# Chemical Biomarkers of Acute Pain in Human Saliva

Thesis

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# ABSTRACT

Pain is an unpleasant sensation that everybody experiences differently, and is affected by a multitude of biopsychosocial factors. In the clinical setting, ineffective pain management and prolonged pain can be both physically and psychologically debilitating. Current pain assessment methods rely on self-reporting, dismissing patients that are unable to provide their input, such as patients with cognitive disabilities or those who are unresponsive. Therefore, a reliable, objective, and non-invasive method of quantifying acute pain intensity is needed. This pilot study explored the relationship between the salivary concentrations of three pain-associated biomolecules (glutamate, substance P and cortisol) and the intensity of experimentally induced acute pain (through the cold pressor task) in twenty healthy volunteers. Due to time constraints, the samples of only ten participants were analysed for this thesis, whilst the remaining samples will be further analysed in the upcoming months to complete the investigation. Results from the study illustrate that there are changes in concentrations of one or more of the three biomolecules following experimental pain induction, but these concentration changes did not exhibit any particular trend, and did not return back to baseline after one hour post-pain. Moreover, participants reporting higher pain scores did not display larger changes in biomolecule concentrations, and vice versa. Statistically significantly lower salivary glutamate levels were observed one hour post-pain compared to baseline values, and significant differences in substance P and glutamate concentrations between certain pain scores, and substance P concentrations between certain age groups were also detected. Furthermore, gender differences in pain-related changes in concentrations of certain biomolecules were noted. Together, the results illustrate that there are changes in biomolecule concentrations with pain, and there are differences in certain biomolecule concentrations for particular variables, such as gender, age and pain score. Further investigation is required to uncover potential trends that may exist, to verify the findings of this current pilot study, and to evaluate the use of saliva as a diagnostic biofluid.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **ANOVA** | Analysis of Variance |
| **CPT** | Cold Pressor Task |
| **ELISA** | Enzyme-Linked Immunosorbent Assay |
| **HPA** | Hypothalamic-Pituitary-Adrenal |
| **MAD** | Median Absolute Deviation |
| **Min** | Minute(s) |
| **NK1** | Neurokinin 1 |
| **NRS** | Numerical Rating Scale |
| **Q-Q** | Quantile-Quantile |
| **SD** | Standard Deviation |
| **SP** | Substance P |
| **VAS** | Visual Analogue Scale |
| **VDS** | Verbal Descriptor Scale |

# 1. INTRODUCTION

Pain is a part of living. It is a personal experience that is not only a physical sensation, but also involves emotional and cognitive responses, making pain multidimensional (Fillingim et al., 2014). In one aspect, pain can be beneficial for our defence systems by enhancing motivation in the accumulation of resources, inhibiting other unpleasant experiences, and eliciting social support and empathy (Leknes and Bastian, 2014). However, generally, pain is an unpleasant experience that, with high intensity and duration, can hinder activities of daily living and reduce quality of life (Morgan, Conway and Currie, 2011). Pain can arise from a variety of causes and manifests in each individual differently depending upon a multitude of factors such as the context, amount of attention, and the individual's expectation of pain (Hansen and Streltzer, 2005).

In spite of the distinctive pain experience each individual undergoes, there is a strong desire to prevent or at least limit pain, particularly in the clinical setting, to enable appropriate pain management and pain rehabilitation approaches that will effectively improve the patient's recovery and increase independence. One element vital to determining the extent of the patient's recovery is the ability to accurately and reliably detect the amount of pain experienced. Presently, all pain assessment tools rely on self-reporting and come in the form of either a scale (predominantly used for acute pain) or a questionnaire (predominantly used for chronic pain) (Breivik et al., 2008). Alongside this, observations of physiological and behavioural indicators using various techniques, including more advanced methods like neuroimaging, are also usually employed to provide a more comprehensive pain profile (Cowen et al., 2015). Although these existing pain assessment methods are well-established and their sensitivity, validity and reliability have been verified in many studies (Breivik et al., 2008), these methods become inadequate when the patient is in an unresponsive or disabled state. In these circumstances, a different reliable way of quantifying pain is necessary.

A promising approach is to look at the biomolecular composition of biofluids such as saliva, and assess pain-related changes in concentrations of certain biomolecules. Saliva is a favourable option because it is easily obtained non-invasively and can be produced in sufficient volumes for testing. Glutamate and substance P (SP) are the primary neurotransmitters responsible for the transmission of pain information evoked by noxious stimulus[1] (D'Mello and Dickenson, 2008) and are found in saliva (Jasim et al., 2018), making them suitable non-invasive salivary biomarker candidates for pain. As well as pain, a slower pain-induced stress response is also usually evoked, producing elevated levels of cortisol, a major regulatory stress hormone (Li and Hu, 2019) that is typically measured in saliva (Kalman and Grahn, 2004). Therefore, salivary cortisol is an additional candidate that may be helpful as a biomarker in acute pain.

Existing research has reported elevated levels of (interstitial) glutamate, (plasma and salivary) SP and (salivary) cortisol with pain in patients with myofascial temporomandibular disorders (Shimada et al., 2016), chronic migraine (Jang et al., 2010) and osteoarthritis (Carlesso, Sturgeon and Zautra, 2016), respectively. Additionally,

[1] Noxious stimulus - harmful, poisonous, or very unpleasant stimulus that can or do cause tissue damage (Treede, 2018)

there is evidence to suggest that serum and salivary levels of these three biomolecules are correlated (Jasim et al., 2018; Vanbruggen et al., 2011). Due to its novelty, the literature regarding pain-associated biomarkers in saliva remains limited. To the best of our knowledge, no study has directly investigated the connection between experimentally induced acute pain and the salivary concentrations of these three biomolecules collectively.

The main aim of this thesis is to carry out an exploratory study on the relationship between salivary concentrations of glutamate, SP and cortisol, and the intensity of experimentally induced acute pain on healthy humans. We hypothesise that (i) the concentration of at least one or more of the three biomolecules will transiently change because of pain and return back to baseline values after around one hour, and (ii) greater changes in concentrations will be seen in individuals reporting higher pain ratings.

# 2. SCOPE

To investigate changes in pain-associated biomarkers in the saliva after an episode of induced cold pain, serving as a proof-of-concept for the feasibility of using biomarkers in saliva to monitor acute pain intensity.

Here, only experimentally induced acute (short duration) nociceptive pain in healthy volunteers is considered. Neuropathic (damage to the nervous system) and chronic (long-lasting) pain are beyond the scope of this study.

The cold pressor task (CPT) was chosen as the pain modality because of the ease of set up, relatively short delay between nociceptive stimulation and perception, and participants having full control over when to start and terminate the nociceptive stimulus.

Only healthy participants with no acute or chronic pain at the time of study were asked to participate. This enables a more direct observation of the correlation between the pain induction and change in biomolecule concentration in saliva, avoiding any influences from additional factors such as central nervous system sensitisation, health state or medication.

Three biomolecules (glutamate, SP, and cortisol) are considered in this thesis even though there are several other biomolecules produced from the signalling cascades in the pain pathway. The number of biomolecules under analysis is constrained by whether they are known to appear in saliva, the availability of assays for that particular biomolecules, as well as the cost of assay kits.

Blood samples were not included as this would add venepuncture pain and potentially stress, which may affect the results and could reduce the number of volunteers. Moreover, the hiring of a qualified person to take blood samples would be required, and this was beyond the available resources for this MSc project.

# 3. LITERATURE REVIEW

## 3.1 The Nature of Pain

Pain is a universal, yet personal, human experience. It is a complex phenomenon that can arise from different causes, and varies in location, strength and unpleasantness (McGrath, 1994). At the most basic level, the body detects potential or actual damaging stimuli and elicits a reflex withdrawal (nociception) through receptors called nociceptors (Sherrington, 1903). However, the degree of activation of these nociceptors do not directly translate to the degree of pain experienced by the individual. Nociception and pain are considered to be two distinct processes, as pain is an experience and nociception is the physical mechanism that produces that experience. Although pain from injury cannot occur without nociception (Sneddon, 2018), the manifestation of, perception of, and reaction to pain in an individual is further influenced by several elements including physical, genetic, psychological and social factors (McGrath, 1994). Accordingly, pain is a subjective sensation that is unique with each recurrence within and between individuals. It is undeniable that pain, regardless of the type, is generally an unpleasant experience, making it desirable to avoid or limit that pain, especially in the clinical setting. Hence, objective pain measurement is important for effective personalised pain therapy.

## 3.2 Current Pain Assessment Methods

Despite the well-established knowledge on the nociceptive pathway that encompasses nociceptive transduction, transmission, modulation and perception (**Figure 1**), no method exists to objectively quantify acute pain intensity. To date, the most conventional tools for assessing the intensity of acute pain are the visual analogue scale (VAS), numerical rating scale (NRS), and the verbal descriptor scale (VDS) (Haefeli and Elfering, 2005) (**Figure 2**). All of these assessments come in the form of a one-dimensional self-reported scale. They have been demonstrated to be valid, sensitive, reliable, and simple indicators of pain intensity in both the research and clinical domain (McDowell, 2006; Jensen et al., 2001), most notably the NRS (Breivik et al., 2000).

However, the self-assessment aspect of those scales is a significant limiting factor affecting effective administration and accuracy of pain measurement in some clinical circumstances, for example to determine the dosage of pain killers during anaesthesia, and for individuals with impaired consciousness and/or cognitive problems (Bendinger and Plunkett, 2016). Self-reporting also dismisses patients who are unable to provide their input, such as babies and young children (Breivik et al., 2008). Under these circumstances, facial expression, and other physiological or behavioural indicators are normally used to assess acute pain intensity (Puntillo et al., 1997). These are also subjective measures and suffer from poor reliability.

Therefore, methods to objectively and accurately measure acute pain are needed, as it has been reported that inadequate pain assessment and management in critical care is linked to increased morbidity and mortality (Shannon and Bucknall, 2003). Reliable pain assessment enables more effective management of a patient's pain, which will improve their quality of life.

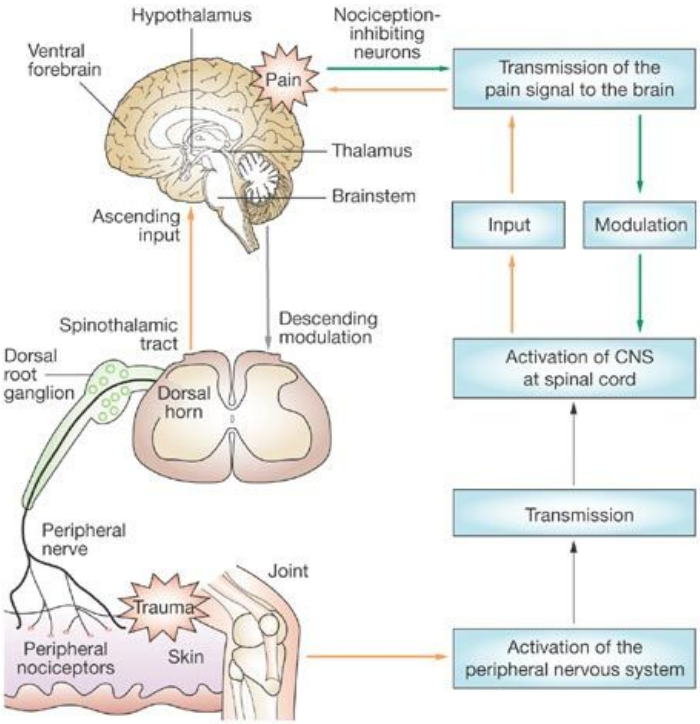


**Figure 1 – The nociceptive pain pathway** (Bingham et al., 2009).

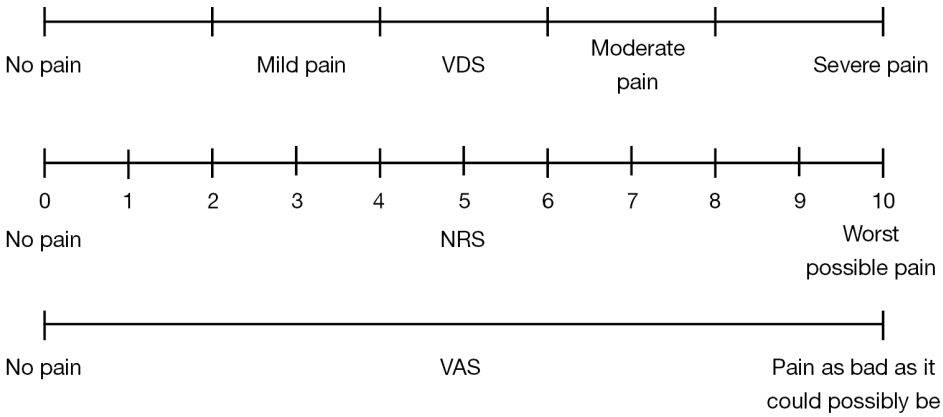


**Figure 2 – Scales for Assessing Pain**. Verbal Descriptor Scale (VDS), Numeric Rating Scale (NRS) and Visual Analogue Scale (VAS) (Woo et al., 2015).

## 3.3 Potential Biomarkers of Pain: Glutamate, Substance P and Cortisol

One feasible technique for quantifying acute pain intensity is to assess changes in concentrations of certain biomolecules associated with the nociceptive pathway, such as neurotransmitters – small molecules used by the nervous system, responsible for transmitting information between neurons at chemical synapses (González-Espinosa and Guzmán-Mejía, 2014). A wide variety of neurotransmitters play a key role in the sensation and transmission of pain from the periphery to the central nervous system (Yam et al., 2018). These neurotransmitters bind to nociceptors and trigger their respective second messenger cascades, eventually eliciting pharmacological effects on pain regulation (Steeds, 2009). Of all the pain-associated biomolecules, glutamate and

substance P (SP) are the principal neurotransmitters related to nociceptive pain sensation (Dubin and Patapoutian, 2010).

Aside from being the most ubiquitous excitatory neurotransmitter distributed throughout practically all circuits within the vertebrate nervous system that is essential for normal brain function (Purves and Williams, 2001), glutamate has additionally been found to play a significant role in the perception of nociceptive pain (Bleakman, Alt and Nisenbaum, 2006). This is due to the fact that glutamate and its receptors are located in the periphery, spinal cord and brain regions involved in pain sensation and transmission (Johnson, 1978; Miller et al., 1988), and glutamate is released upon nociceptive stimulation and nerve or tissue injury (Amaya et al., 2013). In particular, glutamate is the predominant excitatory neurotransmitter utilised by primary afferent fibres to convey nociceptive information from the periphery to the dorsal horn of the spinal cord where this information is integrated and regulated before being sent to higher regions of the brain for cognitive processing and perception (D'Mello and Dickenson, 2008). Experiments using animal models have showed that application of glutamate, or specific glutamate receptor agonists, at the periphery or spinal cord generate nociceptive behaviours (Wang et al., 2010; Calton, Hargett and Coggeshall, 1995). Similarly, electrical stimulation of the primary afferent fibres stimulate glutamate release in the spinal cord (Kangrga and Randic', 1991). In contrast, inhibition of glutamate release or blockage of glutamate receptor activation in the periphery or spinal cord of animal models attenuates acute and chronic pain (Brown and Krupp, 2006; Coderre et al., 2006; Montana and Gereau, 2011). Currently, there are several glutamate receptor-specific agonists that are potential therapeutic options for treating neuropathic pain, but further evaluation of their treatment efficacy is required (Aiyer et al., 2017).

Co-existing with glutamate in a number of primary afferent terminals is SP (De Biasi and Rustioni, 1998), which is a molecule composed of 11 amino acid residues, and is a member of the tachykinin neuropeptide family (Van Der Kleij and Bienenstock, 2007). SP is associated with a large range of processes including wound healing, inflammation and cell survival (Garcia-Recio and Gascón, 2019). Its co-existence with glutamate implicates the additional role of SP as another primary neurotransmitter in pain sensation and perception. This notion is further supported by the evidence that SP and its receptor, neurokinin 1 (NK1) receptor, is also co-expressed in dorsal horn neurons where here, SP enhances glutamate-mediated excitatory neurotransmission following afferent fibre stimulation (Womack, MacDermott and Jessell, 1988; Randić, Hećimović and Ryu, 1990). Given the expression patterns and anatomical locations of SP and its receptors, SP is a neuropeptide that acts as both a neurotransmitter and a neuromodulator (Schwarz and Ackenheil, 2002). Animal models have shown that SP (NK1) receptor antagonists successfully block peripheral inflammatory responses and inflammatory pain (Henrgy, 1993). Regarding the use of SP (NK1) receptor antagonists as a therapeutic approach to alleviate pain, their observed analgesic efficacy in preclinical studies did not persist when tested in the clinical phase, for reasons unknown (Boyce and Hill, 2004).

As pain itself is a potential stressor, a pain-induced stress response may also be elicited in situations where the perception of pain is magnified to the point of being seen as catastrophic or threatening, consequently causing fear and avoidance behaviour of pain-provoking stimuli (Lumley et al., 2011; Crombez et al., 2012). These exaggerated psychosocial responses to pain are maladaptive in chronic pain states, worsening the

pain experience and perpetuate disability. In a typical stress response, the hypothalamic-pituitary-adrenal (HPA) axis is activated upon perceived stress via the amygdala and, after a series of biomolecular and hormonal interactions, leads to the release of cortisol as an end product (Ehlert, Gaab and Heinrichs, 2001). Cortisol is a vital hormone for the maintenance of blood glucose, suppression of non-vital organ systems (so that highly active systems such as the brain are provided with more energy), and for the body's natural anti-inflammatory response (Heim, Ehlert and Hellhammer, 2000). In addition to these important functions, cortisol is also a crucial component of the stress response as it enables the reallocation of necessary energy and substrates required to cope with stress-provoking stimuli or for evading danger (Blackburn-Munro and Blackburn-Munro, 2003). In the short term, stress-induced increase in cortisol is adaptive, however, sustained cortisol secretion may be both physically and psychologically debilitating (McEwen, 2008; Heim, Ehlert and Hellhammer, 2000).

Stress and pain are highly interconnected as they both share significant conceptual and physiological overlaps (Abdallah and Geha, 2017), indicating a close relationship between glutamate, cortisol and SP. As well as being a pain-associated neurotransmitter, SP serves a proinflammatory role (Suvas, 2017) and studies have suggested SP and other members of the tachykinin family as regulators of emotional processing and biopsychosocial stress responses (Ebner, Sartori and Singewald, 2009; Felipe et al., 1998). Furthermore, stress induces the release of glucocorticoids, which rapidly promote glutamate secretion, affecting glutamate neurotransmission in multiple brain regions including the hippocampus, amygdala, and prefrontal cortex (Venero and Borrell, 1999; Groeneweg et al., 2011), implying that stress has the ability to affect cognitive processes. Collectively, all three biomolecules extensively influence the presence of one another, working in combination to regulate the physiological response, and emotional and cognitive processing of stress and pain. Given their respective characteristics and interactions, glutamate, cortisol and SP are therefore attractive biomarker candidates for pain that could be used in combination to give a more complete pain profile.

## 3.4 Existing Research on Pain Biomarkers

### 3.4.1 Normal Biomolecule Concentrations in Blood and Saliva

Using microdialysis[2], animal models have shown that glutamate levels in the brain range from 0.2 to approximately 20 μM (Dash et al., 2009; De Bundel et al., 2011). In healthy adults, blood glutamate levels are maintained in a steady state from around 40 to 60 μM (Bai et al., 2017). From the studies available, normal salivary glutamate baseline concentrations seem to be lower than those in blood, being about 10 to 30 μmol/L (Shimada et al., 2016; Nam et al., 2017).

