Changes in salivary biomolecules in the adult human saliva with acute nociception: a scoping review with additional quantitative focus on cortisol

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Abstract

Background: Understanding the pattern of change of biomolecules involved in nociceptive pathways can aid the development of pain biomarkers that can be used to objectively assess nociception and pain. A systematic search of the literature was conducted to summarise all empirical evidence related to the acute pain effects on the production/variation of salivary biomolecules.

Methods: Five electronic databases were searched: MEDLINE, EMBASE, Web of Science, CENTRAL, and PubMed.

Results:

Conclusion:

Keywords: acute pain, experimental pain, biomarkers, saliva, scoping review

INTRODUCTION

Effective acute pain management is a humanitarian responsibility and is essential to recovery and rehabilitation after surgery and trauma [Levy 2016]. Achieving it relies on robust methods for pain assessment. Pain is by nature subjective [Raja 2020] and current pain assessment methods 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). Other assessment tools for pain and nociception are based on behavioural and physiological indicators and are used when patients are unable to self-report, for example, infants and young children, people under anaesthesia, or those with cognitive disabilities and mobility impairments [Cravello 2019, Varndell 2017]. However, these methods rely on the expertise of healthcare professionals, limiting their reliability [Cowen 2015]. Physiological parameters are not specific for nociception and may be indicators of other physiological responses or pathological processes [Ledowski 2019].

In these circumstances methods for measuring nociception would prove invaluable. One method for doing so could be based on monitoring the biofluid levels of a panel of nociception related biomolecules that are integral to nociceptive signaling and physiological stress. Saliva is a favourable bio-fluid as it can be obtained easily, rapidly and non-invasively.

Several studies have explored change in the salivary concentration of pain related biomolecules in the saliva. In this review we aimed to collate existing data in this field in order to (1) identify saliva-based biomolecules that can serve as useful biomarkers for acute pain, (2) determine whether the biomarker levels are correlated with pain intensity and (3) determine whether the changes in biomarkers are different in men and women.

We have done a more in-depth analysis of cortisol as the most studied biomolecule in the context of acute pain, particularly after controlled exposure to cold pain.

METHODS

Design

This review has been conducted in line with the reporting guidelines for scoping reviews: Preferred Reporting Items for Systematic Reviews and Meta-Analyses Extension for Scoping Reviews (PRISMA-ScR) [Tricco 2018]. The protocol of the review was registered with the Open Science Framework (OSF) on 10th December 2020 (ID:wk8em) [Vounta 2020]. The review has one minor deviation from the registered protocol. This has been explained in the Data Synthesis section.

Information sources and search strategy

A preliminary search was conducted in Medline to help us develop a list of the key search items. A literature search was then performed in five electronic bibliographic databases: Ovid MEDLINE (1946 to July 28, 2020; Appendix A.1), Ovid EMBASE (1980 to 2020 Week 30; Appendix A.2), Web of Science (inception of database to 28/07/2020; Appendix A.3), CENTRAL (inception of database to 28/07/2020; Appendix A.4), and PubMed (inception of database to 28/07/2020; Appendix A.5). These databases were selected as they contain the majority of the literature published in this research field. No previous published reviews or registered review protocols investigating this research topic were found in the preliminary search or afterwards.

Final search terms were grouped to include three concepts related to our research question: a) saliva, b) biomolecules, c) nociception, acute pain, or acute pain conditions. Terms were adapted for use with the selected bibliographic databases and database-specific filters for human studies only were applied. The search strategy was not limited by study design, language, or publication year. Two reviewers (AV and QL) independently searched all five databases for relevant studies according to the search strategy. The reviewers set up email alerts in all five databases to identify new relevant records if one was published after the main search (conducted in July 2020) had ended. The final search strategy along with the dates the searches were last performed, and the total number of records obtained from each database search are reported in the online registered protocol [Vounta 2020], following the PRISMA-S checklist for reporting literature searches [Rethlefsen 2021]. The search strategy is presented in Appendices A.1-5 for reference.

Study selection

All retrieved records from the search of the electronic databases were imported to EndNote X9 software. Two reviewers (AV and QL) removed duplicates and assessed the titles and abstracts for inclusion independently. The full texts of potentially relevant articles were then screened against the inclusion and exclusion criteria for eligibility for final inclusion and reasons for exclusion were recorded. Any disagreements in study selection were resolved after discussion with the other authors (SG and RZ). The reference lists of all included articles were hand-searched to identify any additional and relevant articles.

Criteria for selecting studies for this review

The inclusion and exclusion criteria are listed in Table 1. In summary, studies were included that had adult participants who either had (i) acute nociceptive pain for less than 6 weeks duration secondary to acute pathology or injury or (ii) acute experimentally induced pain. Examples of included acute pain conditions are post-operative pain, burns pain, zoster pain, acute (spinal) disc prolapse, fracture pain, renal colic, and biliary colic. Studies that did not include any pain assessment method were excluded. Studies with patients suffering from chronic pain or chronic painful conditions were included only if acute pain was induced as part of the study or the patients suffered an event that caused acute pain. Included studies were required to have compared the biomolecule levels after the acute pain episode with a form of a reference level for that biomolecule. The reference level could be either the baseline biomolecule concentration before acute pain or the biomolecule concentration of a control group.

Table 1. Summary of the inclusion and exclusion criteria for selecting the sources of evidence.

Attribute	Inclusion criteria	Exclusion criteria

Study population	Human adults (≥18 years): - with experimentally induced acute pain on a background of no pain or a background of a chronic painful condition - with acute pain conditions (such as post-operative pain, burns pain, zoster pain, acute (spinal) disc prolapse, fracture pain, renal colic, or biliary colic) of less than 6 weeks duration	• Age <18 years • Animal studies • Studies with participants who: - had chronic pain, a chronic painful condition, or acute pain that is an exacerbation or flare-up of a chronic or recurrent pain problem (for example migrainous acute headache) - had labour pain or postnatal pain - had a condition that disrupts the normal physiological conditions in the oral cavity (e.g. oral mucositis, oral diseases, or acute dental pain) - had substance dependence or abuse
Type of article	• Peer-reviewed published observational studies in any setting • Studies designed to detect and measure any type of change in nociception-associated biomolecule in human saliva • Studies in any language and with any publication date	Review articles, case reports, conference abstracts, publications without data, letters, and comments
Study outcomes	• Studies that have investigated change in salivary pain-associated biomolecules in acute pain, AND • compared these to a baseline level, either before the onset of acute pain or using a control group	• Studies that did not include a pain assessment method

Data collection and data items

A data-charting form was developed for data extraction. Two reviewers (AV and QL) independently charted data from each eligible article and any disagreements were resolved by discussion between all the authors. The following data were extracted from each of the papers:

(1) article characteristics, including author(s) names, year of publication, study aims;

(2) study design, including (i) study population, sample size, and participant demographics (age, gender, medical history including chronic pain, regular analgesia intake, psychiatric disorders (e.g. clinical depression and anxiety), (ii) type of pain including the type of acute pain condition or the details of pain induction method, (iii) pain assessment, including the pain scale used, time of measurements, and number of pain measurements performed,

(3) study methodology: (i) recruitment method, (ii) power calculation, (iii) biomolecule(s) measured, (iv) sample collection strategy including the saliva collection method, time of measurements, and number of saliva samples taken, (v) sample analysis technique (e.g. type of assay quantification);

(4) outcomes: (i) pain severity scores, (ii) change in salivary biomolecule concentration levels, (iii) patterns of change in salivary biomarkers in response to acute pain (onset of change, correlation of changes with pain intensity and gender), (iv) statistical significance of the observed differences between collection time points and between groups, (v) type of reference level used in the paper (baseline levels or control group).

Data synthesis

Narrative synthesis and structured tabulation of results

A narrative synthesis approach was adopted in which the reported variations in biomolecule levels were analysed and compared [Popay 2006]. The gender-dependent variations and the correlation between biomolecule concentration change and pain severity were included where data were available. The included studies were grouped based on the type of biomolecule studied and the modalities of pain sensation that the study participants experienced. Papers that reported on studies of more than one biomolecule appear in more than one category.

Differences between the registered protocol and this review

In addition to the synthesis methods specified in the study protocol, we opted to do a more in depth quantitative analysis of changes in salivary cortisol concentration after exposure to cold pain. We took this decision because studies investigating salivary cortisol after cold pain were the highest in number overall across different pain induction methods and different biomarkers. The aim of this synthesis was to determine whether there is good evidence for rise in cortisol after cold pain and the pattern of this change.

