyperna. Man kunde även se en stor variation i mängden av de smärtrelaterade substanserna i de olika salivtyperna, och att vissa substanser ökade med högre salivflödet. Utsöndringen av signalsubstanserna nervtillväxtfaktor och neurotrofisk faktor visade sig vara högst på morgonen och mängden minskade sedan under dagen.

Från dessa experiment kunde man dra slutsatsen att det finns mycket stora skillnader mellan olika salivtyper. Det viktigaste lärdomen var att metoden spelade mindre roll, utan det viktigaste är att vara konsekvent och ta provet vid samma tidpunkt och på exakt samma sätt varje gång. Emellertid hade tuggstimulerad helsaliv flera fördelar, varför den metoden valdes för att i kliniska studier samla in saliv från ett fyrtiotal patienter med långvarig käkmuskelsmärta och lika många friska kontrollpersoner.

Resultaten visade att uttrycket av ett tjugotal proteiner skiljde sig åt mellan patienterna och de friska kontrollpersonerna. Elva av dessa är proteiner involverade i ämnesomsättningen, sex deltar i immunförsvaret och de resterande sju är främst involverade i stressreaktioner. Det visar att dessa system sannolikt är av stor betydelse för sjukdomsprocessen. Dessutom så obser-

verades skillnader i proteinuttryck hos patienterna beroende på om smärtan var lokaliserad enbart i käkmusklerna eller om den var spridd även till angränsande vävnader i ansiktet. De patienter som hade en lokal smärta hade högre nivåer av proteiner men rapporterade lägre smärtintensitet och psykisk ohälsa. Motsatsen kunde ses hos de patienter som hade en mer spridd smärta. Dessa skillnader antyder på att mekanismerna mellan dessa patientkategorier kan skilja sig åt och att det föreligger tecken på central sensitisering bland patienter med en spridd smärta.

Vidare analys av vissa specifika smärtrelaterade substanser visade att nivåerna av glutamat var betydligt högre hos patienterna jämfört med de friska individerna. Detta är intressant eftersom tidigare studier har visat förhöja muskelnivåer av glutamat hos patienter med långvarig käkmuskelsmärta. Denna studie är dock den första som visar att dessa skillnader även speglas i tuggstimulerad helsaliv. Signalsubstanserna nervtillväxtfaktor och neurotrofisk faktor var däremot lägre hos patienterna jämfört med de friska individerna och det fanns även ett samband mellan nivåerna av dessa substanser och psykisk ohälsa.

Avslutningsvis antyder resultaten från denna avhandling att det finns stor potential för saliv som ett diagnostiskt hjälpmedel för att studera mekanismerna bakom långvarig käkmuskelsmärta. Potentiella markörer behöver utredas ytterligare och verifieras i en större patientgrupp för att i framtiden kunna användas i kliniken. Dessa markörer kan förhoppningsvis i framtiden kopplas samman med kliniska fynd för att bättre förstå, diagnostisera och behandla långvarig käkmuskelsmärta.

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