A review of the literature shows considerable variability in the reported control serum or plasma-derived SP levels that ranges from 12.25 to 397 pg/mL in humans (Campbell et al., 2006), with each research paper reporting different methods of sample preparation and analysis. On the other hand, a much higher SP serum range of 402 to 1576 pg/mL was reported in an SP assay datasheet that looked at the serum of 22 healthy participants (R&D Systems, 2019). The same source also looked at salivary SP levels in 11 healthy participants and stated a concentration range starting from a non-detectable range to

[2] Microdialysis – a minimally-invasive sampling technique used for monitoring neurotransmitters and other molecules in the extracellular environment (Shippenberg and Thompson, 1997)

1614 pg/mL. Here, the plasma and saliva SP levels seem to be relatively similar. In contrast, one study found lower SP levels in the saliva compared to plasma (Jasim et al., 2018) with salivary levels being around 0 to 600 pg/mL. Yet, several studies have claimed that SP concentrations appear to be considerably higher in the saliva than in plasma (Fischer, Eich and Russell, 1998; Jang et al., 2011; Takeyama et al., 1990). Hence, there does not seem to be a common, consensus range of SP levels in blood and saliva.

The normal, non-stressful secretion of cortisol in adults follow a circadian rhythm, (**Figure 3**). This means that there is a distinct, sharp rise in cortisol at the time of awakening as seen from the figure, followed by a sharp decline by mid-morning, and then a more gradual decrease across the afternoon and evening (Kirschbaum and Hellhammer, 2000). Salivary cortisol levels are significantly lower than plasma levels (Ljubijankić et al., 2008; Jung et al., 2014). According to the referential cortisol values determined in the Laboratory of Clinical Biochemistry at the Institute for Clinical Biochemistry and Chemistry at the University of Sarajevo, serum concentrations in the morning (8 – 9 a.m.) and afternoon (4 – 5 p.m.) are 123.0 to 626.0 nmol/L and 46.2 to 389.0 nmol/L respectively, whilst saliva concentrations are 3.5 to 27.0 nmol/L in the morning and 1.3 to 6.0 nmol/L in the afternoon (Ljubijankić et al., 2008; Jung et al., 2014).

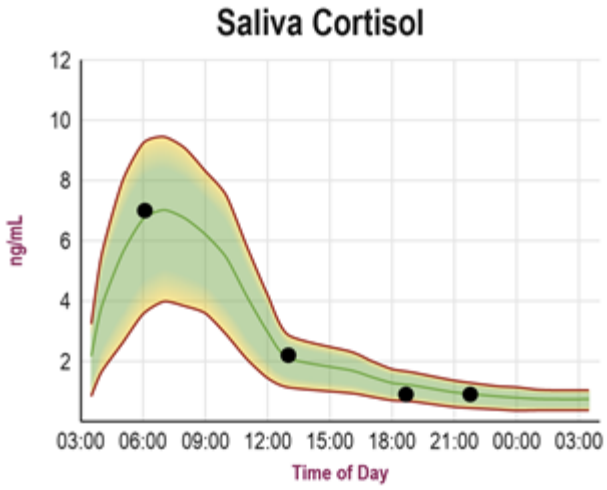


**Figure 3 – Diurnal Cortisol Curve in Saliva** (ZRT Laboratory, 2019).

### 3.4.2 Pain-related Changes in Biomarker Concentrations

A substantial body of evidence has indicated that cortisol concentrations increase with pain. One study looking at the circadian rhythms of plasma cortisol in patients suffering from migraines reported consistently high plasma cortisol levels in 14 of the 25 patients throughout the day, and an overall higher mean plasma cortisol value in patients than controls (Ziegler et al., 1979). Moreover, it was noted that within the migraine patients, the average plasma cortisol levels during the time periods of reported severe pain was higher than periods of no pain. In a separate study of 40 adult patients with nonmalignant, painful conditions, 26 patients demonstrated abnormal serum cortisol concentrations with 12 having higher than normal concentrations, which were reduced to more normal levels following opioid treatment (Tennant and Hermann, 2002). Another investigation exploring the effect of stress on pain perception using 46 healthy

men found that the rating and threshold of pain, induced by electrical stimulation, was increased and decreased respectively in response to experimental stress, achieved by having participants perform a medical test (Choi, Chung and Lee, 2012). Alongside this, the researchers also reported salivary increase in cortisol and decreased testosterone levels. From this, it was suggested that acute clinical pain could potentially be relieved by controlling stress and managing stress-related cortisol and testosterone levels. Similarly, experimental pain induction of 64 male volunteers receiving either controllable (self-administered) or uncontrollable (experimenter-administered) painful electrical stimulation resulted in higher salivary cortisol, subjective helplessness and perceived pain intensity ratings for the uncontrollable conditions than those in the controllable condition (Müller, 2011).

Comparison of salivary cortisol levels between patients with chronic pain conditions such as fibromyalgia, rheumatoid arthritis, and healthy controls in their natural environment found higher than average cortisol levels in both fibromyalgia and rheumatoid arthritis patients (Paananen et al., 2015). Contrastingly, a different study looking at patients with fibromyalgia and controls did not find any significant differences in salivary cortisol between the two groups, but did report a strong relationship between the levels and current pain symptoms at the waking time point and one hour after waking (but not at the later three time points) (McLean et al., 2005). Significant increases in salivary cortisol from baseline values have also been reported in four-month old infants receiving three different immunisations either sequentially or simultaneously (two at once, followed by the third) (Hanson et al., 2010), preterm infants in response to heelstick (Herrington, Olomu and Geller, 2004), and patients with dental pain (Kanegane et al., 2009). A more atypical approach looking at cortisol contents of hair samples belonging to patients suffering from severe chronic pain, receiving opioid treatment for at least one year, revealed increased cortisol contents in relation to controls and higher perceived stress scale scores (Van Uum et al., 2008).

Alternatively, it has been theorised that a dysregulation, rather than just an increased activation, of the HPA axis may actually augment pain perception instead. A study looking at diurnal variations in cortisol and cold pain sensitivity in female twins found that lower diurnal variation of cortisol was associated with higher pain ratings (Godfrey et al., 2013). Another study found that experimentally induced hypocortisolism in healthy volunteers significantly decreased pain detection thresholds and amplified pain elicited by inter-digit web pinching (Kuehl et al., 2010).

Taken together, most existing evidence on cortisol seems to suggest a correlation between pain and elevated cortisol levels in both serum and saliva, but this requires further investigation as many do not consider the direct relationship between experimental acute pain induction and cortisol dynamics.

There are fewer studies that consider SP and pain. One study evaluating serum SP levels in healthy controls and sickle cell disease patients during baseline and acute pain reported higher patient SP baseline levels compared to controls, with even higher SP levels in sickle cell disease patients hospitalised for acute pain (Brandow et al., 2016). The same results were seen in a similar study by a different group of researchers (Douglas, 2008). Additionally, significantly higher salivary SP concentrations have been reported in patients with dental pain compared to healthy controls (Ahmad et al., 2014).

Patients with rheumatoid arthritis were also found to have raised serum SP levels, which were further elevated following critical acute pain in the form of an orthopedic surgery (Lisowska, Siewruk and Lisowski, 2016). These SP elevations correlated with increased pain rating. However, it should be noted that the authors did not find any correlation between SP concentrations in drainage fluid and the severity of postoperative pain, and a healthy control group was not used. Conflictingly, a more recent study observed no significant differences between salivary SP concentrations of chronic neuropathic pain patients and healthy controls (Kallman, Ghafouri and Bäckryd, 2018). The same study also found no correlation between salivary and plasma SP concentrations. The authors go on to conclude that salivary SP, along with beta-endorphin, are not biomarkers of neuropathic chronic pain propensity, and hypothesised that there may be a local production of SP in the salivary glands. It is clear that the feasibility of using SP as a pain-associated biomarker remains debatable, and the establishment of a more definite relationship among SP and pain is desirable.

In parallel, several sources report higher levels of glutamate with the experience of pain. Saliva samples of patients with temporomandibular disorders contained higher concentrations of glutamate than controls (Jasim et al., 2014). Likewise, elevated glutamate levels were seen, via proton magnetic resonance spectroscopy of the posterior insula in fibromyalgia patients, and these levels were negatively correlated with pressure pain thresholds (Harris et al., 2009). Moreover, increased glutamate levels have also been recorded in people who experience chronic migraine compared to those with episodic migraine and controls (Nam et al., 2017), and in tendon dialysate samples belonging to patients suffering from painful chronic Achilles tendinosis (Alfredson, Thorsen and Lorentzon, 1999). Use of time-resolved functional proton magnetic resonance spectroscopy to look at cortical neurotransmitter concentrations showed an overall increase in glutamate concentration with experimentally induced short, acute heat pain in healthy volunteers in relation to the non-stimulus condition (Gusset wet al., 2010). The same observations with glutamate can be seen in animal models. Rats treated with oxaliplatin, a chemotherapy medication that causes painful peripheral neuropathy, had higher baseline glutamate concentrations in the cerebrospinal fluid that were markedly increased with mechanical stimulation of the hind paw (Yamamoto et al., 2017). The literature, though small in volume, suggests that there are raised glutamate levels with pain induction and in painful conditions.

Considering everything as a whole, many gaps exist in the literature concerning pain and the three potential biomarkers, such as how fast the salivary levels of each biomolecule change as a result of pain. All of these needs to be addressed and further clarified.

## 3.5 Saliva as a Window into the Health State of the Body

More recently, saliva has been gaining an increasing amount of attention as a medium for gaining insight into the health state of the brain and central nervous system of the individual. This concept arose through the fact that saliva contains over 2000 proteins, where 27% of those are also found in blood (Loo et al., 2010). It is believed that these proteins enter the saliva through means of passive diffusion through the spaces between the cells (Jusko and Milsap, 1993). Hence, saliva can be viewed as functionally equivalent to serum in the context of reflecting the health status of the human body (Walton, 2018). Moreover, utilisation of saliva as a diagnostic tool is more advantageous than other methods due to its easy, non-invasive, cost-effective collection, and unlimited

reproducibility. As observed from the mentioned literature, some studies, particularly newer ones, have already employed the use of saliva to detect and analyse potential pain-associated biomarkers, though it remains that this method is relatively new and requires more exploration.

## 3.6 Aim, Hypotheses and Vision

Currently, there is no study to date that directly investigates experimentally induced acute cold pain in healthy volunteers and the change in concentrations of a *combination* of pain-associated biomolecules in any biofluid. The main aim of this pilot study is to fill this gap by exploring the relationship between the concentration of three biomolecules – glutamate, substance P and cortisol – in the saliva and the intensity of acute pain.

Our hypothesis is that: *salivary concentrations of one or more of the three biomolecules will change with the presence of acute pain, compared to baseline values, and revert back to baseline after a period of time, such as one hour*. If this initial hypothesis is true, it is further hypothesised that those reporting higher pain intensity ratings in the CPT will exhibit larger changes in biomolecule concentrations compared to those with lower pain intensity ratings.

The null hypothesis is that salivary concentrations of one or more of the three biomolecules will remain unaffected by the presence of pain.

This study hopes to pave the way to a better understanding of pain, potential biomarkers for quantifying acute pain intensity, and the feasibility of using saliva as a non-invasive diagnostic fluid. If positive outcomes are achieved, the long-term vision is to create a wearable system for non-invasive monitoring biomarkers of acute pain from saliva. Such a technology could provide quantifiable data on pain intensity in the clinical setting and ultimately allow personalised and automated acute pain therapy after surgery and trauma.

# 4. MATERIALS AND METHODS

## 4.1 Ethical Approval

This study was approved by the University College London Research Ethics Committee (identification number: 15021/001). The Declaration of Helsinki guidelines were followed. All participants in the study provided both written and verbal consent before taking part.

## 4.2 Participants

Participants were recruited via email advertisement (**Appendix A**) and by word-of-mouth with the inclusion and exclusion criteria displayed in **Table 1**. Those expressing their interest in taking part in the experiment were emailed the consent form and participant information sheet (**Appendix A**), and asked to confirm their participation. Once the participant agreed to take part in the experiment, a convenient date for the participant was chosen.

**Table 1 – Inclusion and exclusion criteria.**

| Inclusion | Exclusion |
|---|---|
| • Have good general health<br>• Aged between 18 to 70 years old<br>• Can maintain oral hygiene on the day of the experiment – refrain from consuming food and drinks (except water) 3 hours before the experiment, and brush your teeth after the meal, but no later than 1 hour before the experiment | Anybody with the following:<br>• Any current pain<br>• Diagnosed with systematic muscular joint diseases<br>• Neurological disorders<br>• Pregnant or lactating<br>• Diagnosed with high blood pressure<br>• Uses tobacco<br>• Take regular medication (includes contraceptives, antidepressants and analgesics)<br>• Poor oral condition (e.g. has dental or oral diseases) |

### 4.2.1 Participants – Experiment

20 healthy participants took part in the study, with equal numbers of males and females.

The mean (± standard deviation (SD)) and median participant age was 27.4 (± 6.8) and 24 years, respectively, with the age range of 21 – 40 years.

From a power calculation, (Kane, 2019) 20 participants were sufficient for detecting a statistically significant difference of at least 20% in biomarker level between samples, with 80% power at a significance level of 5%.

All participants received £10 as reasonable expenses for taking part in the study.

### 4.2.2 Participants – Data Analysis

Due to time constraints, the limited number of wells per microplate, and the cost of biomolecule detection kits, three to four saliva samples of only 10 participants were

analysed in this thesis. The remaining samples will be later analysed to complete the investigation, but will not be included here.

Saliva samples from 5 males and 5 females were chosen to be analysed. The mean (± SD) and median participant age was 28.3 (± 7.4) and 25 years, respectively, with the age range of 22 – 40 years.

## 4.3 Experimental Pain Induction

Methods for inducing pain can largely be grouped into the following categories: mechanical, thermal, electrical, chemical, and muscle stimulation (Reddy et al., 2012). Each approach has its own major shortcomings, such as generating non-specific activation of receptors, variability in individual responses, and the inability to control onset and offset time of pain (Reddy et al., 2012).

The cold pressor task (CPT) was used as the experimental pain modality in this preliminary study as it is a well-established experimental paradigm for experimental pain induction, and has been used in a wide range of studies such as those investigating stress, coronary artery disease and pain (Chang, Arendt-Nielsen and Chen, 2002; von Baeyer et al., 2005; Fulbright et al., 2001). CPT involves submersion of the hand in an ice bath, consequently inducing pain. The associated sympathetic response causes a concomitant increase in heart rate, arterial blood pressure, and other sympathetic physiological responses (Tassorelli et al., 1995). It is known to have high reliability and validity (Edens and Gil, 1995), and can effectively mimic the effects of pain due to its unpleasant sensation (Rainville et al., 1992).

In comparison to other pain modalities, the CPT is relatively easy to set up and does not require specialist equipment, unlike certain types of thermal, mechanical and electrical stimulation. Furthermore, the whole pain-induction process using CPT does not need to account for substantial delays between pain induction and pain perception, which is usually the case for chemical modalities involving injection or topical application of chemicals. Most importantly, using CPT, participants have full control over when to self-induce and terminate pain, ensuring greater comfort and well-being throughout the whole experimental process.

## 4.4 Experimental Design and Setup

The experiment was carried out at the Aspire Create Centre for Rehabilitation Engineering and Assistive Technologies (CREATe) in the Royal National Orthopaedic Hospital, Stanmore.

Three hours prior to coming into the laboratory, participants were requested not to eat or drink anything besides water to prevent a temporary increase in cortisol level (Stachowicz and Lebiedzińska, 2016). Additionally, participants were requested to brush their teeth after having their meal to get rid of any debris in the saliva, but requested to do this at least one hour before providing any samples as this may generate blood contamination in the saliva samples. As toothpaste was not standardised (participants used their own toothpaste) before going into the laboratory, the time window of at least one hour was also given to reduce any potential effects of the toothpaste on the salivary biochemical composition and allow both the composition and salivary flow rate to return back to baseline values (Ligtenberg et al., 2006).

All participants were asked to come into the laboratory between 2 to 3:30 pm on separate days, and each session lasted for a maximum duration of two hours. The experiments were run in the afternoon at the indicated timeframe to account for the cortisol diurnal variation and the fact that cortisol levels remain more stable at a concentration of ~0.1 µg/dL starting from approximately 2pm onwards, two hours after consuming lunch (**Figure 3**).

Acute pain was induced by having subjects submerge their forearm and hand into an ice bath with a temperature maintained between 0 - 5°C for a maximum of 5 minutes (or until the pain becomes unbearable during those five minutes) (Eren et al., 2018). Five minutes was chosen as the upper time limit to avoid any adversary effects (e.g. ice burn or cold stress). The ice bath was comprised of a container with ice, ice packs and cold water, whilst temperature was monitored using a thermometer.

## 4.5 Protocol

The protocol is summarised in **Figure 4**.

Succeeding the participant's arrival, obtainment of written consent, and experimental set up, participants were reminded of the procedure and had it emphasised to them that during the CPT they were allowed to remove their forearm and hand from the ice bath whenever the pain was unbearable, and five minutes was only a maximum limit. It was also stressed to them that the removal of their limb from the ice bath will mark the end of the CPT.

Submerge forearm and hand into ice bath

| **Time (min)** | -2 | -1 | **T = 0** | **T = x** | + 10 | +20 | +30 | +40 | +50 | +60 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Sample #** | S1 | S2 | | S3 | S4 | S5 | S6 | S7 | S8 | S9 |
| | | | | NRS | | | | | | |

**Figure 4 – Experimental Protocol.** Two baseline saliva samples were taken before any pain. Then at T = 0, when participants were ready, the participant's forearm and hand were submerged into the ice bath. At T = x, which is the maximum time of five minutes or when participants removed their limb from the ice bath within those five minutes, another saliva sample was taken and the pain was rated using the numerical rating scale (NRS) provided. Another saliva sample was then obtained every ten minutes afterwards for a total duration of one hour.

After ensuring they understood the instructions, participants were asked to provide two preliminary saliva samples. These two initial saliva samples served as the baseline biomarker values before the induction of pain, and allowed the participants to become familiar with the saliva collection procedure. Then, when participants were comfortable and ready, they were asked to submerge their forearm and hand into the ice bath. After

five minutes, or whenever the participant removed their limb from the ice bath within those five minutes, participants provided another saliva sample and rated the intensity of the pain experienced. Subsequently, a saliva sample was collected every ten minutes for one hour after the termination of the CPT. A total number of nine saliva samples were collected from each participant.

Participant's heart rate and blood pressure was periodically monitored every five minutes throughout the entire experiment to ensure their well-being and to observe the physiological changes induced by the CPT.

## 4.6 Saliva Collection

Saliva was collected in cryovials using Salimetrics Collection Aids® (Salimetrics LLC, State College, PA). During the saliva collection, participants were requested to keep their eyes open and not to speak.

In this study, whole saliva collected by passive drool was collected as it is considered to be a gold standard due to the fact that it eliminates any bias that can arise from factors such as the differentiated contributions of each salivary gland and reflex stimulation (Capelo-Martínez, 2019). As such, the saliva samples obtained using this method are seen to be the purest samples possible, and can furthermore be stored in a "biobank" for future research. Passive drool involves having the participant tilt their head forward whilst drooling down a collection aid attached to a cryovial.

A 1 mL of volume of saliva was collected per sample. In instances where the participant was unable to produce enough saliva before the next sampling time, they were instructed to provide at least 0.5 mL of saliva to ensure that there was enough saliva to test for all three biomolecules.

During the experiment, the collected saliva samples were kept on ice to prevent degradation of sensitive peptides. Immediately after the session, the samples were transported and frozen at -20°C until analysis.

## 4.7 Rating Pain Intensity

Participants rated the maximum pain intensity experienced during the CPT using the NRS provided (**Figure 5**). The maximum pain intensity was requested to determine whether or not the maximum pain experienced correlated with the changes in biomolecule concentrations.

The NRS comprised of a line segmented into ten parts each labelled with the number 0 to 10 in increasing order, as well as a short description above the numbers 0, 5, and 10 indicating "no pain", "moderate pain", and "worst pain" respectively. Participants were to indicate the pain intensity on the numerical scale. Faces expressing a gradual transition from an extremely happy face to crying face at every even number was also included and displayed underneath the numerical scale to help further illustrate the level of unpleasantness the numbers on the scale corresponded to.