For this quantitative analysis, we ran all the included papers that reported salivary cortisol change with pain through an additional set of inclusion/exclusion criteria. Here only studies whose participants were healthy, taking no medications, and who underwent experimentally induced cold exposure shown to be painful as evidenced by significant increase on a validated acute pain scale, were included. Studies were excluded from this data synthesis if the participants had also been exposed to another stressor (for example a cognitive task) in the same experiment. Where there was more than one arm to the study, only participants who met the inclusion criteria and had not been exposed to other stressors were included.

Data synthesis method

After contacting corresponding authors of many of the studies, we were unable to obtain additional data on missing elements such as precise p values and effect size estimates, which limited our options with respect to data synthesis methodology [McKenzie and Brennan 2021]. The selected data synthesis method used is therefore vote counting based on direction of effect [Hilton Boon and Thomson 2020].

Study outcomes were defined as increase in salivary cortisol levels (positive direction of effect), decrease in salivary cortisol levels (negative direction of effect) or no clear effect (NCE) compared with baseline. In order to determine the timing of maximum change using this method, we defined three outcome domains based on the time of the measurements after cold pain induction: change in cortisol levels a) less than 10 minutes, b) between 10 to 20 min and c) more than 20 minutes.

These outcome domains were applied to each study. When all the time points within an outcome domain reported the same effect direction, we reported that direction as the effect direction. In studies that had multiple time points within an outcome domain (for example, measurements at t=+5' and t=+9' minutes after cold pain both falling within the first outcome domain), the effect direction was determined using the method described by Hilton, Boon and Thomson (2020). Where the effect direction varied within an outcome domain, we reported the direction of the clear majority (at least 70%). If less than 70% of outcomes reported consistent effect direction, then we reported "no clear effect" (NCE). The baseline concentration used for comparison was considered to be the pre-CPT salivary cortisol concentration that was taken at the time point closest to the onset of the cold pain task. Statistical significance and size of the effect were not considered in the categorization [McKenzie and Brennan, 2021]. In the effect direction plot (Fig), in each domain, an upwards arrow represents the results for the outcome domain being in a positive direction, and a downwards arrow, a negative direction. A bi-directional arrow indicates no clear effect (or NCE).

Statistical Analysis

Taking the null hypothesis as "there is no difference between baseline and post-pain salivary cortisol levels *or* there is an equal number of positive and negative effects", we used the sign test [Hilton Boon and Thomson,2020] to determine whether there is enough evidence to reject this in each outcome domain. Studies with NCE for any outcome domain were excluded from the analysis for that outcome domain, because they did not represent a clear effect direction (either positive or negative effect). The p-value was calculated for the three domains, separately, using GraphPad (https://www.graphpad.com/quickcalcs/binomial1/) as recommended by Hilton Boon and Thomson (2020). The level of statistical significance for the test was set at 0.05.

Assessment of Study Quality

Risk of bias assessment in the included studies was done independently by two of the authors (RZ and SG). Disagreements were resolved by discussion and consensus. We used

the Risk of Bias In Non-randomized Studies of Interventions (ROBINS-I) [Sterne 2016] and assessed the included studies in the 7 domains of confounding, selection bias, misclassification of interventions, deviations from intended intervention, missing data, outcome measurement and reporting.

Assessment of Heterogeneity

The included studies were assessed for methodological heterogeneity in conducting the cold pressor task and salivary sampling.

RESULTS

Selection of sources of evidence

A PRISMA flow diagram has been provided in Figure 1. The search of the five electronic databases initially yielded a total of 1886 records: 365 in MEDLINE, 633 in EMBASE, 227 in Web of Science, 280 in CENTRAL, and 381 in PubMed. Two other studies were identified through email alerts after the main search had been completed CHECK. After the removal of duplicates, 1023 records remained. After screening the title, abstract, and keywords of the articles for eligibility, a further 962 records were excluded. All the abstracts and papers assessed throughout all the stages of screening were in English, although no language limit was applied to the research. After screening the full-text records of the remaining 61 papers, 26 papers were excluded leaving 35 articles that fulfilled the criteria for inclusion (Table 2). The reference lists of the included articles were also studied for potential studies, but no additional articles were identified. Full citations of the included studies are listed in alphabetical order in Appendix C.

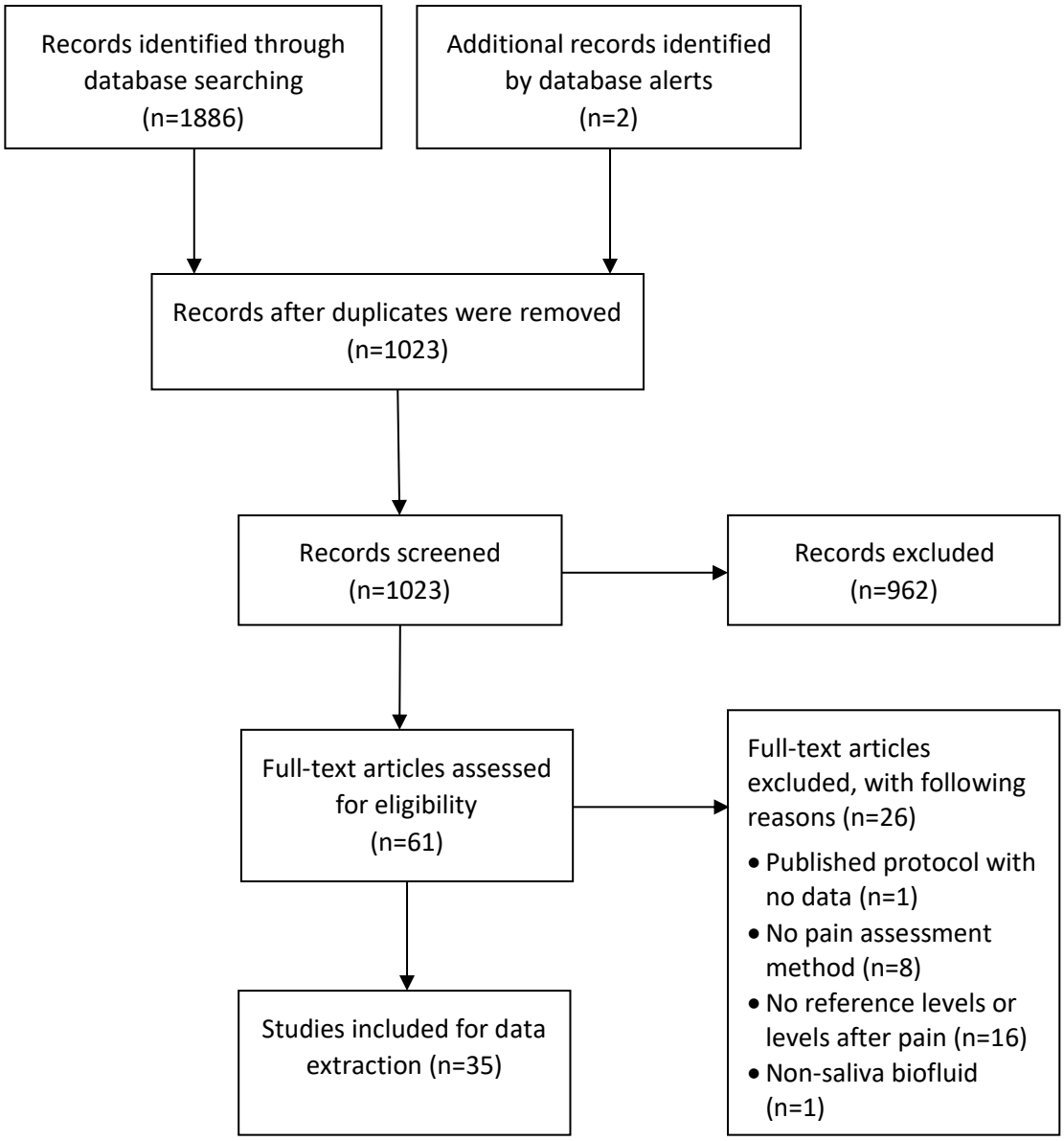

Figure 1. PRISMA flow diagram summarizing the stages of the selection process in this work.

Characteristics of included studies

A summary of characteristics of the included studies such as the type of pain, type of participants, and saliva sampling methods that were used in the included studies, is discussed in this section (Table 3).

Study characteristics (n=35)		Count	Papers
Type of acute pain studied	Acute pain condition	4	P37, P28, P29, P33
	Experimentally induced pain	31	P1-P3, P6, P8-P22, P24-P27, P30-P32, P34-P36, P38, P39
Presence of chronic pain in participants	No	31	P1-P3, P6, P8-P17, P21, P22, P24-P26, P28-P39
	Yes	4	P18, P19, P20, P27
Participants' intake of pain medication	No	15	P38, 36, 35, 20, 25, 26, 29, 31, 32, 27, 14, 10, 9, 6, 1
	Yes	6	P3, P8, P18, P22, P37, P39
	Not reported	14	P2, P11-P13, P15-P17, P19, P21, P24, P28, P30, P33, P34
History of psychiatric disorders	No	17	P3, P6, P9, P10, P14-P17, P20-P22, P25-P28, P36, P39
	Yes	2	P18, P29
	Not reported	16	P1, P2, P8, P11-P13, P19, P24, P30-P35, P37, P38

Type of acute pain in included studies

Of the 35 included articles, the majority (n=31) involved experimentally induced pain [P1-P3, P6, P8-P22, P24-P27, P30-P32, P34-P36, P38, P39]. The remaining 4 studies involved acute pain after surgery or a medical procedure: elective hysterectomy and/or oophorectomy for non-malignant disease [P37], surgery [P28, P29] and gastric or bronchial tube replacement. [P33].

1.1.1.1.Experimental pain induction methods

Six different pain induction methods were utilized in the experimentally induced pain studies. These are, in order of frequency, cold pain, heat pain, mechanical/visceral pain, ischemic

pain, chemical pain and electrical stimulation. More details about the pain induction methods and a list of papers that employed each method are summarized in the Table 4.

Three studies used more than one pain induction method [P25-P27]. Studies involving the most frequently used pain induction technique (the cold pressure task) are presented with a greater level of granularity.

Twenty four of these 31 papers, used clear standardized pain induction methods with citation [P1-P3, P6, P8-P16, P19, P21, P25, P26, P30, P31, P32, P34, P36, P38, P39]. The remaining 7 used either a novel technique that others had not tried or a known pain induction technique but with no cited reference [P17, P18, P20, P22, P24, P27, P35].

Table 4: Summary of the experimental pain induction methods used in the included studies. A short description is given summarizing the main features of each pain task identified across the included studies. 31 out of 35 papers used experimentally induced pain

PUT NUMBERS IN THE TABLE

Type of experimental pain	Pain induction method	Description of method	Included studies (Study IDs)

Cold pain	**CPT** n = 17	Immersion of a body region in a cold water bath for a pre-specified maximum time of cold exposure or for as long as the subject could tolerate it **Water temperature** [a] 0-5 [b] 8-10 **Body region immersed** [c] Hand or arm [d] Foot or feet **Endpoint (sec)** [e] 45 [f] 60 [g] 90 [h] 180 [i] 240 [j] 300	**P1-P3, P6, P8-P12, P25-P27, P30, P34-P36, P39** al' Absi 2003 [a, c, g] al' Absi 2002 [a, c, g] Bachmann 2003 [a, d, h] Goodin 2012 [a, c, j] Hengesch 2018 [a, d, h] Larra-Cinisomo 2015 [a, c/d, h] Nakajima 2011 [a, c, g] Niedbala 2018 [a, c, h] Serrano 2019 [a, c, f] Goodin 2012 [a, c, j] Goodin 2012 [a, c, j] Quartana (2010) [a, c, e] Youssef (2018) [a, c, j] Archey 2019[a, c, j] Burns (2004) [b, c, i] Cruz-Almeida (2004) [b, d, f] Finke (2021) [a, d, h]
	Plunge test n = 1	Intermittent immersion of hand and forearm in a cold water bath of 5°C with duration of immersion and rest periods of 5, 10, or 15 seconds	**P13** Zimmer 2013
Heat pain	**HPT** n = 7	Administration of heat stimuli on the volar forearm with a Peltier device (two thermal stimulators) at a temperature of 35-52°C for 6-50 sec or until pain tolerance was reached	**P14-P18, P27, P31** Gaab 2016 Geva 2014 Geva 2017 Geva 2018 Muhtz 2013 Quartana 2010 Wittwer 2016

	HWT n = 3	Immersion of hand or arm in a bath of circulating hot water (46-47ºC) for 2 min, or for as long as the subject could tolerate it, with a maximum pain exposure of 5 min	**P19, P25, P26** Meeus 2008 Goodin 2012 Goodin 2012
Mechanical / visceral pain	**MPT** n = 6	Application of algometer on several body regions *at an increasing force rate until pain threshold was reached or †at 10N force for 2 minutes	**P20, P21, P27** Geiss 2012* Hoeger Bement 2010† Quartana 2010*
		Balloon rectal distensions placed 5 cm from the anal verge at distension pressure of 2-55 mmHg; six rectal distensions for 30 seconds each	**P22** Icenhour 2020
		Lying on the back on a bed of sharp-edged plastic nails (Shakti-mat) for 20 min.	**P24** Olsson 2011
Ischemic pain	**IPT** n = 2	Modified submaximal effort tourniquet procedure, exercising the hand while blood flow to the arm is occluded for as long as tolerated, with a maximum task duration of 15 min	**P25, P26** Goodin 2012 Goodin 2012
Chemical stimulation	**Saline injection** n = 1	Intra-muscular injection of hypertonic saline solution (0.4 mL) into the masseter muscle over About 30 seconds	**P32** Christidis 2020
Electric stimulation	**Electric shock** n = 1	Electric shock stimuli using a bipolar cutaneous electrode stimulator on the volar forearm; 20-impulse train of electroshocks with a duration of 5 ms to produce pinching pain	**P38** Nelson 2001
CPT: cold pressor task; HPT: heat pain task; HWT: hot water task; PPT: pressure pain task; IPT: ischemic pain task; min: minutes; sec: seconds; ms: milliseconds; cm: centimetres; N: Newton; mmHg; millimetres of mercury, mL; millilitres.			

Reference levels

Thirty-four of the 35 included studies measured baseline levels and compared them with the post-pain levels measured from the same participant group. One study did not collect any

baseline samples and instead used measurements from a control experiment (healthy participants experiencing no pain) as reference levels [P24].

Pain medication (analgesia) intake

Analgesic drugs interfere with nociception mechanisms and the biomolecules involved in nociceptive pathways [Kirkpatrick 2016]. Therefore, information related to pain medication intake was extracted from the included studies. In 15 of the 35 papers (P1, P6, P9, P10, P14, P20, P25-P27, P29, P31, P32, P35, P36, P38) participants with regular analgesia intake had been excluded from the study. Four studies (P3, P8, P22, P39) included participants that occasionally used pain killers. One study (P18) included participants with chronic low back pain who were treated with regular pain medication including opioids and one study (P37), with participants who underwent gynaecological surgery, allowed administration of postoperative pain medication including morphine. The remaining 14 studies (P2, P11-P13, P15-P17, P19, P21, P24, P28, P30, P33, P34) did not report on analgesia intake.

Participants with chronic conditions

Owing to the considerable overlap in biomarkers associated with pain and those associated with stress, chronic conditions, including chronic pain and psychiatric disorders, can affect pain related biomarkers. Of the 35 included studies, 17 studies (P3, P6, P9, P10, P14-P17, P20-P22, P25-P28, P36, P39) specifically excluded participants with psychiatric disorders. In two studies (P18, P29), participants suffered from a psychiatric disorder such as depression or anxiety, while the remaining 16 studies (P1, P2, P8, P11-P13, P19, P24, P30-P35, P37, P38) did not report whether any psychiatric condition was present.

Most studies of experimental pain induction (27 out of 31) excluded potential participants with chronic pain. In the remaining experimental pain studies, the enrolled participants had chronic low back pain (P18), chronic fatigue syndrome with chronic pain (P19), fibromyalgia syndrome (P20), and temporomandibular disorder (P27).

Saliva sampling technique

Table 5 shows the saliva collection procedures in the included studies.

Whole saliva is a mixture of secretions from major (parotid, submandibular and sublingual) and minor salivary glands and also contains several non-salivary components [Kaufman and Lamster, 2002]. Oral mucosal transudate (OMT) is an oral fluid collected from the tissues between the cheeks and the gums and derives from the passive transport of serum components through the oral mucosa into the mouth [Jasim, 2018].