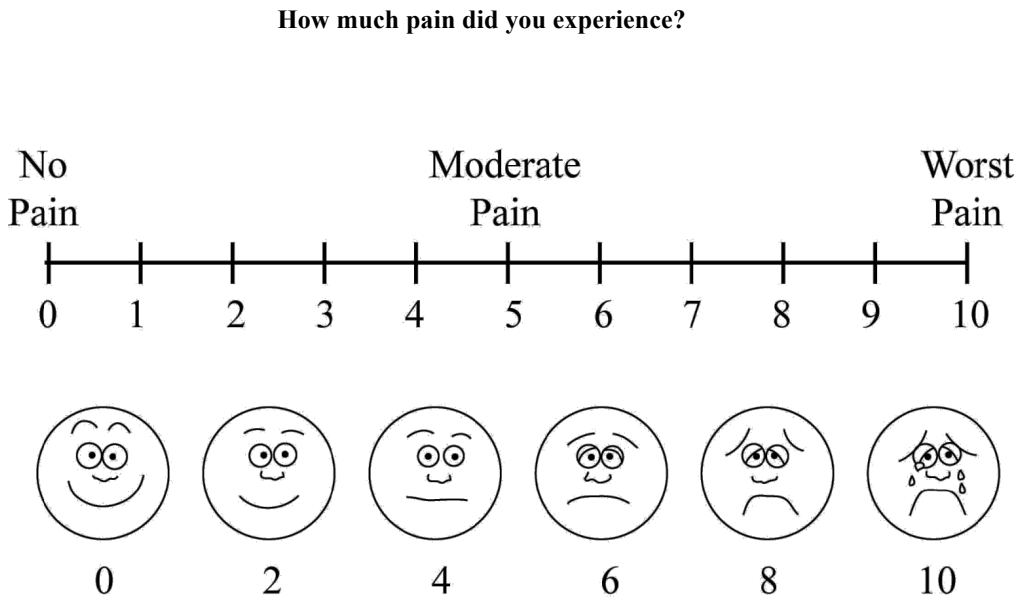


**Figure 5 – Numerical rating scale (NRS).** The NRS provided to the participants to rate the maximum pain experienced from the cold pressor task.

## 4.8 Sample Analysis: Enzyme-Linked Immunosorbent Assay

Glutamate, SP and Cortisol were quantified using enzyme-linked immunosorbent assay (ELISA) kits purchased from Abcam (ab83389, UK) and Stratech (CSB-E08357H-CSB, UK; 1-3002-SAL, UK), respectively. The minimum and maximum value, sensitivity, and maximum percentage error amongst standard duplicates for each ELISA kits is displayed in **Table 2**. Preparation and analysis of saliva samples were carried out in accordance to the manufacturer's recommendations.

**Table 2 – Minimum, maximum, sensitivity and maximum percentage error amongst standard duplicates of the glutamate, substance P and cortisol enzyme-linked immunosorbent assay (ELISA) kit used**

| | Minimum | Maximum | Sensitivity | Maximum Percentage Error Amongst Standard Duplicates (%) |
|---|---|---|---|---|
| **Glutamate** (μM) | NA | 100 | NA | 41.8 |
| **Substance P** (pg/mL) | 6.25 | 400 | 1.56 | 80.7 |
| **Cortisol** (μg/dL) | 0.012 | 3 | 0.007 | 53.3 |

NA = data not available

ELISA is a plate-based assay technique for detecting and quantifying biological substances. This technique generally uses the following principle (**Figure 6**):

1) Specific antibodies are immobilised on high protein-binding plates that usually comes in a 96-well format

2) Standard dilutions and samples are added to the wells, and the plate-bound antibodies will capture the specific biological substances desired
3) To enable the detection of the captured protein, specific biotinylated detection antibody is added to the wells
4) Streptavidin conjugated with alkaline phosphatase is added to the wells, which binds to the biotinylated antibody
5) A colourimetric substrate is added to the wells and a coloured solution will form when it is catalysed by the enzyme
6) Absorbance is measured using a compatible plate reader, and the amount of that biological substance in the sample is determined

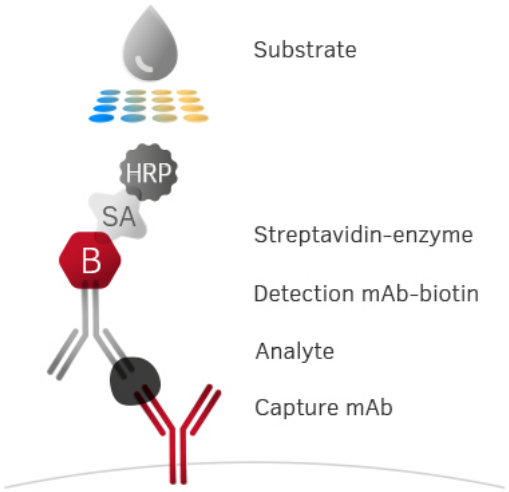


**Figure 6 – The Principles for ELISA**. (Mabtech, 2019)

On the day of analysis, the saliva samples were completely thawed, brought to room temperature, vortexed and centrifuged at 1500 × *g* for 15 minutes to remove mucins and other particles that would interfere with antibody binding and affect results. The supernatant (upper, liquid) portion of each sample was then extracted and pipetted into appropriate microplate wells.

Every assay performed required the use of standards provided in the kit and duplicate readings for every sample. Thus, each data point (for each participant at each time point), is the average value of the two values taken from two wells. Additionally, only three to four saliva samples of the chosen 10 participants could be analysed per 96-well kit. For SP and glutamate, four time points were analysed: Pre, Post +0 minutes (min), Post +20 min, and Post +60 min. For cortisol, three time points were analysed: Pre, Post +0 min, and Post +60 min.

## 4.9 Data Analysis

All data analysis was performed in MATLAB R2018b (The MathWorks, Inc., Natick, MA).

All tests were two-tailed where a *p*-value of $< 0.05$ was used as an indicator of statistical significance, except for the Kruskal-Wallis test where a *p*-value of $< 0.01$ was an indicator of statistical significance instead. One *p*-value is given as an output as a result of each of

the statistical tests, except for the Pearson's correlation coefficient where three different *p*-values are given for the correlation between each combination of biomarker.

### 4.9.1 Normality of the Data

The data for each biomolecule at each time point was tested for normality with the Shapiro-Wilk test as the sample size was less than 50, and the test provides better power than other normality tests (Ghasemi and Zahediasl, 2012). Furthermore, histograms and quantile-quantile (Q-Q) plots were also produced to visually inspect the distribution of the data. Results of the normality tests are in **Appendix B.**

### 4.9.2 Comparison between Time Points

For biomarkers with normally distributed data, the summarised data for each time point is presented in the form of mean (± SD). Non-normally distributed data for each time point is presented in the form of median (± median absolute deviation (MAD)).

The parametric repeated measures analysis of variance (ANOVA) method was used to test for statistical significance between all time points for normally distributed data. The non-parametric Friedman's test was used for data that deviated from a normal distribution.

### 4.9.3 Comparison between Variables

Data of all time points (Pre, Post +0 min, Post +20 min and Post +60 min) were compiled into one dataset before being tested against different variables to see if any differences in the overall range of biomolecule concentrations, regardless of time point, and particular variables exist.

Gender influences were determined using t-test for normally distributed data and the non-parametric Mann-Whitney U test/Wilcoxon rank-sum test was used for non-normally distributed data.

The correlation of NRS and age with biomolecule concentrations were evaluated using the one-way ANOVA and Kruskal-Wallis test for normal and non-normally distributed data, respectively.

Boxplots were produced to visually illustrate the differences in the overall range of biomolecule concentrations and different variables.

### 4.9.4 Correlation between Biomolecules and Categorical Variables

The Pearson correlation coefficient was used to assess any correlation between the three biomolecules. On the other hand, correlations between categorical values were carried out using the Chi-squared test.

# 5. RESULTS

## 5.1 Participants

The mean (± SD) and range of the ten participant's demographics, CPT duration and NRS score is displayed in **Table 3**. The frequency table for the NRS score and age is found in **Table 4**.

**Table 3 – Mean (± SD) and range of participant's demographics, cold pressor task (CPT) duration, and numerical rating scale (NRS) score.**

| Variable | Mean (± SD) | Range |
|---|---|---|
| Age (years) | 28.3 (± 7.4) | 22 – 40 |
| Gender (M/F) | 5/5 | - |
| CPT Duration (min) | 4.09 (± 1.3) | 0.93 - 5 |
| NRS Score | 5.9 (± 1.8) | 3 - 8 |

**Table 4 – Frequency table for each numerical rating scale (NRS) score (left) and age group (right).**

| NRS Score | Frequency |
|---|---|
| 3 | 2 |
| 5 | 1 |
| 6 | 3 |
| 6.5 | 1 |
| 7 | 1 |
| 8 | 2 |

| Age | Frequency |
|---|---|
| 22 | 2 |
| 23 | 2 |
| 24 | 1 |
| 26 | 1 |
| 27 | 1 |
| 36 | 1 |
| 40 | 2 |

The heart rate and blood pressure data of the participants were taken for monitoring purposes only and was therefore not analysed. The data can be found in **Appendix C**.

## 5.2 Change in NRS

Participants were asked to rate the pain intensity using the NRS only once, where the score chosen was to reflect the maximum pain intensity felt during the CPT. It is therefore assumed that the NRS score before and after the CPT was zero.

## 5.3 Changes in Salivary Cortisol

### 5.3.1 Mean Cortisol Levels

The mean (± SD) salivary cortisol concentrations for the baseline (Pre), immediately after experimental pain (Post +0) and one hour post-CPT (Post +60) was 0.184 (± 0.07) µg/dL, 0.216 (± 0.1) µg/dL, and 0.268 (± 0.1) µg/dL, respectively (**Figure 7**). The baseline values were within the normal reported range of cortisol values from the literature (0.04 to 0.2 µg/dL). However, the values at Post +20 and +60 exceeded the upper range given.

No statistical significance was detected between cortisol concentrations between the three time points ($p = 0.397$).

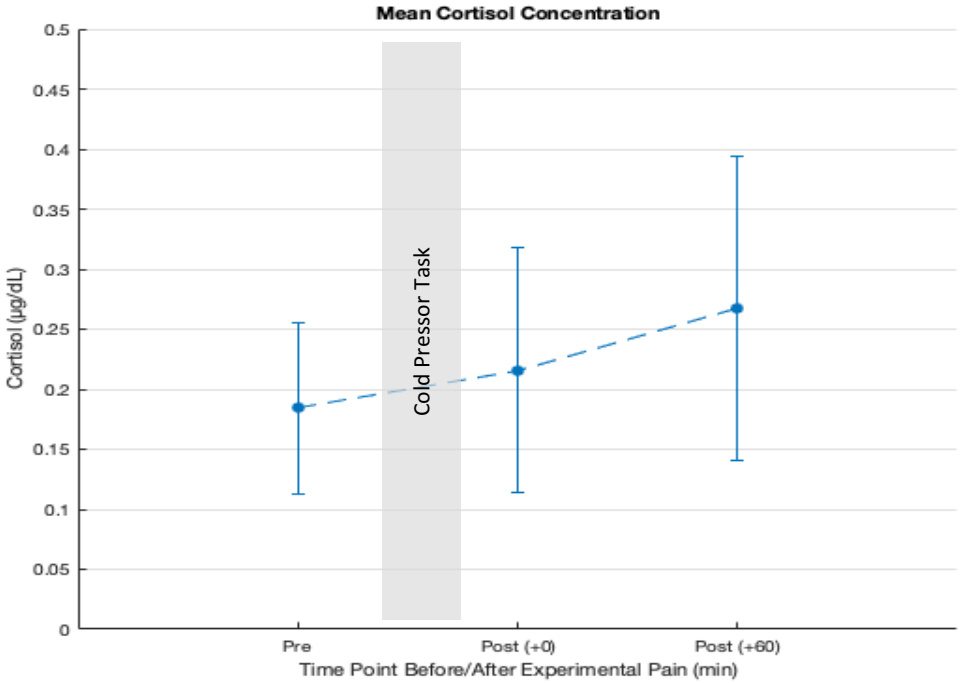


**Figure 7 – Changes in salivary cortisol concentrations.** Mean (± SD) salivary cortisol concentrations for the baseline (Pre), immediately after experimental pain (Post +0) and one hour post-CPT (Post +60).

### 5.3.2 Individual Changes in Cortisol Levels

Individual changes in cortisol levels with each time point is displayed in **Figure 8.** A majority of participants (8/10) exhibited increased cortisol levels at Post +0 from

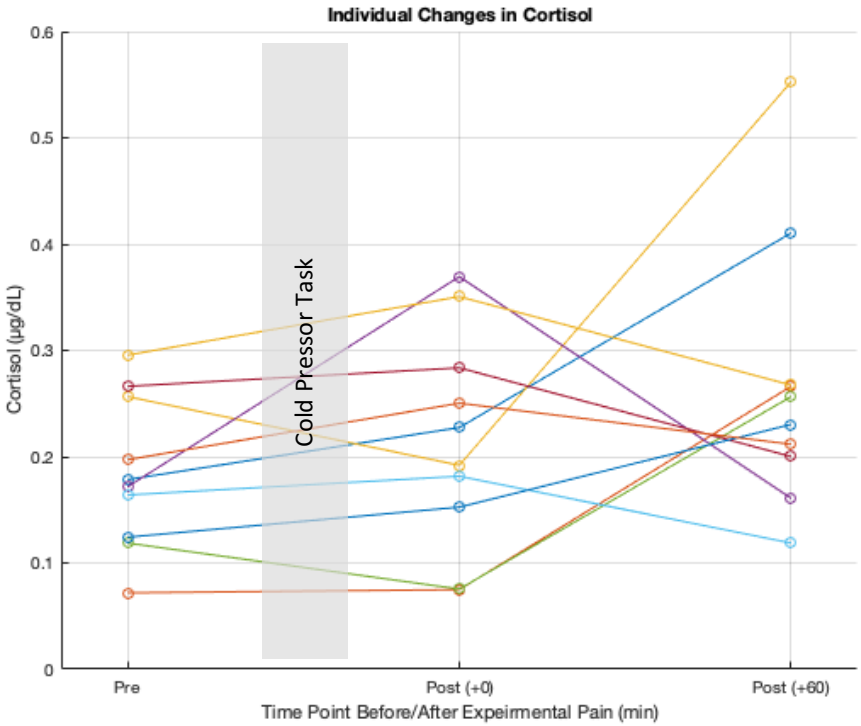


**Figure 8 – Individual changes in salivary cortisol concentrations.** At baseline (Pre), immediately after experimental pain (Post +0) and one hour post-CPT (Post +60).

baseline values. A lower number of participants (5/10) had decreased cortisol concentrations back to (or lower than) baseline concentrations one hour after CPT.

### 5.3.3 Cortisol Changes Categorised by Gender

All five males exhibited an increase in cortisol concentrations after experimental pain (**Figure 9**). Proceeding one hour after pain, four males had cortisol concentrations that declined back towards baseline, with the majority having concentrations even lower than baseline. Only one participant displayed higher cortisol concentrations one hour post-pain.

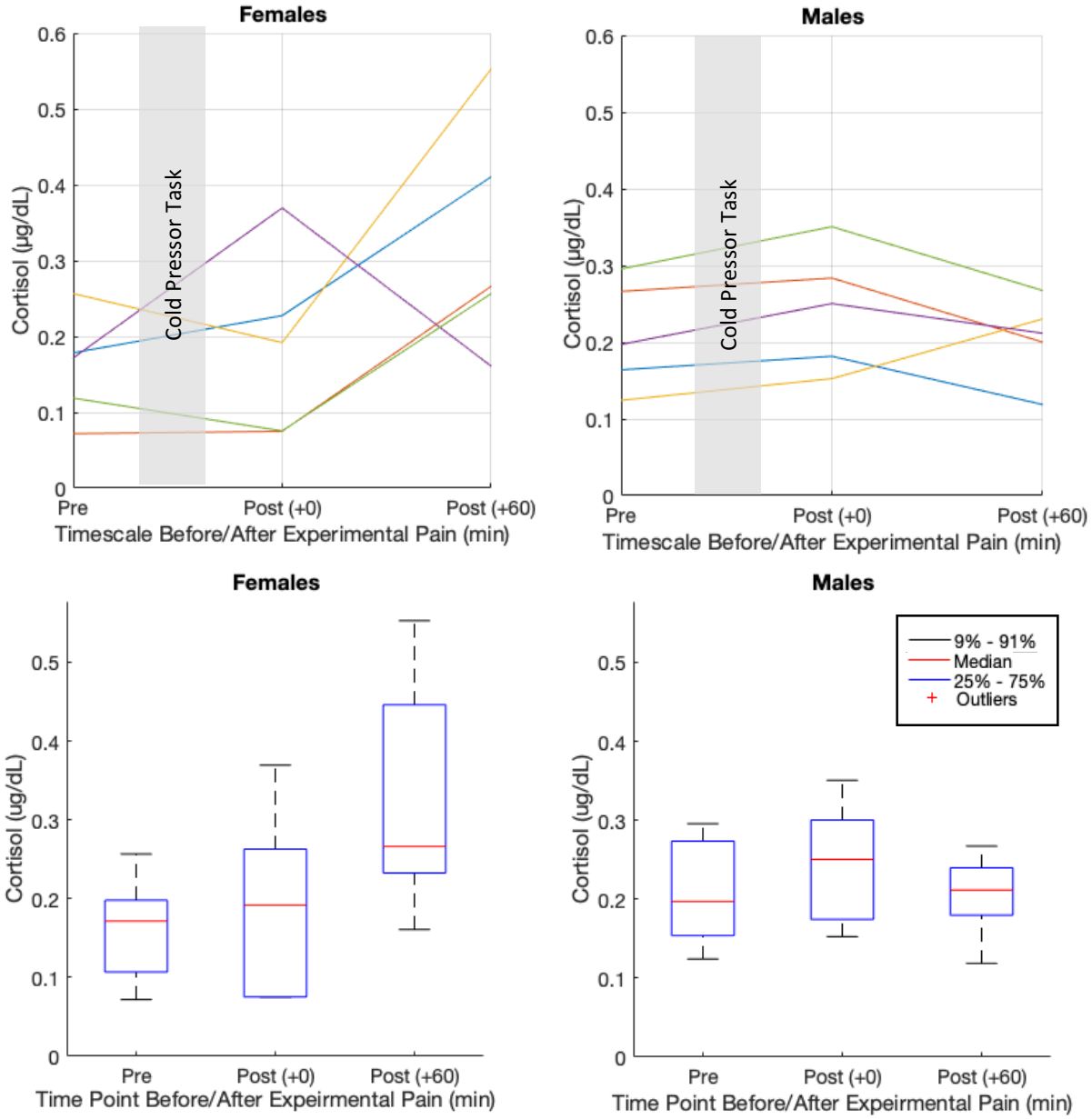


**Figure 9 – Changes in cortisol concentrations and gender.** Individual changes in cortisol concentrations of females (top left) and males (top right) for the baseline (Pre), immediately after experimental pain (Post +0) and one hour post-CPT (Post +60). Boxplots for females (bottom left) and males (bottom right) show the overall range of values for each gender at each time point.

It can be seen from **Figure 9** that three out of five females displayed higher salivary cortisol concentrations +0 after the end of pain induction compared to their baseline, whilst the remaining two females had a decrease from baseline values. One hour after pain, four females had increased cortisol levels from Post +0, whereas the remaining female participant had a decreased cortisol concentration that went back to baseline.

On the whole, females tended to have slightly lower average Pre and Post +0 cortisol values, but higher average Post +60 values than males (**Table 5**).

**Table 5 – Mean (± SD) change in cortisol concentrations for each gender at baseline (Pre), immediately after experimental pain (Post +0) and one hour post-CPT (Post +60).**

| | Mean (± SD) Cortisol Concentration (µg/dL) | | |
|---|---|---|---|
| | **Pre** | **Post +0** | **Post +60** |
| Females | 0.159 (± 0.07) | 0.188 (± 0.1) | 0.329 (± 0.2) |
| Males | 0.209 (± 0.07) | 0.244 (± 0.08) | 0.206 (± 0.05) |

No statistical significant differences were found between cortisol concentrations of males and females for all time points combined ($p = 0.896$). Looking at each time point separately, no statistical significant differences were found between the cortisol values of males and females for Pre ($p = 0.46$), Post +0 ($p = 0.51$) and Post +60 ($p = 0.19$).