Several methods are available for collection of whole saliva or saliva from individual glands [Bhattarai 2018]. Unstimulated parotid saliva or submandibular and sublingual gland secretions can be obtained using a collector at the parotid duct opening opposite the maxillary second molar or at the sublingual ducts at the floor of the mouth. Whole saliva can be collected by tilting the head slightly forward and spitting into a test tube or allowing saliva to dribble from the mouth (unstimulated passive drool). Salivary flow can be stimulated using aqueous 2% citric acid solution applied to the tongue or chewing gum.

The main external factors that affect the salivary concentration of various biomolecules are the method of saliva collection and the degree of stimulation [Helmerhorst & Oppenheim 2007, Jasim 2016]. Although these effects are well recognized [Jasim 2018], reporting of the saliva sampling method is inconsistent. Two studies provided no information on the saliva collection method [P28, P38]. Eight studies did not clearly report whether this was stimulated [P3, P8, P18, P19, P20, P22, P39, P28], although this may be of little consequence where the salivary concentration is known to be independent of salivary flow (e.g. for cortisol). In all the included studies that reported the saliva collection method, a single collection method was used for any single biomarker.

Food, alcohol, nicotine and caffeine intake are known to affect salivary flow [Hildebrandt 2013, Batista 2019]. In 10 of the included studies the experimenters placed a restriction on the intake of all these [P3, P6, P9, P25, P26, P27, P30, P32, P35, P36]. There were 6 studies in which none of these restrictions were reported to have been applied [P12, P18, P22, P28, P34, P38].

The restrictions were variably applied and the level of these also varied considerably. The range for food restriction was 30 - 120 minutes (mode 60 minutes) and for caffeine 1 - ≥12 hours (mode 12 hours). Smoking and alcohol were even more variably restricted, sometimes as length of time, and sometimes as dose. One of the included studies was specifically designed for investigating responses to cold pain in smokers [10]. Three included participants if they smoked less than 5 cigarettes a day [P39, P3, P9], one included those smoking less than 15 a day [P8] and 4 only asked participants to not smoke on the day of the experiment or in the preceding 12-24 hours [P11, P19, P21, P24]. The rest excluded smokers. Alcohol was restricted as the number of units or "drinks" a day in 7 studies [39, 1, 2, 8, 10, 14, 33] and by asking participants to avoid intake for anything between 1 hour to 24 hours prior to the experiment in 14 studies [3, 6, 9, 19, 21,25, 26, 27, 30, 31, 32, 35, 36, 37] . The remaining 14 studies did not report or collect data on alcohol intake. [38, 11, 12, 13, 15, 16, 17, 18, 20, 22, 24, 28, 29, 34].

Seven of the studies included a restriction of 0.5 – 3 hours on tooth brushing as well as eating to avoid blood contamination from micro-injuries to the mucosa [6, 21, 25, 26, 31, 32, 36].

Table 4. Saliva collection techniques used in the included studies (n=35). The number of studies that used each collection method and a brief description of each technique is presented in the table.

Saliva collection device		**Description**	**Type of saliva collected**		
			Unstimulated	**Stimulated**	**Not clear**
Swab device	**Salivette®** 25	Whole saliva collected by chewing on a cotton swab and placing into a tube, later centrifuged to recover clear saliva for analysis.	P1, P2, P13-P17, P27, P31, P36	P6, P10, P21, P24, P30, P35	P3, P8, P18-P20, P22, P39
	OraSure® 2	Oral mucosal transudate (OMT) collected by a swab placed between the lower cheek and gum	-	P25, P26	-

	Q-tip® 1	Whole saliva collected by a pre-weighed, cotton-tipped applicator rolled and placed under the tongue for a maximum of 5 min	-	P37	-
Monitor device 1		A hand-held monitor device with a disposable test strip which consisted of a collecting sheet attached to collecting papers. Sample collection after placing the collecting papers under the tongue	-	P33	-
Passive drool 5		Whole saliva accumulation in the mouth and dripping off the lower lip into a funnel attached to a collection vessel	P12	P11, P29, P32, P34	-
Spitting 1		Whole saliva accumulation on the floor of the mouth and collection into tubes after expectoration	-	P9	-
Not reported 2		Saliva collection method not reported	P38	-	P28

Synthesis of results:

The biomolecules that were measured across the included studies were: melatonin (n=1) (P38), kallikrein (n=1) (P37), pro-inflammatory cytokines (interleukins, IL-6, IL-8, IL-10, IL-4) (n=2) (P36, P20), immunoglobulin A (IgA) (n=2) (P29, P35), testosterone (n=2) (P29, P34), soluble tumor necrosis factor-alpha receptor II (TNFαR-II) (n=3) (P25, P26, P29), a-amylase (n=8) (P9, P12, P14, P29-P33) and cortisol (n=26) (P1-P3, P6, P8-P22, P24-P29, P39). A summary of the findings on each biomolecule is presented in a narrative synthesis in this section.

Melatonin

Melatonin is a neurohormone that has a major role in the regulation of the circadian rhythm in vertebrates. It has been shown to have anti-inflammatory properties. In animal models, melatonin has been shown to reduce hyperalgesia Responses to noxious stimuli are different in day time compared with night time with higher nociceptive thresholds during darkness. In surgical and cancer patients pain intensity and analgesia use are higher in the day time. However, an analgesic response in humans has not been definitively demonstrated and the mechanism through which this might occur remains unclear [Xie 2020].

P38 et al investigated the relationship between salivary melatonin levels and nociception in 18 healthy people, who took no analgesia, after acute pain induced by electric stimulation. Melatonin levels changed less than 5 minutes after the pain stimulus with an initial decrease followed by a rise. There was then a reduction until levels similar to those anticipated for the time of day was reached and the levels did not change after this. Correlation between pain ratings or gender effects and melatonin levels were not analysed [P38].

Kallikreins

Kallikreins are a family of proteases that are responsible for several physiological functions including blood pressure regulation and inflammation [Prassas 2015]. P37 et al reported a significant increase in salivary kallikrein levels after elective hysterectomy with or without oophorectomy for benign disease. Compared with baseline preoperative levels, salivary kallikrein increased significantly at two, four, and six hours after surgery, but not at one hour. The peak increase (8x higher than pre-operative levels) was at the 4 hour time point. The reported pain levels did not follow this pattern. Pain levels peaked at one hour and declined after this point [P37]. No analysis by gender was done.

Pro-inflammatory Cytokines and soluble tumour necrosis factor α receptor II (sTNF-αRII)

Pro-inflammatory cytokines including interleukins and tumour necrosis factor (TNF) are known to have a role in the development of the sequelae of nerve injury and chronic pain conditions [Totsch 2017]. P36 et al measured change in a panel of cytokines (IL-6, IL-8, IL-10, IL-4) in the saliva and blood of healthy participants who took no analgesia, after CPT or after a thermal water task that was not painful [P36]. The non-painful task was done on the same participants with and without venepuncture. IL-6, IL-10, and IL-4 concentrations peaked 60 minutes after CPT and IL-8 peaked at 45 minutes. In contrast, no significant changes were reported in participants who completed a non-painful thermal water task. Venepuncture had no significant effect on the cytokine levels. The relationship between change in the cytokine levels and gender was not analysed. In this study, the time course of the peak levels of cytokines in the CPT session was nearly identical in saliva and plasma. In another study, salivary IL-6 levels were measured alongside salivary cortisol in female patients with fibromyalgia and compared with levels in healthy pain-free women [P20]. Pain was induced by measuring pressure pain thresholds in 8 defined anatomical points and salivary samples were taken over the next 6.5 hours. In women with fibromyalgia, IL-6 (and also cortisol) increased significantly after measuring pain pressure thresholds but this did not happen in the healthy subjects. The relationship between pain intensity and IL-6 increase was not analysed.

The membrane TNFα receptor 2 (TNFαR-II) has a neuroprotective role. Soluable TNFα receptor 2 (sTNFαR-II) is the circulating form of this membrane bound receptor and it has a role in the regulation of TNFα signaling. Two of the three studies on salivary sTNFαR-II [P25, P26] were aiming to assess salivary pro-inflammatory cytokines after acute pain induction and chose to measure this biomolecule because it is more stable than TNFα and can be measured more reliably. All three studies [P25, P26, P29] that analyzed the changes in salivary sTNFαR-II levels after acute pain showed significant reduction in the levels. Two of these [P25 & P26] were studies of pain induction in healthy volunteers taking no analgesia after exposure to multiple pain modalities (cold, heat and ischaemic pain). A significant

reduction was observed immediately after pain induction in one study and [P26] and 25-35 minutes later in the other [P25]. P29 et al showed significant reduction in sTNFαR-II levels one hour after corneal surgery. There was no significant correlation with pain ratings in the 2 studies that analysed this [P25 & P29]. None of the studies reported analysis by gender.

Secretory Immunoglobulin A (sIgA)

There is known relationship between stress, including that induced by CPT and fall in sIgA. Change in sIgA in the saliva has been studied in relation to pain in a number of experiments although the mechanisms that would explain the association between sIgA and pain remain unclear. We found two studies [P29, P35] that had analyzed salivary IgA changes and also measured acute pain. In a study of healthy participants taking no analgesic medication, P35 et al found that sIgA levels fell significantly after first exposure to CPT, but not after second exposure in the same participants' other arm [P35]. In this study, correlation of salivary IgA levels with pain intensity and gender were analysed and no significant differences were found.

In an investigation of healthy participants taking no analgesia who underwent corneal surgery, [P29] et al measured sIgA at two sampling time points one hour and 72 hours post-surgery and found significant increase at one hour after surgery compared to pre-operative baseline levels. The sIgA levels fell back to baseline at 72 hours post-surgery. There was a significant positive correlation between pain intensity and change in sIgA one hour post-operatively compared with baseline. Gender differences were not analysed.

Testosterone

There is evidence from animal studies that testosterone has a role in analgesia and anxiolysis and may have a protective effect in the development of long term pain [Lesnak 2020]. Two studies [P29, P34] investigated salivary testosterone levels before and after acute pain. P29 et al did not find a significant change in testosterone levels post-operatively in their study of biomarkers after corneal surgery. In a study that was specifically designed to examine the

role of testosterone in female pain perception and how this might be different to males, P34 et al did not find a significant difference in salivary testosterone between males and females after undergoing a cold pressor task in healthy participants who did not take analgesic medication. Look up discrepancy with page 12 table (analgesia intake).

Salivary alpha-amylase

Salivary alpha-amylase (sAA) is a salivary enzyme synthesized mostly by the parotid gland. Numerous studies have showed that sAA increases in response to sympathetic nervous system over-activity and psychological stress [Nater and Pohleder 2009], leading investigators to examine change in sAA in painful conditions.

We found 8 studies that have measured change in sAA after a pain stimulus [P9, P12, P14, P29, P30, P31, P32, P33]. In 4 studies, pain was induced in healthy participants not taking any analgesic medication: cold pain in 2 studies [P9, P30 discrepancy with page 12], heat pain in one study [P31] and hypertonic saline muscle injection in one study [P32]. The cold and heat pain induction experiments reported significant rise in sAA. A significant positive correlation between sAA activity and pain intensity was reported in the study with heat pain induction [P31] but this was not analysed in the cold pain studies. No change in sAA was found after hypertonic saline injection.

In a study designed to observe the impact of psychosocial stress on heat pain perception in healthy men (P14), heat pain alone was not associated with significant change in sAA, while psychosocial stress was.

P12 et al compared the influence of the catechol-O-methyltransferase (COMT) Val158Met polymorphism on change in sAA after CPT and found significantly greater change in sAA in COMT Met allele carriers compared with Val homozygotes at the 20 minute post-CPT time point. Pain ratings increased significantly immediately after CPT but were not affected by

COMT polymorphism. The authors concluded that the COMT genotype influences the stress response to painful stimuli. The correlation of sAA with pain intensity was not studied

P33 et al measured sAA in people with severe motor and cognitive disabilities undergoing medical procedures and found rise in sAA that correlated with pain intensity. However, acute pain after corneal surgery was not associated with rise in sAA [P29].

Two of the included studies analyzed their data to look for potential sex differences in sAA variation [P9, P32]. Neither found any significant differences in sAA change response cold between the sexes.

Cortisol

Cortisol is the most significant glucocorticoid released as a result of hypothalamic-pituitary-adrenal axis (HPA) stimulation. The paraventricular nucleus (PVN) of the hypothalamus regulates HPA activity and is affected by signals from the spinothalamic tract as well as by inflammatory cytokines and TNFα [BJA 2019]. Cortisol levels would therefore be expected to rise in response to pain stimuli.

Salivary cortisol changes quickly in step with plasma cortisol and this is independent of salivary flow rate [El-Farhan 2017]. Cortisol is the most studied salivary biomolecule in relation to acute pain, measured in 18 of the included studies. Twenty six of the included studies (P1-P3, P6, P8-P22, P24-P29, P39) in this review have measured the salivary cortisol response to acute pain and in most of these (n=22) (P1-P3, P6, P8-P11, P13-P18, P21, P24-P29, P39) pain was experimentally induced.

The results of the salivary cortisol studies are discussed below, categorized by the type of pain that was experienced by the participants.

Only 6 studies (out of 26) reported a separate analysis of changes in cortisol levels by gender (P2, P6, P8, P9, P13, P21). From these, five studies found no significant difference in the cortisol levels between men and women throughout the experimental session (P2, P6, P8,

P9, P21). One study reported a significantly greater increase from baseline in men at 20 minutes after the plunge test (P13).

In 3 studies a significant positive correlation was found between cortisol change from baseline and pain intensity ratings (P6, P13, P25). One study that included patients with CFS found a significant negative correlation between cortisol change from baseline in the group and pain intensity (P19) CHECK. Three studies found no correlation (P14, P18, P27) and two out of them included participants with chronic pain conditions (P18, P27). The rest of them did not report any analyses of correlation (P1-P3, P8-P12, P15, P16, P20-P22, P24, P26, P28, P29) Consider taking these out.

Post-operative Pain: We found 2 studies [P28, P29] that measured salivary cortisol and pain intensity before and after surgery. P28 et al assayed salivary cortisol in participants undergoing skin surgery under local anaesthesia with low to moderate reported pain intensity. There was a significant rise in salivary cortisol 30 minutes post-surgery compared with 1 week before the operation, but not when compared to 30 minutes pre-operatively. Furthermore, there was no significant difference between 30 minute preoperative and intra-operative levels. Cortisol levels remained elevated one week after surgery. In their study of salivary biomarkers after ocular surgery P29 et al also reported rise in cortisol in the contradiction here with another page immediate pre-operative period compared to baseline with a further rise 1 hour after surgery. In this study there was no relationship found between pain severity and cortisol change. These results would indicate that a significant proportion of cortisol rise may be secondary to pre-operative stress and anxiety rather than the surgical trauma and pain. Neither study analysed correlation with pain intensity or gender.

Heat pain: In two [P25 & P26] studies, healthy volunteers taking no analgesia were exposed to multiple pain modalities including the hot water task (HWT), as well as CPT and ischaemic pain task (IPW). Salivary cortisol and sTNFαR-II levels were measured. P25 et al found that the HW task did not induce significant change in salivary cortisol (and neither did IPW, though CPT did) [P25]. In the other study, the results were not separated for different pain modalities

and salivary cortisol elevation was observed after the participants underwent a battery of pain inducing tasks for heat, cold and ischaemic pain [P26]. Correlation with pain intensity or gender were not analysed.

P18 et al assayed salivary cortisol before and after heat pain in healthy participants taking no analgesia, people with chronic pain and people with depression. No statistically significant increase in cortisol levels were observed in the three groups [P18]. Similarly, in a study where healthy pain free subjects or those with chronic fatigue and chronic pain were exposed to heat pain, there was no change in salivary cortisol either the healthy participants or those with chronic fatigue and pain [P19].

In three studies that were designed to assess the effect of acute psychosocial stress on pain modulation [P15, P16 and P17], healthy male participants were subjected to heat pain before and after a psychosocial stress task. Salivary cortisol was measured at 4 time points. Change in salivary cortisol after heat pain was not significant before the stress task. In contrast, cortisol levels increased significantly in response to heat pain after the participants had done the stress task. Correlations with pain intensity and gender were not analysed.

In another study of the impact of acute psychosocial stress on heat pain perception in healthy men, heat pain was not associated with significant change in cortisol, while psychosocial stress was. This study is also referred to in the sAA section and the pattern of cortisol change was similar to sAA [P14].

Mechanical & visceral pain:

Mechanical pressure pain was induced in 4 studies of salivary cortisol [P20, P21, P24, P27]. P24 et al induced mechanical pain by asking healthy participants to lie on a bed of nails (Sakti-mat) and compared this to lying on a soft bed. They found no increase in salivary cortisol despite the participants on the Sakti-mat reporting rapid and significant rise in pain [P24].