### 5.3.4 Cortisol Concentrations Categorised by NRS

The overall cortisol concentrations grouped by NRS score is displayed in **Figure 10**.

There were no statistically significant differences in cortisol concentrations for each NRS score ($p = 0.477$).

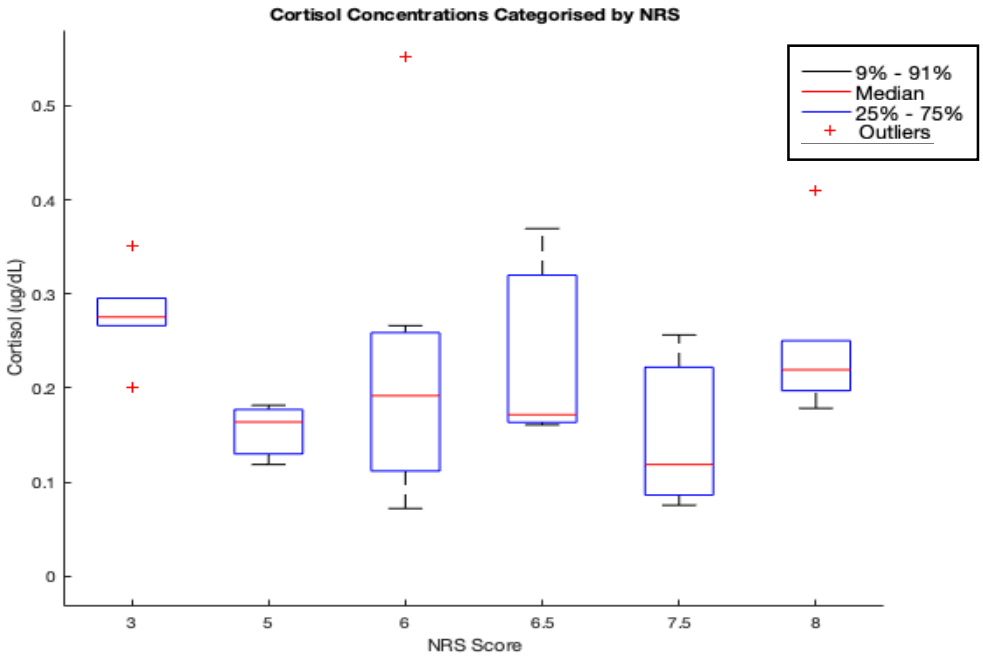


**Figure 10 – Cortisol concentrations and different numerical rating scale (NRS) scores.**

### 5.3.5 Cortisol Concentrations Categorised by Age

Similarly, no statistically significant differences in cortisol concentrations for each age group was found ($p = 0.422$). The overall cortisol concentrations according to each participant's age is displayed in **Figure 11**.

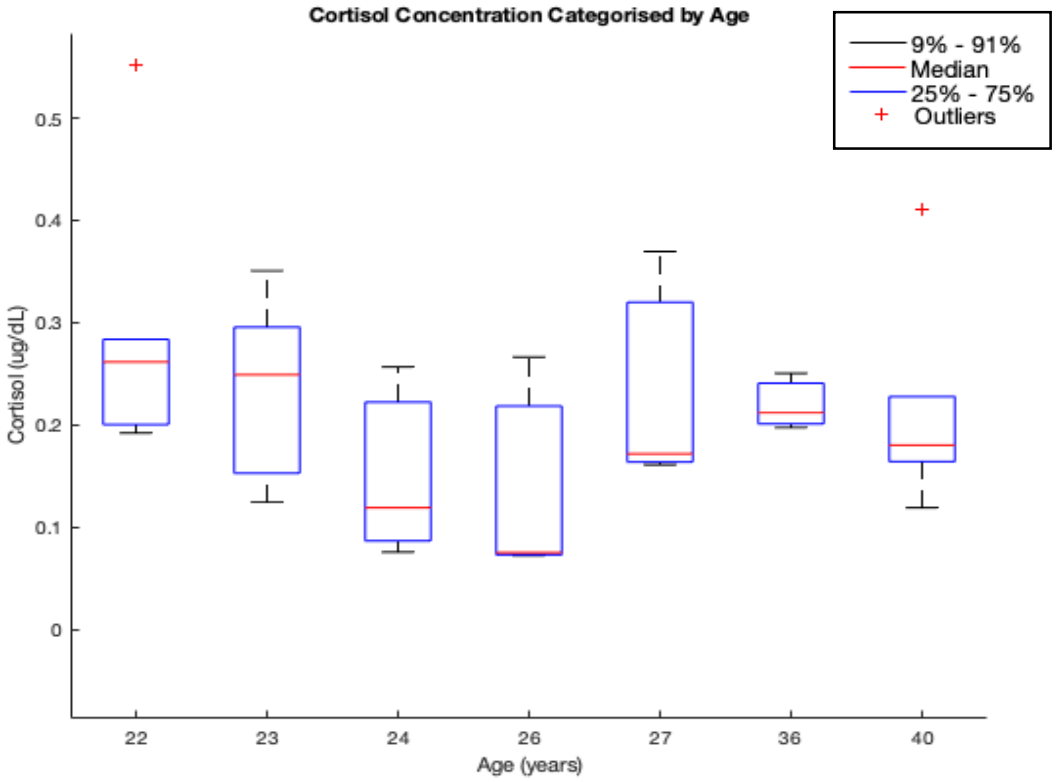


**Figure 11 – Cortisol concentrations and different age groups.**

## 5.4 Changes in Salivary SP

### 5.4.1 Median SP Levels

The median (± MAD) salivary SP concentrations for the baseline (Pre), immediately after experimental pain (Post +0), twenty minutes post-CPT (Post +20) and one hour post-CPT (Post +60) was 50.3 (± 28) pg/mL, 32.6 (± 25) pg/mL, 21.1 (± 11) pg/mL, and 20.5 (± 11) pg/mL respectively (**Figure 12**). The SP values for baseline and all other time points fell within the SP salivary concentration ranges reported in the literature (0 to 1614 pg/mL).

Statistical analysis revealed no significant differences between the SP concentrations at each time point ($p = 0.118$).

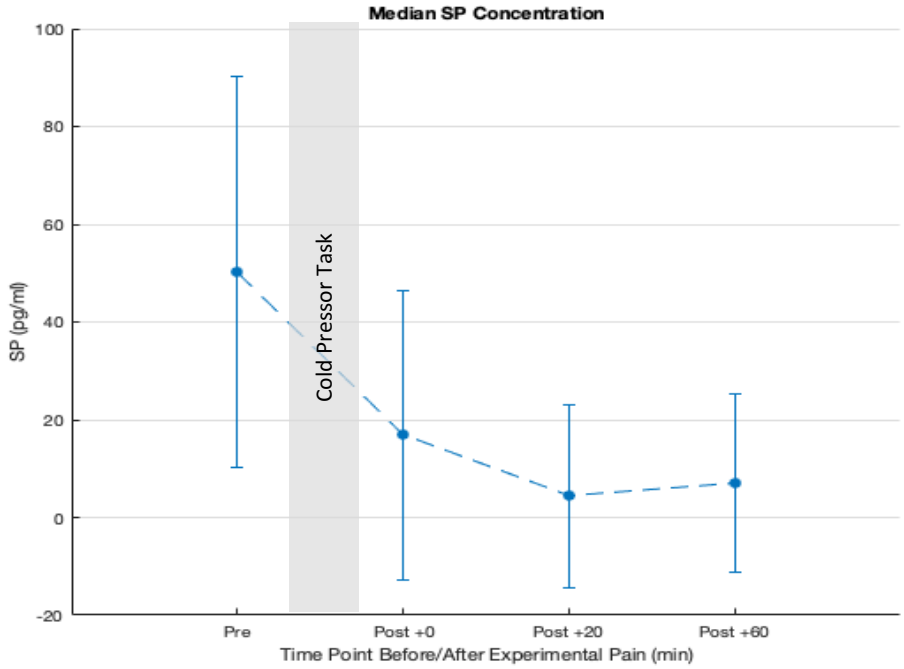


**Figure 12 – Changes in salivary SP concentrations.** Median (± MAD) salivary SP concentrations for the baseline (Pre), immediately after experimental pain (Post +0), twenty minutes after pain (Post +20), and one hour after pain (Post +60).

### 5.4.2 Individual Changes in SP Levels

Looking at each participant individually, 6/10 participants had subtle increases in SP concentration from baseline values (**Figure 13**), whilst 5/10 participants showed

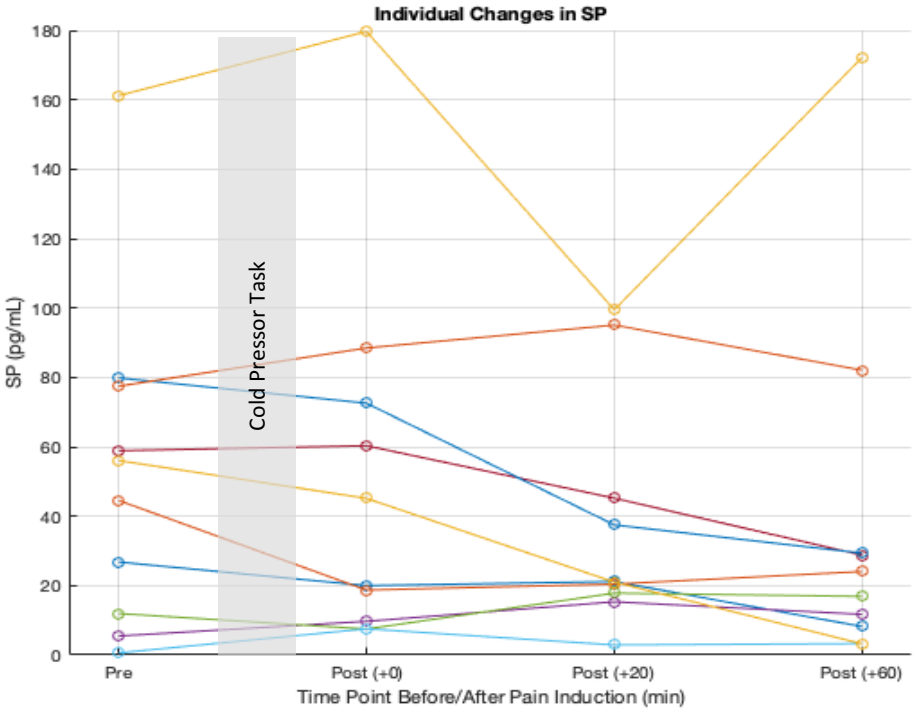


**Figure 13 – Individual changes in salivary SP concentrations.** At baseline (Pre), immediately after experimental pain (Post +0), twenty minutes after pain (Post +20) and one hour after pain (Post +60).

decreases from Post +0 to Post +20, and from Post +20 to Post +60 instead. Four participants showed very minor fluctuations in SP levels between each time point.

### 5.4.3 SP Changes Categorised by Gender

Males generally displayed higher SP baseline values compared to females (**Table 6**), but after one hour post-pain the values between both genders became more closely clustered together (**Figure 14**). At Post +20, the SP values of both genders were the closest. Apart from this, male participants seemed to cover a larger overall range of change in SP concentration with each successive time point, whilst females barely showed any changes.

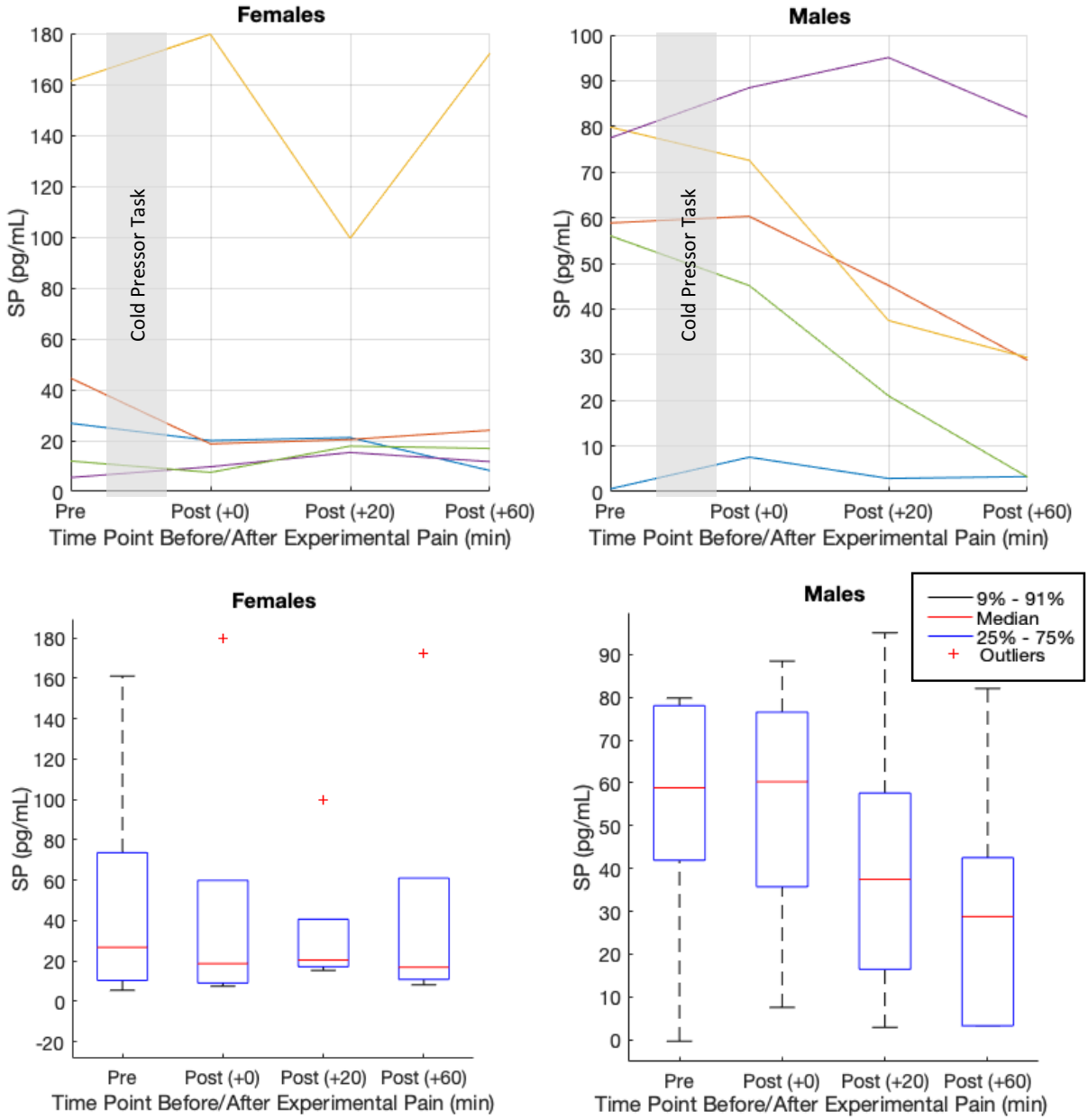


**Figure 14 – Changes in SP concentrations and gender.** Individual changes in SP concentrations of females (top left) and males (top right) for the baseline (Pre), immediately after experimental pain (Post +0), twenty minutes after pain (Post +20) and one hour after pain (Post +60). Boxplots for females (bottom left) and males (bottom right) show the overall range of values for each gender at each time point.

**Table 6 – Median (± MAD) change in SP concentrations for each gender at baseline (Pre), immediately after experimental pain (Post +0), twenty minutes after pain (Post +20) and one hour after pain (Post +60).**

| | Median (± MAD) SP Concentration (pg/mL) | | | |
|---|---|---|---|---|
| | **Pre** | **Post +0** | **Post +20** | **Post +60** |
| Females | 26.8 (± 18) | 18.7 (± 9) | 20.4 (± 2.6) | 16.9 (± 7.2) |
| Males | 58.9 (± 19) | 60.3 (± 15) | 37.5 (± 16) | 28.8 (± 25) |

Gender differences were not observed between overall SP levels of males and females ($p = 0.351$). Examination at each time point separately showed no statistically significant differences between males and females for Pre ($p = 0.55$), Post +0 ($p = 0.42$), Post +20 ($p = 0.69$) and Post +60 ($p = 1$).

### 5.4.4 SP Concentrations Categorised by NRS

The overall SP concentrations grouped by NRS score is displayed in **Figure 15**.

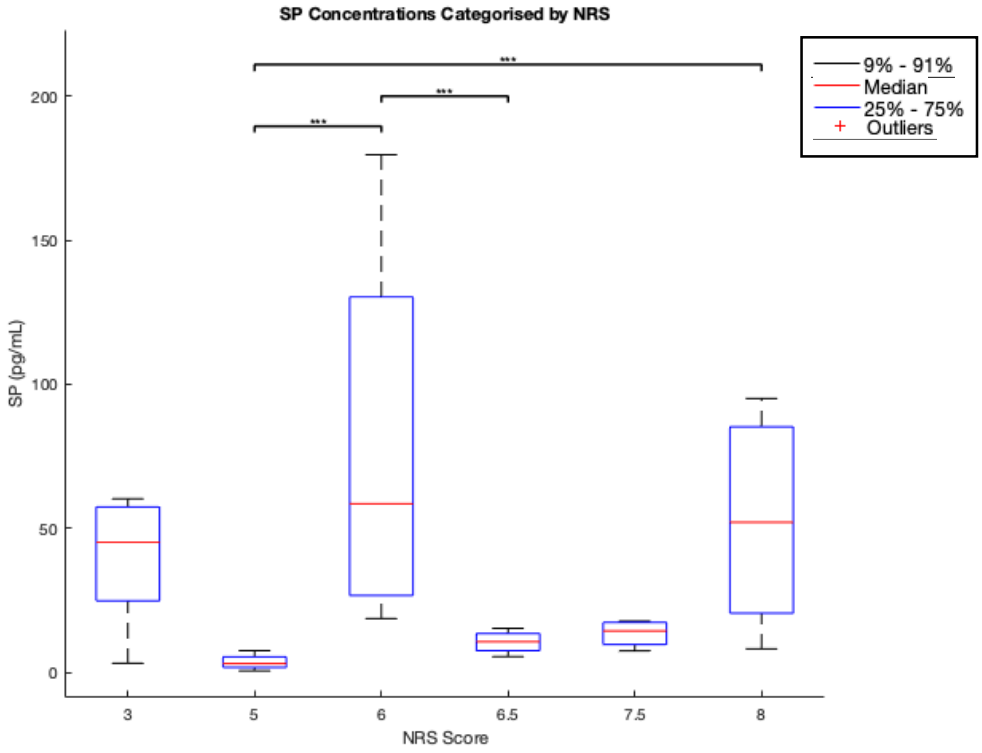


**Figure 15 – SP concentrations and different numerical rating scale (NRS) scores.** Significant differences between groups are indicated by the asterisks (***).

A statistically significant difference was detected between the SP concentrations of some NRS scores ($p = 4.85\times 10^{-4}$). More specifically, participants indicating a NRS score of 6 collectively had significantly higher SP concentrations for all time points compared to the concentrations of participants that rated the pain as 5 and 6.5. Additionally, participants

with an NRS score of 8 also had significantly higher SP concentrations than those with a score of 5.

### 5.4.5 SP Concentrations Categorised by Age

A statistically significant difference was also detected between SP concentrations and some age groups ($p = 9.06\times 10^{-5}$). Here, the overall SP concentrations of participants aged 22 years were significantly higher than concentrations of those aged 24, 27 and 40 years, and participants aged 36 years had significantly higher SP concentrations than those aged 27 and 40 (**Figure 16**).

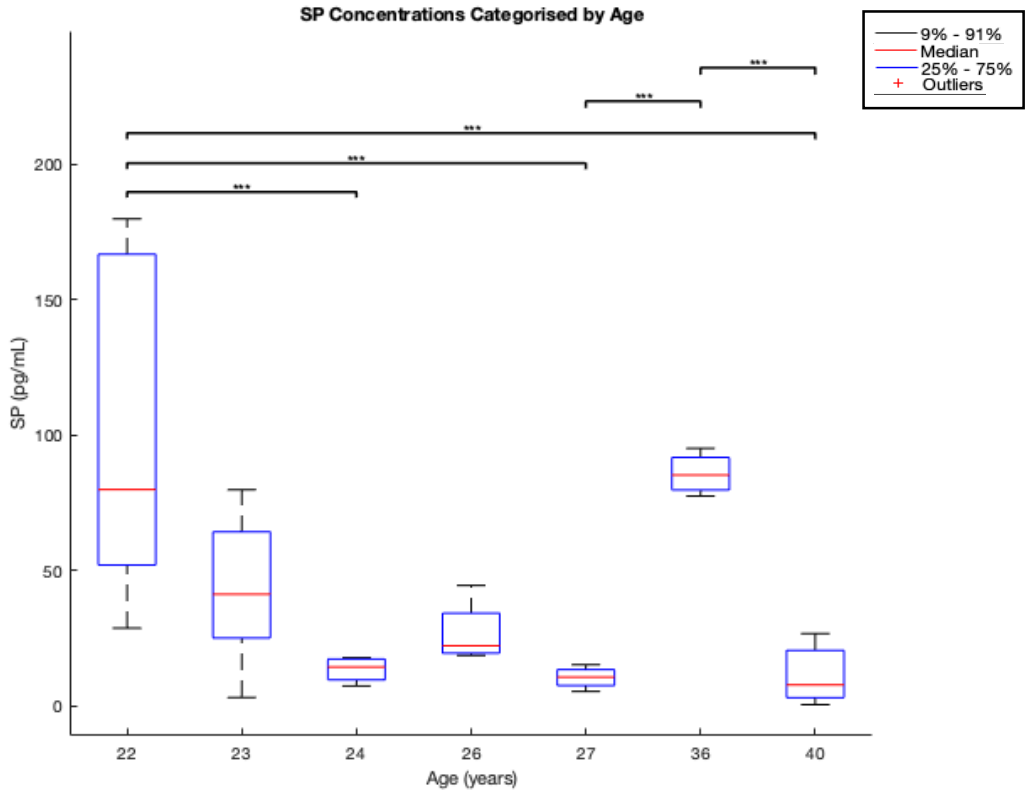


**Figure 16 – SP concentrations and different age groups.** Significant differences between groups are indicated by the asterisks (***).

## 5.5 Changes in Salivary Glutamate

### 5.5.1 Median Glutamate Levels

The median (± MAD) salivary glutamate concentrations for the baseline (Pre) was 7.51 (± 2.5) ng/µL, immediately after experimental pain (Post +0) was 7.43 (± 4.2) ng/µL, twenty minutes after pain was 5.86 (± 0.59) ng/µL and one hour post-pain (Post +60) was 5.14 (± 1.5) ng/µL (**Figure 17**). Glutamate salivary concentrations at every time point, except at Post +60, were higher than the concentrations reported in the literature (1.47 to 4.41 ng/µL).

Statistical analysis revealed significantly different glutamate concentrations between the time points ($p = 0.03$) whereby there was significantly higher Pre values than values Post +60 values.

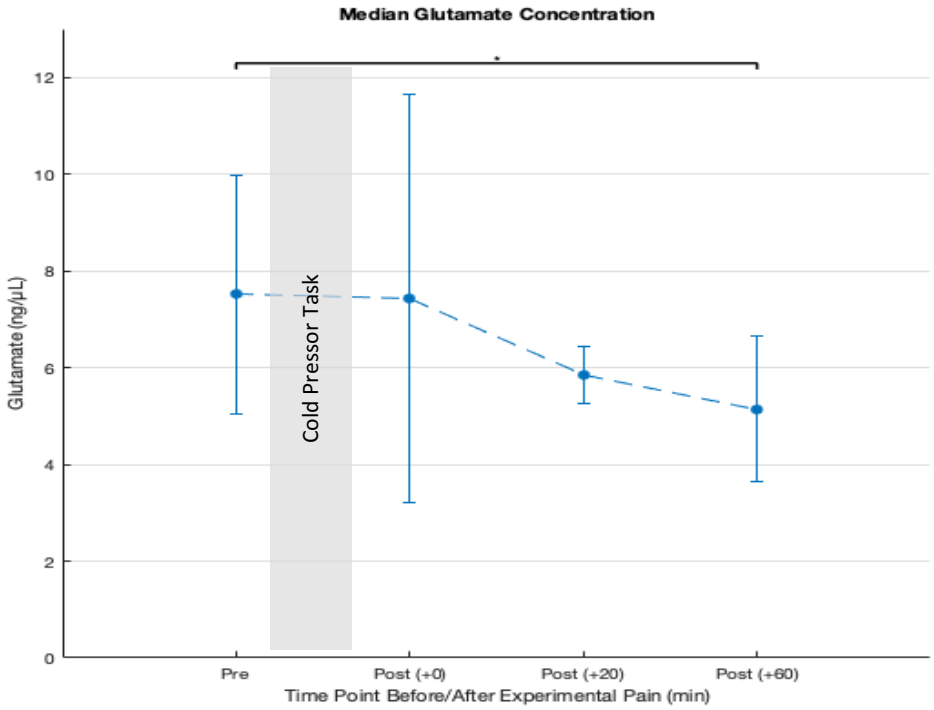


**Figure 17 – Changes in salivary glutamate concentrations.** Median (± MAD) salivary glutamate concentrations for the baseline (Pre), immediately after experimental pain (Post +0), twenty minutes after pain (Post +20) and one hour after pain (Post +60). Significance is indicated by the asterisks (*).

### 5.5.2 Individual Changes in Glutamate Levels

Individually, four out of ten participants exhibited increases in glutamate concentration after the CPT (**Figure 18**). A larger majority of the participants (7/10) went on to have

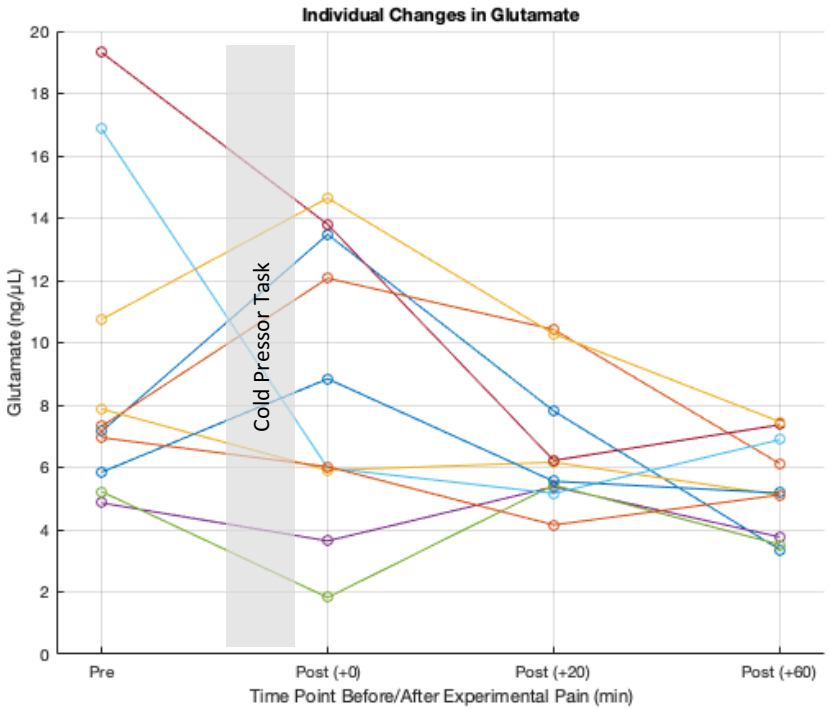


**Figure 18 – Individual changes in salivary glutamate concentrations.** At baseline (Pre), immediately after experimental pain (Post +0), twenty minutes after pain (Post +20) and one hour after pain (Post +60).

even lower glutamate values at Post +20, which further decreased in seven different participants at Post +60. From the figure, it can be seen that eventually the glutamate concentrations of each individual became more clustered together one hour after experimental pain.

All ten participants had lower glutamate levels one hour post-pain compared to their starting baseline values.

### 5.5.3 Glutamate Gender Changes

Two females exhibited the same glutamate pattern throughout the entire time scale (**Figure 19**): their glutamate levels rose Post +0, which then gradually declined back down to below baseline levels at Post +60. On the other hand, the remaining three females also displayed similar changes in glutamate concentrations but these participants had decreases at Post +0 instead, which all increased at Post +20 and all decreased below baseline at Post +60. The glutamate concentrations of all females decreased from Post +20 to Post +60 and the value at Post +60 was lower than their starting baseline values.

In a similar manner, two males exhibited the same change in glutamate (**Figure 19**), where there was an increase at Post +0 that then decreased at Post +20 and Post +60. The other three males had decreases at Post +0 from baseline and at Post +20, and increased glutamate concentrations at Post +60.

**Table 7** displays each gender's median (± MAD) glutamate concentration for each time point.

**Table 7 – Median (± MAD) change in glutamate concentrations for each gender at baseline (Pre), immediately after experimental pain (Post +0), twenty minutes after pain (Post +20) and one hour after pain (Post +60).**

| | Median (± MAD) Glutamate Concentration (pg/mL) | | | |
|---|---|---|---|---|
| | **Pre** | **Post +0** | **Post +20** | **Post +60*** |
| Females | 7.15 (± 1.9) | 5.91 (± 4.1) | 6.16 (± 0.8) | 3.76 (± 0.4) |
| Males | 10.7 (± 4.9) | 8.84 (± 2.9) | 5.55 (± 0.7) | 6.88 (± 0.6) |

*** -** statistical significance difference detected between genders

No statistically significant differences were found between the overall glutamate concentration between males and females ($p = 0.105$). Looking at each time point individually no significant differences were found between males and females for Pre ($p = 0.22$), Post +0 ($p = 0.22$), and Post +20 ($p = 0.55$). However, a statistically significant difference was found between males and females at Post +60 ($p = 0.0317$), with males having much higher glutamate concentrations.

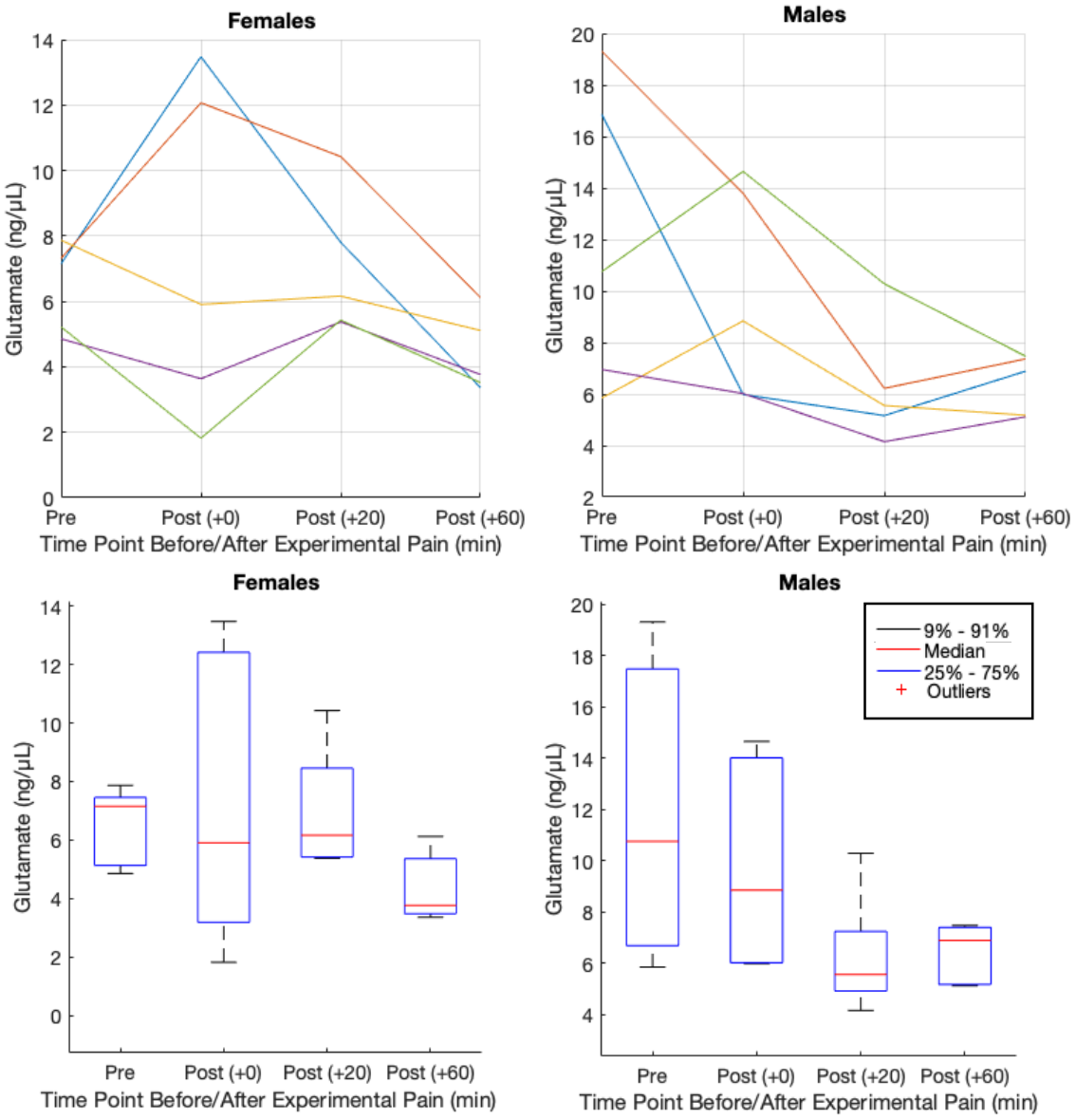


**Figure 19 – Changes in glutamate concentrations and gender.** Individual changes in glutamate concentrations of females (top left) and males (top right) for the baseline (Pre), immediately after experimental pain (Post +0), twenty minutes after pain (Post +20) and one hour after pain (Post +60). Boxplots for females (bottom left) and males (bottom right) show the overall range of values for each gender at each time point.

### 5.5.4 Glutamate Concentrations Categorised by NRS

Analysis revealed a statistically significant difference in NRS score and overall glutamate concentrations ($p = 0.002$) whereby participants reporting a NRS score of 3 had significantly higher values than those reporting an NRS score of 6.5 and 7.5 (**Figure 20**).

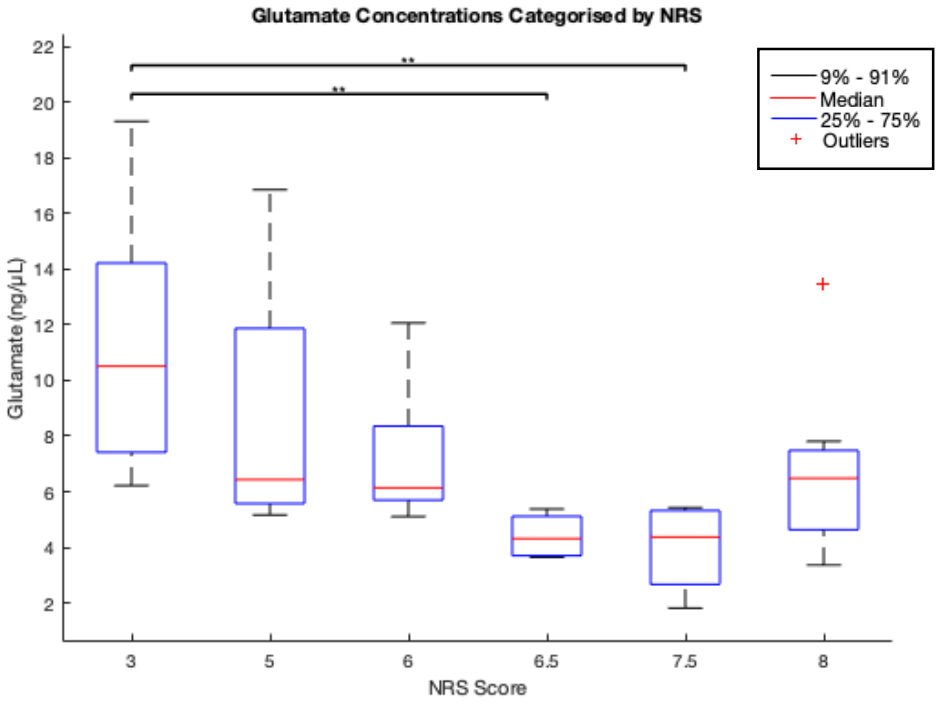


**Figure 20 – Glutamate concentrations and different numerical rating scale (NRS) scores.**

### 5.5.5 Glutamate Concentrations Categorised by Age

No significance was detected for all glutamate concentrations between different ages ($p = 0.013$) (**Figure 21**).

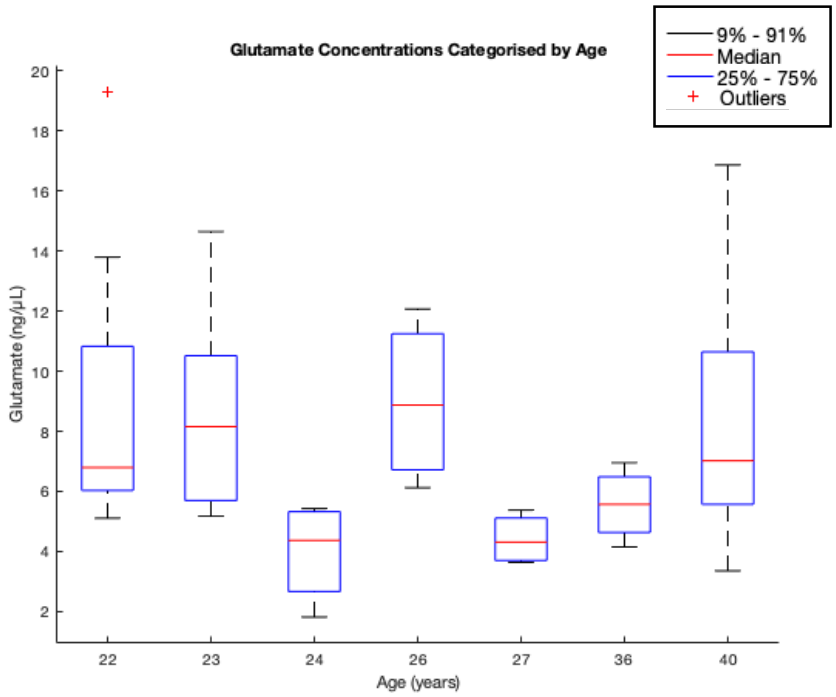


**Figure 21 – Glutamate concentrations and different age groups.**

## 5.6 Change from Baseline Values to Post +0 and Pain Intensity Rating

The change from baseline values to Post +0 values for all biomolecules were calculated and plotted according to the pain rating for each participant (**Figure 22**).