In an all-female study, P20 et al compared patients with fibromyalgia with healthy women who had no pain. Pain pressure thresholds were measured in 8 defined anatomical points. In women with fibromyalgia salivary cortisol (as well as IL-6 reported above) increased significantly 10 minutes after the measurements but this did not happen in healthy subjects [P20]. Correlation with pain intensity was not analysed.

In a cross over study designed to observe the effect of cognitive stress on pain perception, healthy participants were subjected to pressure pain to the right index finger with or without doing a 'stressor' mental mathematics task. Salivary cortisol levels were measured 20 minutes after each pain induction. The salivary cortisol levels did not change from baseline when the participants did not do the stressor task. In contrast, there was significant rise in cortisol after pain induction when participants were due to do the stress task. The authors concluded that rise in cortisol is related to anticipation of the stressor and not to pressure pain induction [P21].

P27 compared people with temporomandibular disorder and healthy pain free controls. Pain pressure thresholds were measured at defined anatomical sites, along with heat pain threshold and cold pain during 45 minutes of pain testing procedures. There was no difference in cortisol response from baseline to post-pain between the people with TMD and healthy people. In a separate analysis of the same experiment, in people who had a tendency to catastrophizing there was a reduction in salivary cortisol immediately and 20 minutes after pain compared to baseline.

P22 et al measured salivary cortisol in response to visceral pain induced by rectal distension in healthy individuals who took no analgesia CHECK? Contrast?. Participants completed a validated questionnaire that put them in either an elevated perceived chronic stress or a low perceived chronic stress group. Cortisol levels were significantly higher throughout the experiment in those with higher perceived stress but there was no rise in cortisol in either group on measuring rectal pain thresholds [P22].

Cold pain: The majority of studies that measured salivary cortisol after acute pain (14 of 18) induced cold pain by using the cold pressor task (CPT) [P1-P3, P6, P8-P12, P25-P27, P39] or plunge test [P13]. Five of these analysed correlation with gender (2, 6, 8, 9,13) and four analysed correlation with pain intensity (6,13,25,27). Four found no correlation with gender (2,6, 8, 9) and P13 found that there was rise in cortisol after the plunge test which correlated with pain intensity and also gender with men showing greater rise in cortisol than females. P6 and P13 also found correlation between cortisol rise and pain intensity.

Ten of the cortisol cold pain studies met the criteria set for a quantitative data synthesis and have been included in a more in-depth analysis of the pattern of change in salivary cortisol after cold exposure. Four did not meet the criteria. Reasons for exclusion were: (1) the experimental design included an emotional manipulation or cognitive task as well as CPT in all participants [P11, P8], (2) pain was induced by a combination of noxious stimuli with no separate analysis of cold pain [P26, P27]. These studies will be considered here. P11 found that when participants subjected to cold pain were put in a situation that allowed a positive appraisal of pain, the cortisol response was inhibited compared with controls, even though they did not report less pain.

P8 found that in people with early life adversity (ELA) the cortisol response after CPT combined with a cognitive task (the Paced Auditory Serial Addition Task, PASAT) was blunted compared with people who did not have a history of ELA while there was no difference in reported pain intensity between the groups. The results of these studies would suggest that the salivary cortisol response to cold pain can be affected independently from pain intensity.

In the study by P1, 76 of the 152 recruited healthy, pain free participants were exposed to social stress (public speaking) and the others were not. Both reported pain intensity and salivary cortisol increased in both groups after CPT. Participants in the social stress arm of the experiment reported less pain but salivary cortisol rise was greater. The 76 participants of

this study who were not exposed to a stress task, met the criteria for inclusion in the quantitative analysis.

Quantitative synthesis of cortisol variation with cold pain

A quantitative synthesis of studies that have investigated salivary cortisol after pain cold (following the method described in section 2.5.3) is given in this section. Ten articles [P1-P3, P6, P9, P10, P12, P13, P25, P39] met the inclusion criteria described in section 2.5.3 (Table). In one of these, hand CPT was compared with foot CPT in the same patient group and this study was therefore entered as 2 experiments, giving eleven experimental study groups for this analysis [P9]. The effect direction plot is summarized in Table 6 for the three outcome domains. The outcome of the sign test for each outcome domain are as follows.

Table 6. Effect direction plot summarizing direction of changes in mean or median salivary cortisol levels from studies of experimental cold pain (CPT and plunge test).

First author (Date)	Study ID	Sample size	Cold stimulus	Cold limb	Cortisol levels from baseline (min after cold pain)		
					<10	10-20	=>20
al'Absi (2003)	P1	76[a]	CPT	hand	▼	▲	No data
al'Absi (2002)	P2	62	CPT	hand	▼	▲	No data
Bachmann (2018)	P3	27	CPT	feet	No data	▲	▲[4]
Goodin (2012)	P6	40	CPT	hand	▲	▲[2]	▲[3]
Lara-Cinisomo (2015)	P9a*	22	CPT	hand	▼	▼	▼[4]
	P9b*	22	CPT	feet	▲	▲	◀▶[4]
Nakajima (2011)	P10	91	CPT	hand	▼	▲	No data
Serrano (2019)	P12	86	CPT	hand	▼	No data	No data

Zimmer (2003)	P13	76	Plunge test	hand & forearm	No data	▲	No data
Goodin (2012)	P25	10	CPT	hand	◄►	▲[3]	◄►[2]
Finke (2021)	P39	14	CPT	feet	◄►	▲	◄►[2]
Included: 11 experiment groups, total of 526 cold pain experiments in 504 participants							
Positive Effect (upwards arrow)					2	9	2
Negative Effect (downwards arrow)					5	1	1
No Clear Effect (sideways arrow)					2	0	3
No Data in the Outcome Domain					2	1	5
Two-tailed p-value (sign test for positive effect direction)					0.453	0.022	1.000

Effect direction:

▲ ▲ ▲ = increased levels from baseline

▼ ▼ ▼ = decreased levels from baseline

◄ ► ◄ ► ◄ ► = no change/mixed effects/conflicting findings

Sample size in each group:

large arrow, ▲ ▼ ◄ ► (orange colour) >50;

medium arrow, ▲ ▼ ◄ ► (green arrow) 25–50;

small arrow, ▲ ▼ ◄ ► (blue colour) <25.

The sample size corresponds to the final number of participants included in the analysis.

Number of time points in each outcome domain is 1 unless indicated with a number beside the effect direction arrow.

'No Data' indicates no measurement in the time frame of the outcome domain

CPT: cold pressor task; NCE: no clear effect.

Less than 10 minutes outcome domain: Nine of the 11 included experiments had data in this outcome domain. In two there was no clear effect. Seven experiments contributed to the sign test (5 where there were reductions from baseline and 2 where there were increases from baseline). The calculated two-tail p-value for the sign test for this outcome domain is 0.453 and the null hypothesis is accepted.

10 - 20 minutes outcome domain: One study had no data in this outcome domain. The remaining 10 experiments were included in the sign test (1 where there was reduction from baseline and 9 where there was a rise from baseline). The two-tail p-value is 0.022, and the null hypothesis is rejected for this outcome domain.

20 minutes or greater outcome domain: Five studies had no data in this outcome domain. Three had no clear effect. Therefore only 3 experiments were included in the sign test (2 positive and 1 negative). The calculated two-tailed p-value for the sign test for this outcome domain is 1.000. It is clear that small number of non-NCE effects in this outcome domain makes the power of the sign test very limited.

Risk of bias in included studies

Table 7

	Funding & Conflicts of interest	Confounding	Selection of participants	Classification of exposure	Departures from intended exposure	Missing data	Measurement of outcomes	Selection of reported result
Al Absi 2003	Low	High	Moderate	Low	Moderate	Low	Low	Moderate
Al Absi 2002	Low	High	Moderate	Low	Moderate	Low	Low	Moderate
Bachmann 2018	Low	Moderate	Moderate	Low	Low	Moderate	Low	Moderate
Goodin 2012	Low	Moderate	Moderate	Low	Moderate	Low	Low	Moderate
Lara 9a 2015	Low	Moderate	High	Low	Low	Moderate	Low	Moderate
Lara 9b 2015	Low	Moderate	High	Low	Low	Moderate	Low	Moderate
Nakajima 2011	Low	High	Moderate	Low	Moderate	Low	Low	Moderate
Serrano 2019	Low	High	Moderate	Low	Moderate	Low	Low	Moderate
Zimmer 2003	Low	Moderate	Moderate	Low	Moderate	Moderate	Low	Moderate
Goodin 2012	Low	Moderate	Moderate	Low	Moderate	Low	Low	Moderate
Finke 2021	Low	Moderate	Moderate	Low	Low	Low	Low	Moderate

Risk of bias was assessed in relation to the cold exposure related experiments within the articles. We used the Risk of Bias in Non randomised Studies of Interventions (ROBINS I) to

assess bias in seven domains: confounding, bias in selection of participants into the study, bias in the classification of exposure, bias due to departures from intended exposure, bias due to missing data, bias in measurement of outcomes and bias in selection of the reported result [Sterne 2016]. In each domain one of the four categories (low, moderate, high or critical) of risk of bias judgement was assigned.