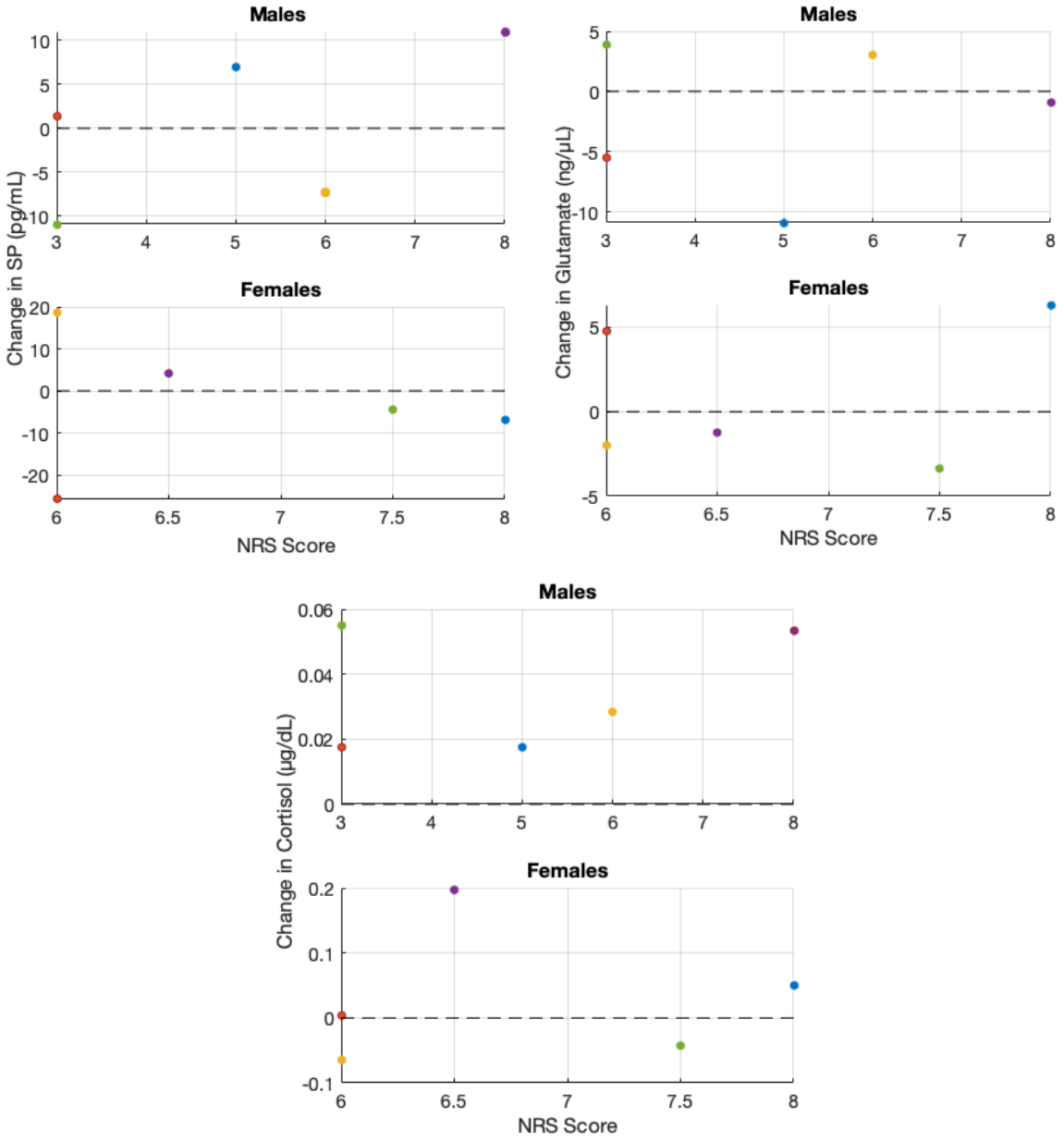


**Figure 22 – Changes from baseline values to Post +0 values according to the pain intensity rating.** Change in SP concentrations (top left), change in glutamate concentrations (top right), and change in cortisol concentrations (bottom). The dashed line (---) signifies no change in biomolecule concentration.

Smaller changes in biomolecule concentrations were not always observed in participants with lower pain ratings compared to other participants that reported higher ratings. At the same time, participants with higher pain ratings did not always exhibit the largest change in biomolecule concentrations. No specific pattern was observed between the NRS score and amount of change in each biomolecule and between each biomolecule concentration immediately after CPT.

## 5.7 Correlation between Biomolecules

No correlation was found between the concentrations of all three biomolecules for all time points combined – SP and glutamate: $p = 0.996$, SP and cortisol: $p = 0.08$, and cortisol and glutamate: $p = 0.92$. From the scatter graphs (**Figure 23**), there are no positive or negative correlations between each biomolecule.

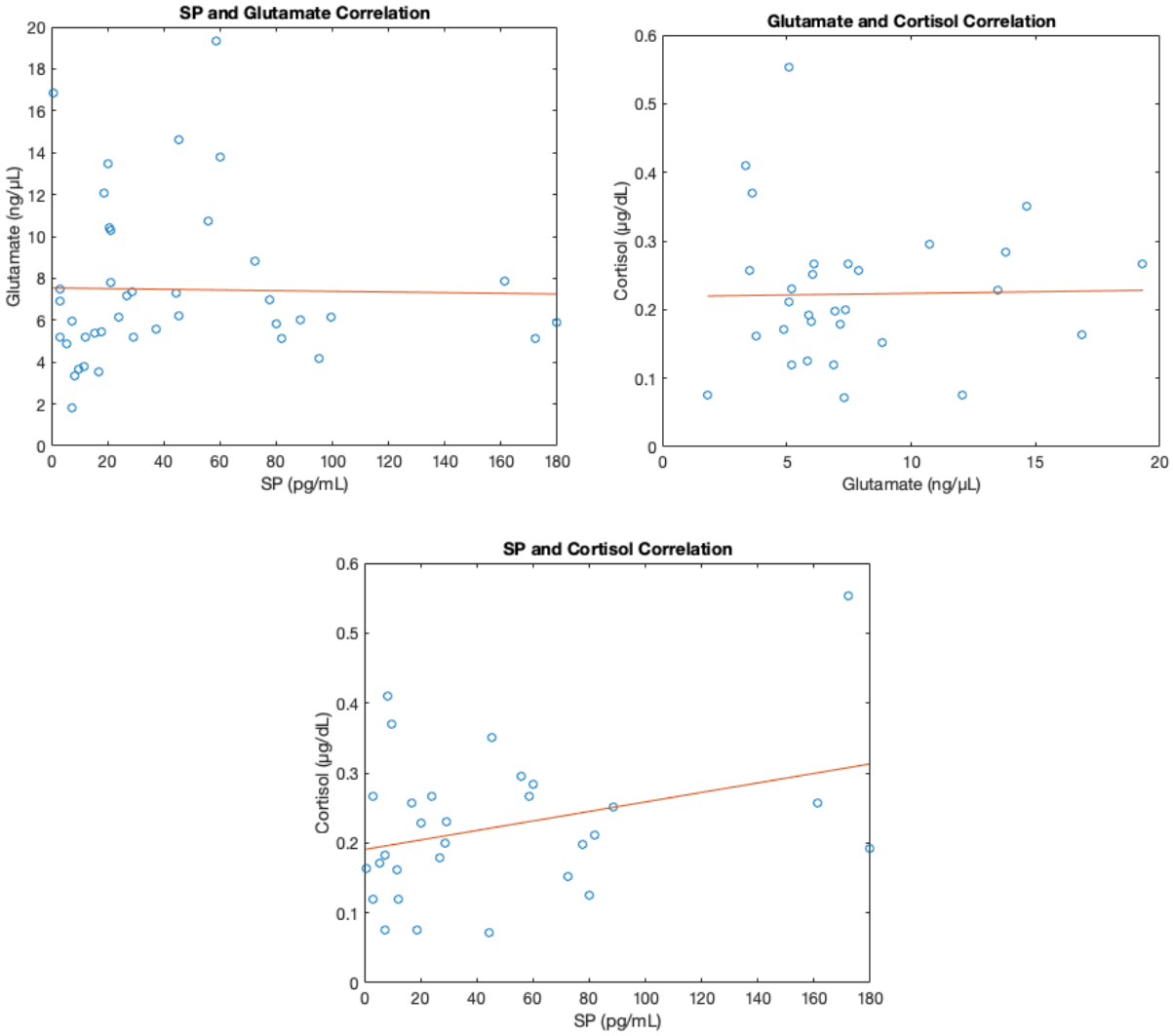


**Figure 23 – Correlation between the three biomolecules.** Correlation graph between SP and glutamate (top left), glutamate and cortisol (top right), and SP and cortisol (bottom) for all time points. The line represents the line of best fit.

## 5.8 Correlation between Categorical Variables

There was no correlation between age and NRS ($p = 0.274$), and none between gender and NRS ($p = 0.377$).

# 6. DISCUSSION

The main aim of this pilot study was to explore the relationship between the concentrations of glutamate, substance P and cortisol in the saliva and the intensity of acute pain. Here, no statistically significant changes in SP and cortisol concentrations were observed after experimentally induced pain. However, for glutamate, it was found that Post +60 values were significantly lower than baseline values. There was no correlation was found between the three biomolecules.

It is important to point out that even though statistical tests were carried out, they hold fairly low power as the sample size is very small (Razali and Wah, 2011). Small sample sizes make it hard to draw any definite conclusions, and may increase the chance of assuming a false premise as true (Faber and Fonseca, 2014). Nevertheless, the data obtained will be regarded as a preliminary insight into the effect of experimentally induced acute pain on pain-associated biomolecule concentrations, and the remaining is to be later analysed for completion.

## 6.1 Hypothesis

Prior to the experiment, it was hypothesised that salivary concentrations of one or more of the three biomolecules will change from baseline values with the presence of acute pain, and eventually revert back to baseline values after some time, at around one hour. From the results, it is concluded that on the whole, the concentration of all biomolecules did indeed change with pain, but not all reverted back to baseline values and thus, the original hypothesis is rejected. However, we also fail to accept the null hypothesis that salivary biomolecule concentrations remain unaffected by the presence of pain, because changes in biomolecule levels were still observed, though without a clear pattern. Therefore, more research is needed to gain a better understanding of pain-related changes in biomolecule concentrations in saliva.

In addition, it was further hypothesised that if biomarker levels changed after experimentally induced pain, participants reporting higher pain ratings would present larger changes in biomolecule concentrations, and vice versa. Results from this study do not support seem to support this notion, and no clear pattern between the extent of change in biomolecule concentrations after induced pain and pain intensity ratings could be established.

With regards to the literature, some participants displayed the expected trend whereby the biomolecule concentration increased from baseline values following acute pain induction, and eventually went back to baseline after around one hour. However, this trend was not seen in all three biomolecules at the same time for any of the participants.

A probable explanation as to why collective, transient increases in SP, cortisol and glutamate concentrations were not observed with experimental pain in healthy volunteers on the whole, as seen in many of the available literature, is perhaps the fact that each biomolecule also has important roles during normal processes, not just during pain, where their concentrations naturally fluctuate and are continually altered to maintain the body's natural state. For example, SP-containing nerves are also present in the airways, skin and around blood vessels (Van Der Kleij and Bienenstock, 2007),

glutamate is involved in vital brain processes such as learning, memory and the maintenance of consciousness (McEntee and Croo, 1993), and cortisol participates in the regulation of the immune system and the metabolism of protein, carbohydrate, and adipose (Katsu and Iguchi, 2106). As a dense bed of capillaries surround the salivary glands, many blood components from various areas of the body can easily pass through the capillary walls into the saliva glands (Salimetrics, 2019). Additionally, there may also be local productions of those biomolecules in the salivary glands, as it has been suggested for SP (Kallman, Ghafouri and Bäckryd, 2018). Therefore, pain-related increases in SP, cortisol and glutamate may have been masked by changes in those biomolecules for other normal, physiological processes.

In contrast, perhaps a dysregulation, rather than an increase, in biomolecule concentrations may be a hallmark for pain instead, as it was suggested for cortisol. This can be seen from the results where the median concentrations of both SP and glutamate displayed a continuous decrease after experimental pain induction with the progression of each time point. At the same time, a continuous increase in cortisol concentrations was observed after experimental pain induction. Nevertheless, the normal pain-free salivary profile of glutamate, SP and cortisol of each participant is required to see and compare whether the changes in biomolecule concentrations after pain is regarded as a dysregulation.

Another component that may have affected the pain-associated changes in biomolecules is the fact that the blood pressure and heart rate of the participants were taken every five minutes. When taking the blood pressure and heart rate using the cuff, the cuff will squeeze the arm very tightly before slowly reducing the grip. Although the cuff was placed on the opposite arm to the arm that was submerged in the ice bath, it can still be a relatively painful procedure, particularly if taken at the same area for a prolonged period of time. Thus, this may explain why levels of some biomolecules may still be elevated even after one hour post-pain.

## 6.2 Statistical Significances Detected

One statistically significant finding was that glutamate concentrations at Pre were significantly higher than glutamate concentrations at Post +60. Interestingly, at Pre, all participants started with a relatively large range of glutamate values, which then all changed at varying degrees immediately after experimental pain. Then at Post +20 and Post +60, all the participant's glutamate concentrations started to converge to lower, more similar values. From this, glutamate levels are somewhat affected by pain, but it is unclear as to why there are much higher glutamate values at Pre and much lower glutamate levels at Post +60. Possible explanations for the higher Pre glutamate values is the potential link between glutamate and reward anticipation (Faure, Richard and Berridge, 2010), as well as between glutamate and threat processing or pain anticipation (Roots et al., 2016). As such, the participants may have been anticipating pain from the CPT or could be motivated to carry out and complete the CPT, leading to the increase in glutamate levels at baseline before the pain induction. More saliva samples need to be taken at longer periods Pre-pain and Post-pain to draw proper conclusions about this significant difference.

The statistically significant differences in glutamate and SP concentrations between participants rating different NRS scores (glutamate – pain rating of 3 $>$ 6.5 and 7.5; SP –

pain rating of 6 > 5 and 6.5, and pain rating of 8 > 5) and for SP concentrations between different age groups (22-year-olds > 24, 22 and 40-year-olds, and 36-year-olds > 27 and 40-year-olds) is most likely due to the fact that there was only one participant for a particular NRS score and age group. Accordingly, there is a very small biomolecule concentration range for that single participant, making that data more isolated than the rest of the data, leading the statistical test to detect significance. More data is needed to ensure that the statistical significances and statistical insignificances detected are valid and true.

Nevertheless, the detectable difference could imply that there may be quantifiable differences in pain responses for certain variables. It is interesting to observe that for glutamate, significantly higher glutamate levels were detected in participants reporting a NRS score of 3 compared to those with higher pain scores of 6.5 and 7.5. At the same time, although not significant, cortisol and SP concentrations of the same participants that reported a NRS of 3 were also higher than participants with higher pain intensity ratings, including the highest rating of 8. These higher salivary concentrations for lower pain ratings may suggest an inverse relationship between salivary biomolecule concentration and pain intensity instead.

On the other hand, it could be possible that the quantity of the molecule may be independent from the pain intensity perception. It has been observed that higher opiate receptors in the brain were correlated with higher pain tolerance (Brown et al., 2015). Opioids have a mechanism of action by either inhibiting the release of SP or through reducing excitation of second-order neurons to SP (Aicher, Punnoose and Goldberg, 2000). This implies that in high tolerance subjects, the actual concentration of SP, cortisol and glutamate may not be substantially reduced, but the brain's *response* to those molecules are modulated instead, making the presence and quantity of the pain-associated neurotransmitters independent from the actual perceived pain intensity.

In addition to this, another study found that increased pain tolerance significantly correlated with the rostral anterior cingulate cortex activation (Yilmaz, et al., 2010), which contains spindle neurons that do not express or have receptors for glutamate, SP or cortisol. The same concept can be applied to pain sensitisation where nociceptor neurons have increased responsiveness to normal or even subthreshold afferent inputs due to multiple biophysical and transcriptional mechanisms that produce changes in membrane properties, making it easier to elicit an action potential (Pinho-Ribeiro, Verri and Chiu, 2017). Hence, looking at the extent of change in biomolecule concentration as a direct indicator for pain intensity may not be practical.

Another significant finding was that participants aged 22 had significantly higher SP concentrations than participants aged 24, 27 and 40, and the participant aged 36 had significantly higher SP concentrations than 27- and 40-year-old participants. A systematic-review and meta-analysis of literature concerning age effects on pain and tolerance thresholds found that ageing decreases sensitivity for pain of low intensity, but has no effect on pain tolerance (Lautenbacher et al., 2017). This decreased sensitivity for pain is perhaps reflected in the decreased range of SP values with an increase in age for this current thesis. However, it does not account for the fact that the 36-year-old participant had much higher SP concentrations than some of the younger and older age groups. Additionally, the lower biomolecule concentrations for older participants were

not observed for cortisol and glutamate. Accordingly, this significant finding may be due to the fact that many of the age groups only had one participant. Further investigation is needed to determine any effects of age on pain-related changes in biomolecule concentrations.

## 6.3 Gender

A notable difference in change in biomolecule concentrations after pain induction was found between males and females for cortisol. Four out of five females displayed minor increases or decreases in cortisol values at Post +0, which then all increased at Post +60. In contrast four out of the five males all displayed a minor increase in cortisol levels at Post +0 that then all decreased lower than baseline at Post +60. These findings support the notion that there are gender differences in stress reactivity due to different HPA response patterns (Balhara, Verma and Gupta, 2012). It has been reported that males were found to have significantly greater cortisol responses than females (Uhart et al., 2006). This same observation can be found in this current study where, on the whole, male participants had higher baseline and Post +0 cortisol values than females. On the other hand, female sex hormones have been reported to attenuate HPA responsiveness, leading to delayed containment of the stress response or slow cortisol feedback on the brain (Balhara, Verma and Gupta, 2012), which could explain why a difference in response was seen in females and why the cortisol concentrations increased at Post +60. However, as we did not take into account the current stage of the menstrual cycle of the female participants, it is uncertain as to which response reflects the effect of which stage of the menstrual cycle. More data points at more time points are needed to get a clearer picture of differences in male and female responses to pain.

With regards to gender and SP, it can be seen that males generally displayed larger ranges and more changes in SP concentrations at each time point compared to females who barely presented any differences in SP concentrations. For glutamate, males had decreased glutamate levels post-pain whilst females had post-pain glutamate levels that remained higher than males. At Post +60, males had significantly higher glutamate concentrations than females. Our study demonstrates differences in pain-related changes in biomolecule concentrations between genders, and potentially imply that pain perception in females may be independent of SP concentration. To the best of our knowledge there is no literature available to explain why these gender-specific changes in SP and glutamate concentrations are exhibited. Further research needs to be conducted to see if these gender-related changes hold true.

## 6.4 The Effect of Motivation

All participants, except for one, pushed themselves to five minutes for the CPT. It was already stressed to the participants before the start of the experiment and during the CPT that they were allowed to remove their limb from the ice bath whenever the pain felt unbearable, but participants insisted that they could bear with the pain. Several participants commented that the initial submersion of the limb into the ice bath was the most excruciating part, but after some time their forearm and hand felt numb and they could keep it submerged for the maximum set time. In a situation like this, additional factors such as motivation and persistence most likely comes into play and could have influenced the concentrations of the biomolecules. It has been reported that cortisol may be able to promote reward-driven or approach-motivated affective state as exogenous cortisol increased risky decision making in healthy participants (Putman et al., 2009).

This could be what is reflected in the results where the rise in mean cortisol levels rose after experimental pain is a combination of both the stress-induced pain and the participant's motivation.

The remaining participant that did not keep their limb submerged in the ice bath for five minutes reached a CPT duration of 45 seconds. This participant did somewhat display ideal curves for SP and cortisol where the concentrations of both molecules increased at Post +0 or Post +20, and went back down to baseline by Post +60. However, the same pattern was not observed for glutamate. Perhaps there is a difference in salivary biomolecule concentration profiles in pain that immediately terminates after eliciting the withdrawal reflex, compared to pain that engages higher cognitive processes that require the participant to mentally engage and push themselves through the pain without withdrawing from the painful stimuli.

# 7. LIMITATIONS AND FUTURE WORK

## Incomplete Analysis of All Saliva Samples and Small Sample Size

Nine saliva samples were originally taken from each participant to see the change in biomolecule concentrations at small time intervals. However, due to the limited number of samples that can be analysed per ELISA kit and the delay in procurement and delivery of more kits, only three (for cortisol) or four (for SP and glutamate) time points could be analysed. This meant that the results achieved from the analysis was incomplete and inspection of biomolecule changes at smaller time intervals were not possible. Furthermore, only 10 out of the 20 original participants had their data analysed because of the time constraints, limited number of samples that could be analysed, and the cost of the assay kits. Reducing the number of participants reduces the power of the statistical tests and the power of the study. Consequently, an overall trend could not be established as possible increases in biomolecule concentrations at earlier and later time points with respect to Post +20 min could have been omitted, and individual differences were more prominent than potential trends. Therefore, the remaining saliva samples have been stored and will be analysed in the upcoming months. To ensure that the study has greater power, more volunteers should be recruited.