The relevant confounder in this data synthesis would be co-exposure to psychological stress induced during the cold exposure experiment. This would falsely create or amplify the effect cold pain on salivary cortisol. All experiments with no neutral water or warm water control were judged as at least moderate in risk of confounding. If no steps were taken to minimise participant stress the risk was judged as high. A reasonable step that would minimise stress was considered to be the participant being specifically aware that they could withdraw from the experiment at any time. Where there was a neutral / warm water immersion control, the study was judged to be at low but only if stress and anxiety was showed to be equivalent in cold water and control groups at the start and also not rise in the control group after the task and if this was not shown, the risk was judged to be moderate.

Selection bias in the included studies was principally related to the recruitment of participants through communication restricted to university students and staff in all but one of the studies (in which there was also some community recruitment). Selection bias was therefore judged to be at least moderate in all the experimental groups in the data synthesis. It was judged as high when it was unclear how the participants were approached whether all respondents to the call for participation had had an equal chance of being included. This bias creates issues of generalizability or transferability to other populations, and could be classified as sampling bias (rather than selection bias). Nonetheless, we made the decision to include it in the risk of bias table because of a concern that it would be ignored by many researchers: only 3 of the 10 included papers considered this sampling bias in their discussion section.

Risk of bias related to classification of exposure was judged low in all of the studies because the exposures to cold and the control procedures were well defined at the start of the experiments and prior to the assessment outcome.

The method for exposure to cold was described well in all the included studies. However we considered interactions between experimenters and participants to be an important co-exposure that could affect acute change in cortisol concentration. On this basis, risk of bias due to departures from intended exposure was judged to be moderate when these interactions were not clearly standardised for example if it was not clear whether the experimenter was in the room with the participant during CPT.

There was low risk of bias due to missing data in six of the included studies as there was no missing data in relation to salivary cortisol measurement [P1, P6, P10, P12, P25, P39]. Where there was missing data, risk of bias was considered low in the study that accounted for this in the analysis [P2] and moderate when participants with missed data were not accounted for in the analysis [P3, P9, P9, P13]. In these 5 experimental groups, the median percentage of missing data as a proportion of individual experiments done was 8.3%. Overall 542 individual experiments were done of which 16 (2.95%) had missing data with 13 (2.4%) of these having missing data that was excluded by the study authors from the salivary cortisol analysis.

The method of salivary cortisol measurement was described by all of the included studies, both in terms of saliva collection and in terms of the cortisol assay. All papers reported storing the samples at -20, -70 or -80°C before they were defrosted and analysed in bulk, some time later, by one of the researchers using standardised assays. On this basis, although none of the papers specifically described blinding of the researchers in performing the sample analysis, we considered it unlikely that there would be performance bias in sample analysis and the risk of bias in measurement of outcomes was judged as low in all cases.

We were not able to access pre-specified study protocols for any of the included studies. In all of the included studies only one method of salivary cortisol measurement and results analysis was used and values were not selected from multiple outcomes. We therefore judged the risk of bias in selection of the reported result as moderate in all cases.

All included studies provided a statement on funding sources. All studies were funded by non-profit organisations, university funding bodies or national institutes and were deemed at low risk of bias from the funding point of view. Two included papers also specified the role

on the funding body in the conduct of the research and its publication, both reporting no role [P9, P25].

Overall the risk of bias was judged to be high in 5 of the included studies, that is, in 6 of the experiments and moderate in 4 of them. The high risk was due to possible confounding in 4 of the studies and due to possible bias in the selection of participants one study that included two cold pain experiments [9].

Heterogeneity in studies of salivary cortisol change in response to cold pain

Methodological heterogeneity in the included studies can be broadly divided into differences in saliva collection, differences in salivary cortisol assays and differences in conducting the pain induction test.

Differences in saliva collection

Timing of saliva collection: All the studies included in the cold - cortisol analysis conducted their experiments in a particular part of the day. However, there is variability in the time chosen by the researchers. Three chose the morning (1, 2, 10) and 7 chose afternoon times (3, 6, 9, 12, 13, 25, 39). In 4 of the studies, the researchers gave no reason for their choice of timing (1, 2, 9, 10), in two studies the researchers stated that afternoon times are associated with greater cortisol responses (6, 25) and therefore an afternoon time for the experiments had been chosen. The rest gave the reason simply as control for diurnal variation (3, 12, 13, 39).

Saliva collection method: In 10 of the included experiments, whole saliva was collected for the cortisol assay while one collected oral mucosal transudate [P25]. Given that salivary cortisol closely follows free serum cortisol, this is unlikely to be significant. In 7 of the 11 experiments the device used involved a cotton swab which is later centrifuged to release liquid for analysis [P1, P2, P3, P6, P10, P13, P25, P39]. This method could yield a different cortisol concentration compared to saliva obtained by passive drool [El Farhan 2017]. Though this difference would not be expected the effect the outcome of our data synthesis because it is based on the pattern of cortisol change, it becomes important when absolute values of cortisol concentration are being compared.

Participant preparation: In the studies included in the cortisol- cold pain data synthesis, restrictions to food, alcohol, smoking and caffeine were variably applied. Most of the included studies placed restrictions on all of these substances [P6, P25, P3, P9a, P9b, P1, P2, P39]. One study (P12) placed no restrictions, P10 restricted drinks only and P13 placed only a smoking restriction. Precautions to reduce the risk of blood contamination due to bleeding from gums were taken in five experiments [P6, P25, P3, P9a, P9b] by placing restrictions on food consumption that can cause minor mucosal abrasions. In two studies P6 and P25 brushing teeth at least 2 hours prior to the start time of the experiment was also restricted. Restriction on acute exercise was placed in 4 of the included experiments for at least 2 hours prior to starting. None of the studies collected data on longer term exercise.

Differences in assays of salivary cortisol concentration

Cortisol levels vary between 5 and 25 mcg/dL or 140-690 nmol/L. Immuno-assays with high sensitivity were used to measure cortisol in all the experiments included in the quantitative synthesis. Intra and inter-assay coefficients of variation were reported in 7 of the experiments with values ranging 4-12%. Overall the assay heterogeneity in the included experiments is likely to be insignificant when observing the pattern of cortisol change.

Differences in conducting the cold pain induction tests

Water temperature was 0-5 °C in all the studies. Eight of the 11 experiments were of upper limb immersion and 3 were feet immersions. Overall the experiments included in the data synthesis had little heterogeneity with respect to the conduct of the CPT.

DISCUSSION

Summary of main results

A number of salivary biomolecules have been studied in experimental designs that involve pain induction or in acute pain settings. Researchers' rationale for selecting these biomarkers varies. Melatonin, pro-inflammatory cytokines and testosterone have been selected because there is evidence for their involvement in modulation of noxious stimuli even though the mechanism through which their effects occur remain unclear. Rise in kallikriens and salivary

alpha amylase and fall in sIgA have been selected for investigation in acute pain conditions primarily because these biomolecules are stress biomarkers. Cortisol has been measured as a physiological stress marker and also because it is released in response to acute pain through activation of the HPA axis.

Most researchers have chosen to induce acute pain under controlled conditions. Here, cold pain induced by CPT has been the most studied modality in relation to acute pain.

Taken together these results would suggest that cortisol rise after acute pain is modality dependent. Heat pain alone did not result in rise in salivary cortisol in any of the studies.

Collectively the findings of this review indicate that much of cortisol change in acutely painful conditions happen as a result of the stress associated with the events rather than pain. In all studies where participants have been exposed to acute stress, there is a rise in cortisol.

Change in salivary biomolecules in healthy individuals after controlled pain induction

In healthy people taking no medications, salivary biomarkers that change with stress would be expected to change in the same manner when acute pain is induced. In this context, sAA has been shown to increase in response to both heat and cold pain [9. 30, 31] but not hypertonic saline injection [32]. IgA has been shown to fall after cold pain induced by CPT [35] but this has no relationship with pain intensity. Kallikriens have not been studied in healthy participants in controlled settings.