## Duration and Use of One Pain Modality

The maximum duration of the CPT was set to five minutes, which may be too short for some participants with higher pain tolerance or may be insufficient in producing marked changes in biomolecule concentrations. Thus, a longer pain induction time could be implemented to achieve a larger variety in CPT duration, and to see the effect of pain duration on biomolecule concentrations and participant's pain scores. As well as this, cold pain was the only pain modality used in this study, which may not necessarily trigger the release of all the three biomolecules. Cold pain also does not reflect the same type of pain that patients would experience after surgery or trauma. Hence, the use of other pain modalities such as mechanical pain or chemical pain along with the CPT could give a better understanding of the changes of each biomolecule under different types of pain, and may help in the identification of certain biomolecules as being a biomarker of specific pain-types.

## Three Pain-Associated Biomarkers

Three major biomolecules were examined in this study. Even though glutamate, SP and cortisol have been associated with pain, all the three biomolecules are abundant and play key roles in a wide variety of other physiological processes. As such, it is unrealistic to assume that the changes seen in their concentration is only due to experimentally induced pain alone. To circumvent this uncertainty, examination of more pain-specific biomolecules could be useful, such as neurokinin A, which also serves as a neurotransmitter in primary afferent C fibres (Otsuka and Yanagisawa, 1990). However, it should be noted that it should be ensured that the biomolecule(s) chosen can be detected in saliva or to identify and use other biofluids that can be sampled pain-free.

## Absence of a Control Group

There was a lack of a control group, thus, the changes in biomolecule concentrations observed may have been due to the experimentally induced pain, but it could have also

been due to normal fluctuations as well. A control group where the same number of participants were to sit and provide saliva samples at the same time points for the same duration, without any induced pain, would be ideal. To make the investigation even more controlled, the same participants who did the pain experiment could come back the next day and carry out the control condition themselves, as this would prevent any mismatches in biomolecule levels.

### Relatively Short Time Duration Examined

The baseline concentrations were collected a short period of time before experimental pain induction. Additionally, collection of saliva samples terminated only one hour after the CPT. Collecting more saliva samples for a longer period of time before and after experimental pain may provide a more sufficient understanding of how the biomarkers change. Taking samples at a longer time before inducing pain may reveal more realistic baseline values as there may be stress, concern or pain expectations at time points closer to when the participant was meant to induce pain, affecting the biomolecule concentration. Furthermore, some people may also require a longer time for their biomolecule concentrations to return back to baseline as their body may produce a large, prolonged reaction to the pain experience compared to others.

### No Continuous NRS Measurement

Only one pain rating was taken, meaning that it was assumed that before and after experimental pain, the pain experienced by the participant immediately increased from and decreased to zero, instead of having a gradual increase and decrease in pain. It is possible to experience sudden pain that reverts back to zero instantly, however, with the CPT, the pain should be more gradual as the stimulus is known to produce slowly mounting pain from mild to moderate intensity (von Baeyer et al., 2005). This was not done in the current study as initial participants found it hard to bear with the physical pain, rate the pain, and have their blood pressure and heart rate taken all at once. Taking NRS continually, especially every minute as soon as the forearm and hand was submerged into the ice bath until at least ten minutes after the end of the CPT may give a more comprehensive view of the dynamic changes of an individual's pain perception, which may be further reflected with the same pattern of changes in biomolecule concentrations in the saliva.

# 8. CONCLUSION

This study measured and analysed salivary glutamate, substance P, and cortisol concentrations using enzyme-linked immunosorbent assays in ten participants at different time points before and after experimentally induced acute pain using the cold pressor task. It was found that there were changes in the concentrations one or more of the three biomolecules in healthy volunteers at progressive time points following acute pain. However, the concentration changes of these biomolecules did not follow any specific trend, and did not revert back to baseline after around one hour post-pain. As well as this, larger changes in biomolecule concentrations from baseline were not seen with higher pain scores, and vice versa. Statistically significantly lower salivary glutamate concentrations one hour after pain compared to baseline values were observed, and statistically significant differences were also detected in glutamate and substance P concentrations between certain pain scores, and in substance P concentrations between certain age groups. Furthermore, females had significantly higher glutamate concentrations one hour post-pain compared to males. Although no significant differences were identified between salivary concentrations of cortisol and SP before and after pain, and there was no correlation between the three biomolecules, this study demonstrates that pain-induced changes in biomolecule concentrations exist, even though the trend may be currently unclear. Moreover, this study also presents gender differences in pain-related changes in biomolecule concentrations, along with some differences in concentration between certain age groups and pain scores, implying that there may be quantifiable differences in pain responses for certain variables. More research is required to obtain clearer trends, to confirm the statistical significances and statistical insignificances detected here, and to validate the method of measuring these salivary biomarkers as a tool for quantifying acute pain intensity.

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# APPENDIX A

**ADVERTISEMENT EMAIL**

## HEALTHY VOLUNTEERS NEEDED FOR PAIN RESEARCH

what

- **acute pain will be induced** via. submerging your forearm and hand in an ice bath for a maximum of 5 minutes - *Initiation and termination of pain will be entirely under your control*
- you will **provide us with saliva samples** before and after the pain induction → two samples before, one immediately after removal from ice bath, and additional samples every 10 minutes, for an hour.
- you will be presented with a numeric visual analogue scale to **rate the intensity of pain** experienced
- your **heart rate and blood pressure will be monitored** throughout the whole experiment.
- the whole process should not take longer than 1 hour and 30 minutes.
- later, your saliva samples will later be analysed in the lab for the concentration of biomolecules glutamate, substance P, and cortisol
- **£10 compensation** will be given for your participation.

who
If you:

- have good general health
- aged between 18 to 70
- can maintain oral hygiene on the day of the experiment - **refrain from consuming food and drinks (except water) 3 hours before the experiment, and brush your teeth after the meal, but no later than 1 hour before the experiment**
- **DO NOT** have the following:
    - any current pain
    - diagnosed with systemic muscular joint diseases
    - neurological disorders
    - pregnant or lactating
    - diagnosed with high blood pressure
    - use tobacco
    - take regular medication (includes oral contraceptives, antidepressants and analgesics)
    - poor oral condition (e.g. has dental or oral diseases)

Then you are eligible to take part in this study.

when

- **3.30-5pm** on any day that is suitable for you

where

- At the **UCL Aspire CREATe Lab** at the Royal National Orthopaedics Hospital, Stanmore

why

- pain is crucial to existence but it is complex and subjective
- The ability to objectively assess pain in the clinical setting will enable the study of pain and better pain treatment
- most conventional pain assessment methods rely on self-reporting, but this has very limited reliability and dismisses patients who are unable to provide their input (e.g. babies and children, or those in an induced coma or anesthesia)
- our main objective is to explore the relationship between the concentrations of certain biomolecules in saliva and the presence and intensity of pain.
- This research is a foundational study that paves the way to developing non-invasive saliva-based tools to objectively measure pain.

Please contact arisara.amrapala.18@ucl.ac.uk if you would like to participate in the experiment or would like more information.

**Aspire Create**
**Centre for Rehabilitation Engineering and Assistive Technology**

## CONSENT FORM FOR ADULT VOLUNTEERS IN RESEARCH STUDIES

**Please complete this form after you have read the Information Sheet and/or listened to an explanation about the research.**

**Title of Study:** Chemical Biomarkers of Acute Pain in Human Saliva
**Department:** Division of Surgery & Interventional Sciences
**Name and Contact Details of the Researcher(s):** Arisara Amrapala (arisara.amrapala.18@ucl.ac.uk)
**Name and Contact Details of the Principal Researcher:** Dr Sara Ghoreishizadeh (s.ghoreishizadeh@ucl.ac.uk)
**Name and Contact Details of the UCL Data Protection Officer:** Lee Shailer l.shailer@ucl.ac.uk
**This study has been approved by the UCL Research Ethics Committee: Project ID number:** 15021/001

Thank you for considering taking part in this research. The person organising the research must explain the project to you before you agree to take part. If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to join in. You will be given a copy of this Consent Form to keep and refer to at any time.

**I confirm that I understand that by ticking/initialling each box below I am consenting to this element of the study. I understand that it will be assumed that unticked/initialled boxes means that I DO NOT consent to that part of the study. I understand that by not giving consent for any one element that I may be deemed ineligible for the study.**

| | | Tick Box |
|---|---|---|
| 1. | *I confirm that I have read and understood the Information Sheet for the above study. I have had an opportunity to consider the information and what will be expected of me. I have also had the opportunity to ask questions which have been answered to my satisfaction. | |
| 2. | *I understand that I will be able to withdraw my data at any time preceding the analysis of my data. | |
| 3. | *I consent to participate in the study. I understand that my personal information (that consist of: my age and my gender) will be used for the purposes explained to me. I understand that according to data protection legislation, 'public task' will be the lawful basis for processing. | |
| 4. | **Use of the information for this project only**<br><br>*I understand that my data gathered in this study will be stored anonymously and securely. It will not be possible to identify me in any publications. | |
| 5. | *I understand that my information may be subject to review by responsible individuals from the University for monitoring and audit purposes. | |
| 6. | *I understand that my participation is voluntary and that I am free to withdraw at any time before my data is analysed without giving a reason.<br><br>I understand that if I decide to withdraw, any data I have provided up to that point will be deleted unless I agree otherwise. | |
| 7. | I understand the potential risks of participating and the support that will be available to me should I become distressed during the course of the research. | |
| 8. | No promise or guarantee of benefits have been made to encourage me to participate. | |
| 9. | I understand that the data will not be made available to any commercial organisations but is solely the responsibility of the researcher(s) undertaking this study. | |
| 10. | I understand that I will receive £10 compensation for taking part in this study. | |
| 11. | I agree that my anonymised research data may be used by others for future research. [No one will be able to identify you when this data is shared.] | |
| 12. | I understand that the information I have submitted will be published as a report and I wish to receive a copy of it. Yes/No | |
| 13. | I consent to give saliva samples during the study and understand that the samples will be stored anonymously, and will be used for specific research purposes only. | |
| 14. | I hereby confirm that I understand the inclusion criteria as detailed in the Information Sheet and explained to me by the researcher. | |
| 15. | I hereby confirm that:<br><br>(a) I understand the exclusion criteria as detailed in the Information Sheet and explained to me by the researcher; and<br><br>(b) I do not fall under the exclusion criteria. | |
| 16. | I have informed the researcher of any other research in which I am currently involved or have been involved in during the past 12 months. | |
| 17. | I am aware of who I should contact if I wish to lodge a complaint. | |
| 18. | I voluntarily agree to take part in this study. | |
| 19. | Use of information for this project and beyond<br><br>I would be happy for the data I provide to be archived at UCL.<br><br>I understand that other authenticated researchers will have access to my anonymised data. | |

**If you would like your contact details to be retained so that you can be contacted in the future by UCL researchers who would like to invite you to participate in follow up studies to this project, or in future studies of a similar nature, please tick the appropriate box below.**

| | | |
|---|---|---|
| | Yes, I would be happy to be contacted in this way | |
| | No, I would not like to be contacted | |

| | | |
|---|---|---|
| _____ | _____ | _____ |
| Name of participant | Date | Signature |
| _____ | _____ | _____ |
| Researcher | Date | Signature |

***THIS IS A GUIDANCE DOCUMENT AND MUST BE TAILORED TO MEET THE NEEDS OF YOUR STUDY. ONLY USE THE CLAUSES THAT ARE APPLICABLE FOR 'YOUR' STUDY**

**Participant Information Sheet For Adult Volunteers**

UCL Research Ethics Committee Approval ID Number: 15021/001

**YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET**

**Title of Study:** Chemical Biomarkers of Acute Pain in Human Saliva

**Department:** Division of Surgery & Interventional Sciences

**Name and Contact Details of the Researcher(s):** Arisara Amrapala (arisara.amrapala.18@ucl.ac.uk)

**Name and Contact Details of the Principal Researcher:** Dr Sara Ghoreishizadeh (s.ghoreishizadeh@ucl.ac.uk)

1. **Invitation Paragraph**
   You are being invited to take part in a research project. Before you decide whether or not to participate, it is important for you to understand why the research is being done and what participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

2. **What is the project's purpose?**
   Pain is crucial to existence, but it is complex and subjective. It is necessary to measure pain in the clinical setting to enable successful management of the pain experienced by patients. To date, the most conventional methods for assessing the intensity of acute pain rely on self-reporting. This self-assessment gives the measures very limited reliability, and dismisses patients who are unable to provide their input (e.g. babies and children, those in an induced coma or anesthesia, or those with communication and cognitive impairments). As such, methods to objectify acute pain are needed. There is evidence to suggest that the levels of certain biomolecules such as cortisol, glutamate, and substance P in bodily fluids (blood and saliva) are associated with pain [1].

   The main aim of this project is to explore the relationship between the concentration of these biomolecules in saliva, and to investigate how they change after a short period of induced pain. This is a foundational study that paves the way to developing non-invasive saliva-based analysis to objectively measure pain.

   In this study, we will be testing the following hypothesis: biomarker levels in the saliva change when participant undergoes a period of pain.

   [1] Hajer Jasim, Anders Carlsson, Britt Hedenberg-Magnusson, Bijar Ghafouri & Malin Ernberg, Saliva as a medium to detect and measure biomarkers related to pain, Nature, 2018, DOI:10.1038/s41598-018-21131-4

3. **Why have I been chosen?**
   We are looking for people who:
   - Have good general health
   - Between the age of 18 and 70 years
   - Free of fever or cold
   - Can maintain oral hygiene on the day of the experiment
   - Do *not* have any current pain, diagnosed with systematic muscular joint diseases, have neurological disorders, is pregnant or lactating, has high blood pressure, uses tobacco, regularly uses medication (includes oral contraceptives, antidepressants and analgesics), or has poor oral condition

4. **Do I have to take part?**
   It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep (and be asked to sign a consent form). You can withdraw at any time without giving a reason and without it affecting any benefits that you are entitled to. If you decide to withdraw you will be asked what you wish to happen to the data you have provided up to that point.

5. **What will happen to me if I take part?**
   If you agree to take part, we will ask you to sign a consent form. You will then be invited to visit our lab in the Royal National Orthopaedic Hospital on one occasion for about two hours. We will also invite you to visit the UCLH Institute of Neurology for about two hours on a different day.

   During each session, up to 10 samples of your saliva (using passive drool) will be taken before, during and after inducing pain. The pain will be induced for a few seconds to a couple of minutes using hot or cold temperature methods on your forearm. You will either be asked to immerse your hand and forearm in cold water (first session), or a small device (this is a heater/cooler) will be connected to your forearm (second session). This device is connected to a medically approved equipment that controls the contact temperature between $5^{o}$ to $50^{o}$ C. The duration of the experiment and all temperatures will be completely under your control. You will be using a machine that enables you to control when to initiate the pain induction and when to terminate the stimulus, using a single button, when you feel uncomfortable. You can remove your hand from the ice bath whenever you want.

   We have set a maximum time for applying the pain stimulus to be less than 5 minutes to avoid any skin burn effects.

   We will ensure your well-being by monitoring your heart rate and blood pressure periodically throughout the experiment, and will communicate with you to make sure that you feel comfortable during the entire session.

   After the experimental pain induction, you will be given a numeral visual analogue scale (VAS) to rate the amount of pain experienced. The session will end after this.

   After each session, we will anonymise all data and the saliva samples. We will later measure the levels of certain biomarkers in your saliva samples using the commercially available Enzyme-linked immunosorbent assay (ELISA) kit and compare the findings with the pain ratings reported.

6. **What are the possible disadvantages and risks of taking part?**
   You will have to go through a short period of induced pain. However, you have complete control over when to induce and terminate the pain induction, and the intensity of the pain. The machine also has several built-in safety mechanisms that will automatically stop at dangerous

stimulus. Furthermore, we have set a maximum time for applying the pain stimulus to be less than 5 minutes to avoid any skin burn effects.

You will have to provide us with several saliva samples. However, this will be done in a protected, sanitary environment, using a non-invasive saliva collection method (passive drool).

7. **What are the possible benefits of taking part?**
Whist there are no immediate benefits for those people participating in the project, it is hoped that this work will lead to a better understanding of the biomolecules involved with the experience of pain and their behaviour with respect to pain intensity. Accordingly, we hope to use this work as a foundational study to pave the way towards developing a non-invasive saliva-based analysis to objectively measure pain.

8. **What if something goes wrong?**
If this study has harmed you in any way or if you wish to make a complaint about the conduct of the study you can contact the Principal Researcher:

Dr Sara Ghoreishizadeh,
Aspire CREATe, UCL – IOMS, Brockley Hill, Stanmore, Middlesex HA7 4LP.
0208 9542 300-3787 / s.ghoreishizadeh@ucl.ac.uk

If you are not satisfied with the resolution of your complaint, you can contact UCL using the details below for further advice and information:

The Chair,
UCL Research Ethics Committee,
Academic Services
UCL
Gower Street London
WC1E 6BT

ethics@ucl.ac.uk

9. **Will my taking part in this project be kept confidential?**
All the information that we collect about you during the course of the research will be kept strictly confidential. You will not be able to be identified in any ensuing reports or publications.

10. **Limits to confidentiality**
Confidentiality will be respected subject to legal constraints and professional guidelines.

11. **What will happen to the results of the research project?**
The anonymised data and results of the project will be written up in educational reports (undergraduate and postgraduate dissertations). The findings may also be presented at scientific meetings and contribute towards a paper publication in a journal. We will provide you with an electronic copy of the results of the study and the dissertations if you wish.

12. **Local Data Protection Privacy Notice**

**Notice:**
The controller for this project will be University College London (UCL). The UCL Data Protection Officer provides oversight of UCL activities involving the processing of personal data, and can be contacted at data-protection@ucl.ac.uk

Your personal data will be processed for the purposes outlined in this notice.

The lawful basis used to process your personal data will be 'Public Task' for personal data.

***Your personal data will be processed so long as it is required for the research project.*** If we are able to anonymise or pseudonymise the personal data you provide we will undertake this, and will endeavour to minimise the processing of personal data wherever possible.

If you are concerned about how your personal data is being processed, please contact UCL in the first instance at data-protection@ucl.ac.uk. If you remain unsatisfied, you may wish to contact the Information Commissioner's Office (ICO). Contact details, and details of data subject rights, are available on the ICO website at:
https://ico.org.uk/for-organisations/data-protection-reform/overview-of-the-gdpr/individuals-rights/

13. **Who is organising and funding the research?**
This research study is sponsored and funded by UCL

14. **Contact for further information**
Dr Sara Ghoreishizadeh,
Aspire CREATe, UCL – IOMS, Brockley Hill, Stanmore, Middlesex HA7 4LP.
0208 9542 300-3787 / s.ghoreishizadeh@ucl.ac.uk

You will be given a copy of the information sheet, and a signed consent form to keep if you decide to take part in the study.

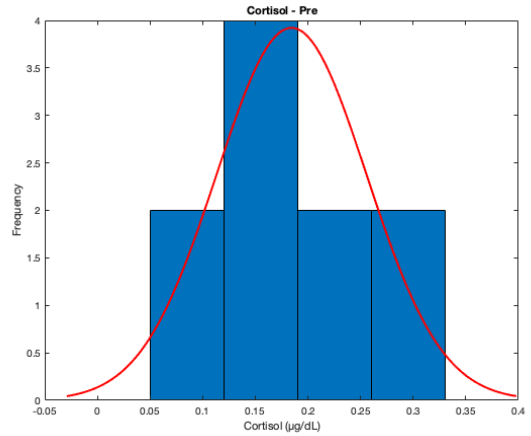
**Thank you for reading this information sheet and for considering to take part in this research study.**

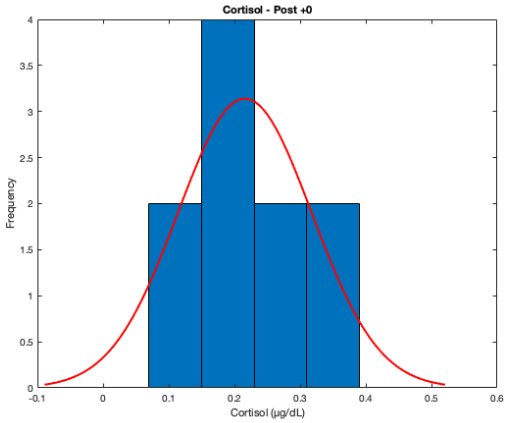
# APPENDIX B

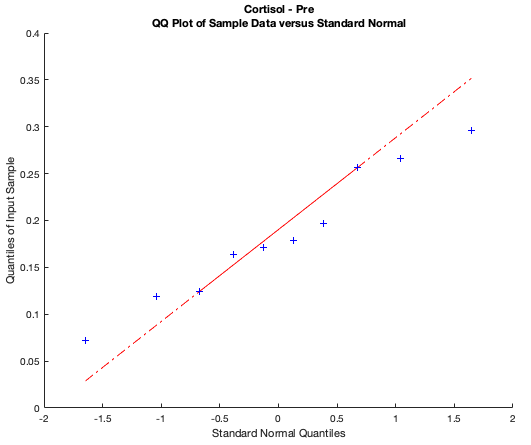
| | *p* – value (distribution) | | | |
|---|---|---|---|---|
| | **Pre** | **Post +0** | **Post + 20** | **Post + 60** |
| **Cortisol** | 0.87 (N) | 0.74 (N) | - | 0.088 (N) |
| **SP** | 0.17 (N) | 0.02 (NN) | 0.02 (NN) | 0.001 (NN) |
| **Glutamate** | 0.014 (NN) | 0.3 (N) | 0.044 (NN) | 0.29 (N) |

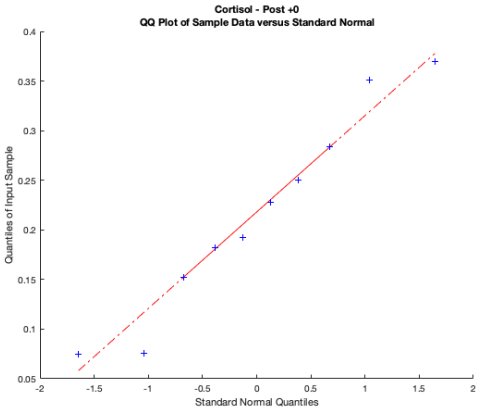
N – normal, NN – non-normal

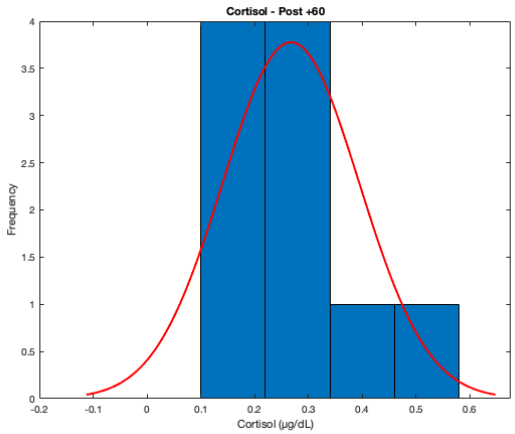
**CORTISOL**

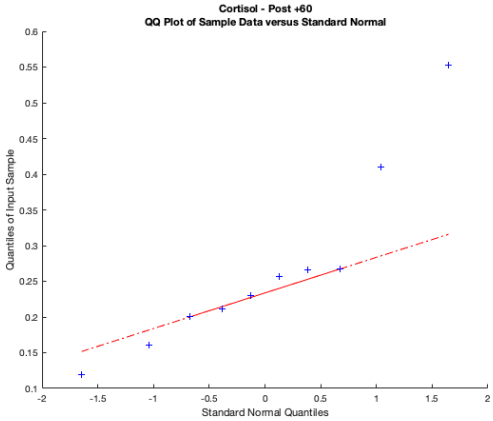


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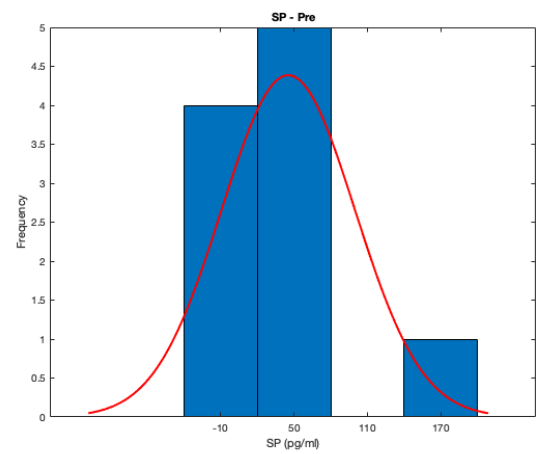
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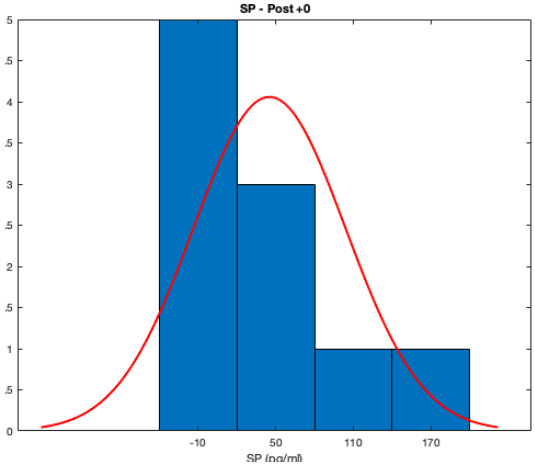
Frequency
SP (pg/ml)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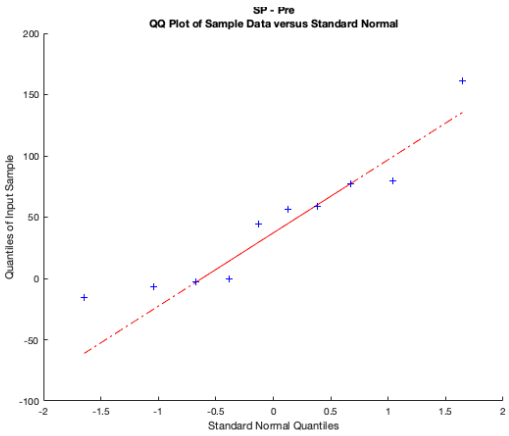
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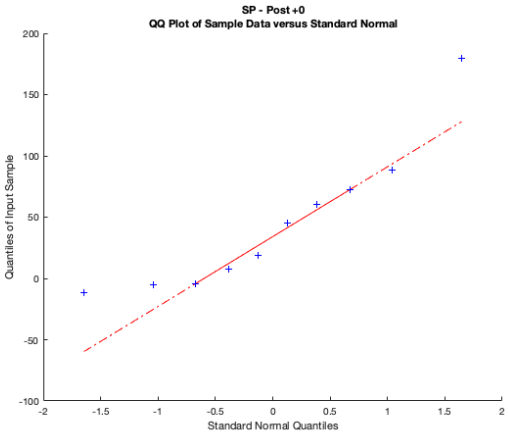
Quantiles of Input Sample
Standard Normal Quantiles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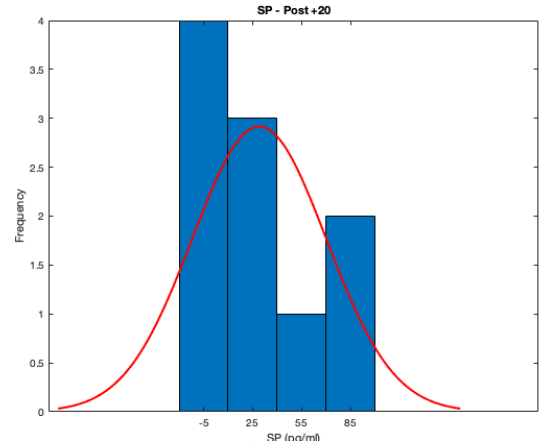
Quantiles of Input Sample
Standard Normal Quantiles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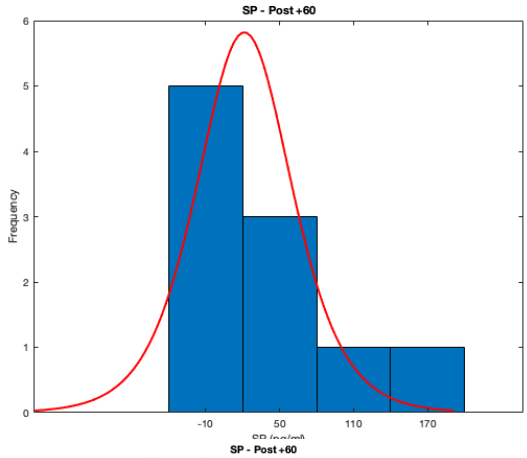
Frequency
SP (pg/ml)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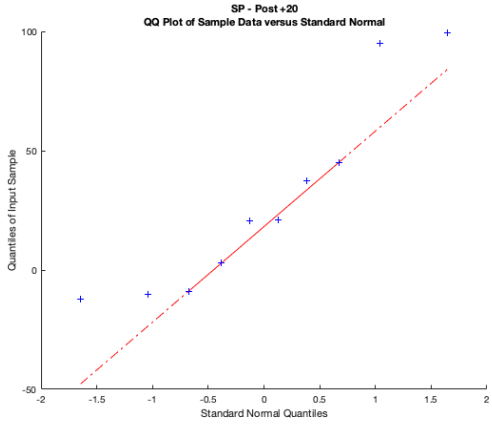
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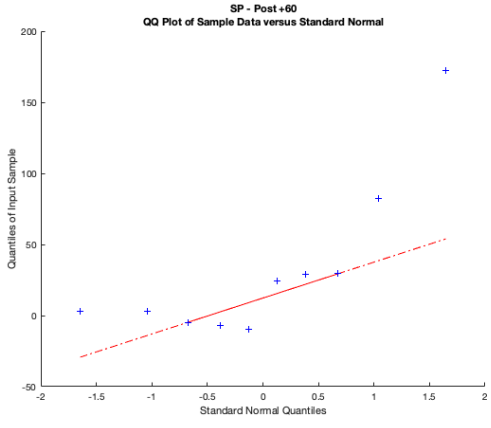
Quantiles of Input Sample
Standard Normal Quantiles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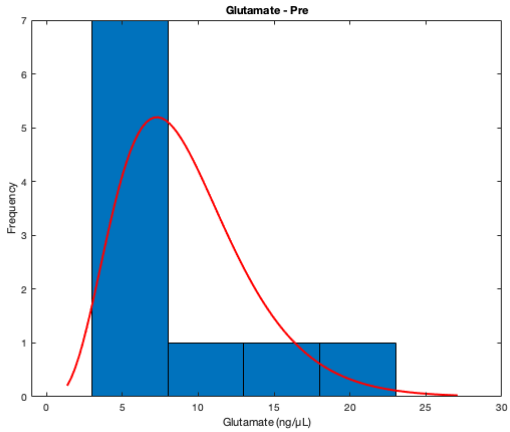
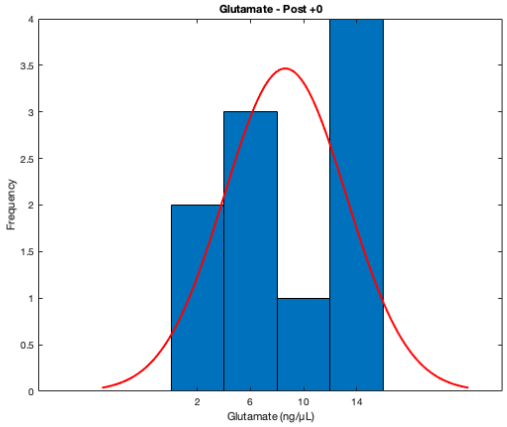
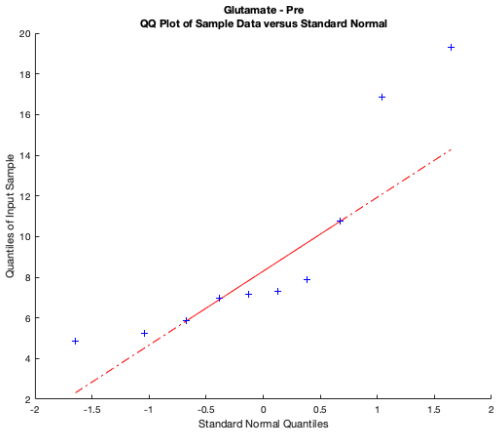
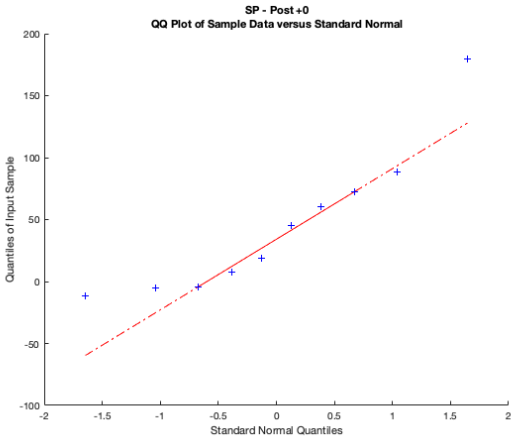
Quantiles of Input Sample
Standard Normal Quantiles

## GLUTAMATE


Glutamate - Pre
Frequency
Glutamate (ng/µL)


Glutamate - Post +0
Frequency
Glutamate (ng/µL)


Glutamate - Pre
QQ Plot of Sample Data versus Standard Normal
Quantiles of Input Sample
Standard Normal Quantiles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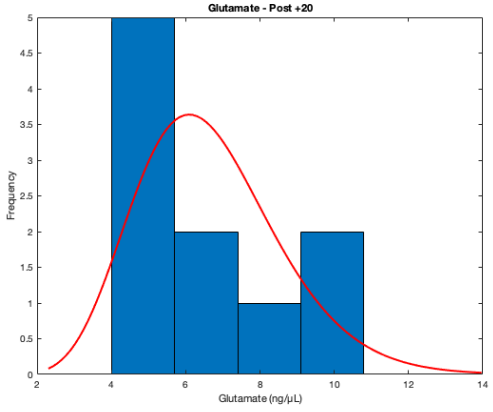
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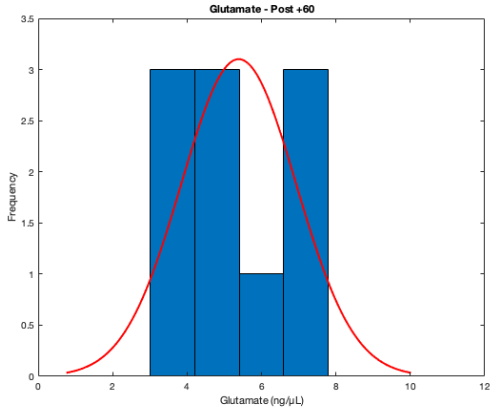
Frequency
Glutamate (ng/µL)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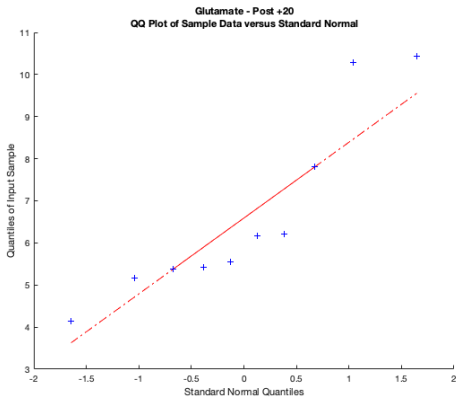
Frequency
Glutamate (ng/µL)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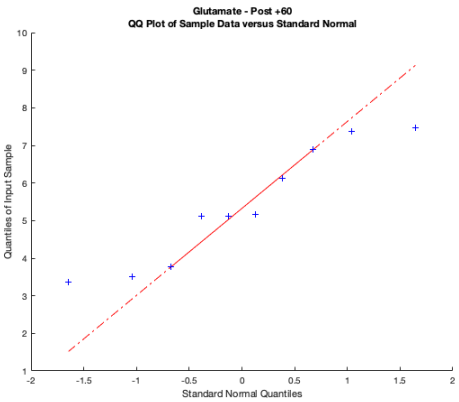
Standard Normal Quantiles


Glutamate - Post +60
QQ Plot of Sample Data versus Standard Normal
Quantiles of Input Sample
Standard Normal Quantiles

# APPENDIX C

## PARTICIPANT'S BLOOD PRESSURE (BP) AND HEART RATE (HR)

| | T -2 & T-1 | | T = 0 | | T = X | | T = X + 5 | | T = X + 10 | | T = X + 15 | | T = X + 20 | | T = X + 25 | | T = X + 30 | | T = X + 35 | | T = X + 40 | | T = X + 45 | | T = X + 50 | | T = X + 55 | | T = X + 60 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BP | HR | BP | HR | BP | HR | BP | HR | BP | HR | BP | HR | BP | HR | BP | HR | BP | HR | BP | HR | BP | HR | BP | HR | BP | HR | BP | HR | BP | HR |
| **P3** | 113/80 | 58 | 141/91 | 70 | 108/68 | 48 | | | 112/76 | 57 | | | 108/78 | 51 | | | 109/74 | 54 | | | | | | | | | | | | |
| **P5** | 132/85 | 66 | 150/98 | 69 | 126/92 | 72 | 122/81 | 67 | 113/80 | 68 | 121/74 | 56 | 123/81 | 62 | 114/87 | 62 | 124/84 | 61 | 121/84 | 66 | 125/90 | 70 | 112/81 | 64 | 118/77 | 68 | 117/79 | 69 | 112/73 | 61 |
| **P7** | 127/86 | 77 | 144/96 | 80 | 129/77 | 78 | 121/79 | 74 | 123/79 | 83 | 127/74 | 70 | 124/82 | 76 | 130/84 | 77 | 121/77 | 75 | 126/77 | 78 | 121/79 | 78 | 120/72 | 71 | 121/76 | 75 | 124/76 | 69 | 119/80 | 77 |
| **P8** | 93/70 | 74 | 117/86 | 88 | 99/67 | 69 | 109/88 | 67 | 94/66 | 68 | 96/67 | 70 | 100/72 | 68 | 111/70 | 60 | 98/73 | 76 | 102/74 | 68 | 97/67 | 67 | 95/68 | 64 | 109/89 | 67 | 110/69 | 66 | 95/70 | 70 |
| **P9** | 100/77 | 68 | 136/100 | 66 | 100/75 | 61 | 109/79 | 62 | 109/78 | 64 | 106/80 | 61 | 103/77 | 57 | 102/78 | 60 | 125/106 | 58 | 112/76 | 61 | 102/79 | 60 | 103/72 | 62 | 101/79 | 59 | 106/90 | 73 | 122/73 | 54 |
| **P14** | 124/89 | 75 | 135/99 | 79 | 125/85 | 78 | 116/83 | 66 | 121/85 | 77 | 125/80 | 63 | 119/94 | 77 | 119/80 | 68 | 116/82 | 75 | 124/88 | 73 | 106/82 | 74 | 118/92 | 72 | 123/93 | 75 | 119/83 | 67 | 118/87 | 70 |
| **P15** | 121/77 | 61 | 138/89 | 73 | 118/99 | 66 | 122/82 | 64 | 105/69 | 64 | 108/86 | 71 | 114/70 | 65 | 113/76 | 64 | 110/74 | 66 | 98/62 | 62 | 111/75 | 69 | 111/80 | 65 | 106/70 | 59 | 100/72 | 63 | 109/74 | 64 |
| **P16** | 105/85 | 71 | 125/99 | 78 | 96/77 | 72 | 101/82 | 69 | 104/90 | 71 | 101/87 | 75 | 112/95 | 82 | 101/87 | 72 | 100/81 | 73 | 109/94 | 69 | 105/92 | 71 | 107/85 | 68 | 97/81 | 73 | 113/95 | 72 | 106/92 | 76 |
| **P17** | 111/78 | 69 | 123/85 | 76 | 112/78 | 70 | 110/75 | 72 | 111/84 | 70 | 123/90 | 65 | 116/83 | 71 | 106/82 | 72 | 114/87 | 73 | 120/87 | 65 | 112/80 | 68 | 108/74 | 78 | 118/87 | 73 | 109/85 | 67 | 111/81 | 66 |
| **P19** | 126/84 | 72 | 154/95 | 74 | 136/84 | 69 | 130/81 | 67 | 135/90 | 74 | 134/93 | 74 | 126/82 | 76 | 122/85 | 69 | 129/89 | 76 | 117/83 | 72 | 128/85 | 80 | 136/84 | 71 | 140/90 | 83 | 125/85 | 76 | 126/87 | 76 |