Among salivary biomolecules that have been shown to have a role in pain signal modulation, pro-inflammatory cytokines have been shown to increase after CPT but IL6 did not rise after measuring pressure pain thresholds. Secretory TNFalphaRII has been shown to fall significantly after exposure to multiple pain modalities (cold, heat and ischaemic pain). Melatonin has been shown to change after electrically stimulated pain.

In healthy adults, our quantitative data synthesis shows that salivary cortisol rises after cold pain induction. This rise also occurs after pain induction with multiple modalities when these include cold (P25, P26). However no rise in salivary cortisol has been demonstrated after induction of other types of pain such as heat pain (P25, P18, P19), ischaemic pain (P25), mechanical pressure (P20, P21, P24) or visceral pain (P22).

There is a striking difference between the effect of cold pain, which is always followed by a rise in cortisol and inflammatory cytokines, and other pain types which consistently are not. This is considered in greater detail further below.

Change in salivary biomarkers in healthy, acutely stressed individuals after controlled pain induction

In studies where healthy participants were exposed to a form of acute stress in the form of a cognitive or psychosocial task as well as heat pain, stress resulted in rise in sAA and cortisol (heat: P14, P15 16 17) while heat pain alone did not. Similarly, in a study by P21, pressure pain alone did not cause a rise in cortisol but this did happen with stress. In contrast, P11 showed that positive appraisal of cold pain reduced the stress response, including a lack of rise in salivary cortisol.

Change in salivary biomarkers in people with a chronic condition after controlled pain induction

Cortisol and IL6 levels increased after pressure pain was induced in fibromyalgia but not in healthy pain free participants [20]. Salivary cortisol did not rise after heat pain in people with CP [18], CP and fatigue [19] or depression [18].

Change in salivary biomarkers after surgery or medical procedures

There were mixed results for biomarkers that change with stress: In healthy people having a corneal surgery there was no rise in sAA but there was a rise in sIgA 1 hour after surgery. However, in a study of people with severe disability having medical procedures, there was rise in sAA which correlated with pain intensity [REF]. Kallikrein was shown to rise after elective gynaecological surgery, though this rise was not in step with change in reported pain severity, probably reflecting a post-surgical stress response.

With respect to biomarkers that have a role in pain signal modulation, testosterone did not rise but TNFaRII dropped after elective corneal surgery [REF].

Cortisol rise was more timed to pre-operative stress than surgical trauma or pain in two studies of skin surgery and ocular surgery [REF].

Pain biomarker level correlations with gender and pain intensity

There are complex relationships between gonadal hormones and physiological and biochemical functions related to pain processing (Mogil 2020). This would include effects on biomarker concentrations and related molecules. The effect of testosterone has been previously described in this article and oestrogen is known to increase the levels of cortisol binding globulin (CBG). Although current evidence shows that menstrual cycle phase has no effect on the perception of pain in healthy women (Iacovides 2015), menstrual cycle phase, use of HRT, the oral contraceptive pill and pregnancy could all influence cortisol concentration. Some researchers have circumvented these issues by recruiting only male participants.

Of the 35 studies that have been included in this review, only 10 analyzed the relationship between change in biomolecule concentration and pain intensity and 7 analyzed the relationship with gender (Table). These two groups were highly heterogenous and it is not possible to draw reliable conclusions with respect the relationship between biomarker changes and pain intensity or gender.

Cortisol after cold pain

Ten studies, including 11 separate experiments, measured cortisol change after cold pain induction in healthy people. These were included in a separate data synthesis in this review. There is moderate certainty evidence that in healthy people taking no medications salivary cortisol rises 10-20 minutes after cold pain.

We have taken a strict approach to determining the risk of bias and included an assessment of bias related to the 7 domains of ROBINS-I as well as funding and conflicts of interest.

Five of the studies,

6 of the experiments, are judged to be at high risk of bias though only one domain carries this high risk in each of these. Across all the included experiments the risk is judged to be high in 6 domains, moderate in 38 domains and low in 44 domains of a total of 88 domains examined for risk of bias.

The included studies in the cortisol – cold pain synthesis are relatively homogenous in terms of methodology (table). This was partly achieved by only including studies that had specified that the participants were in good health. Health conditions, both physical and mental, as well as acute illness affect cortisol binding globulin (CBG) levels as well as HPA axis function (V M-S, BJA 2019).

One of the heterogeneities in the included studies are restrictions on substances that affect salivary flow. Alcohol, nicotine and caffeine intake are known to affect salivary flow [Hildebrandt 2013, Batista 2019] but as salivary cortisol is not affected by flow, this change would not affect the results of this data synthesis. It is, however, important for researchers to bear in mind that other biomarkers could be affected. A number of heterogeneities were identified in the included studies that are unlikely to affect the outcome of this data synthesis but could influence the magnitude of the change in cortisol levels. These include differences in the timing of saliva collection, the saliva collection method, restriction on substances that blunt or enhance the cortisol stress response, blood contamination of saliva, exercise, assays of cortisol, conduct of the cold pain induction.

Although the influence of the circadian rhythm on the pattern of change in salivary cortisol in response to pain is not known, the researchers in all the included studies considered a possible effect and chose to plan the experiments in defined times of the day. Cortisol is integral to circadian physiological control with a well-recognized diurnal change pattern that is very well described: it starts to rise between 02:00 and 04:00, reaches a peak at around 08:00 (cortisol awakening response) and then falls during the day reaching its minimum at midnight. Most researchers chose to do the salivary cortisol – cold pain experiments in the afternoon while cortisol levels are in decline. Regardless of the chosen timing, most did not explain the reason for this choice further than to take account of circadian variability and may therefore have been influenced by convenience factors such as availability of the researchers, participants or lab space. There is little evidence on whether this would influence the size of the observed changes.

The effects of alcohol, caffeine, nicotine and also food on cortisol release into the circulation are potentially more important. Caffeine is a stimulator of the HPA axis (Patz 2006, Rohleder

2006) and its intake results in serum cortisol rise lasting many hours. Caffeine has been shown to enhance the cortisol response to mental stress [Lovallo 2006].

Nicotine also stimulates the HPA axis [Kirschbaum and Hellhammer 1994] though in habitual smokers, there is blunting of the cortisol stress response [al' Absi 2012]. Alcohol consumption is associated with higher daily circulating cortisol levels in general [Badrick 2008] though the physiological response to stress is blunted in people with high alcohol intake and alcohol dependency. The impact of nicotine, caffeine and alcohol is therefore for the most part the blunting or enhancement of the cortisol stress response. It is unlikely that they change the pattern of the response and therefore result of this data synthesis in this study.

The effect of exercise on serum cortisol change varies depending on whether it is regular or done in acute bouts [Gerber 2009].

The CPT was developed as a stimulus for measuring vasomotor reactions in the study of hypertension [Hines 1936] and researchers continue to use it as a protocol to activate the sympathetic stress response [Shilton 2017]. Over the years, variations to the original design have been used in experiments, including immersion of the non-dominant hand, hand and forearm immersion and submersion of one or both feet [Bachmann 2018, Larra 2015] and single finger [Sendowski 1997]. All have been shown to induce a physiological response, there is some evidence for a relationship between systemic and local responses and the size of the skin area exposed to cold [Sendowski 1997, Larra 2015]. The impact of these variations on experimental outcomes have scarcely been considered. McGinley and Friedman studied the differential effect of lateralized cold afferent input in 73 healthy right handed university students using facial cooling to create vagal stimulation and foot CPT to create sympathetic activation. They found that left foot CPT resulted in greater sympathetic responses in terms of rise in blood pressure and skin conductance. Although the cortisol response has not been studied in this way, some researchers have argued for bilateral feet CPT to avoid any laterality bias (P9 Larra 2015, P3 Bachmann 2018, P8 Hengesch 2018). This has the added advantage of keeping both arms free to be used for other aspects of the experimental design (e.g. taking blood pressure, blood sampling or motor tasks). CPT is also

widely used as an experimental pain stimulus [Johansen 2014]. We have found it to be the most commonly used stimulus for assessing the salivary cortisol response to acute pain. This may in part be due to it being controllable by participants and easy to administer with minimal equipment.

Some of the experimental designs made attempts to try to separate pain from stress. In these experiments stress was a greater determinant of rise in cortisol than pain. In the experiments involving CPT, the cortisol response may be primarily secondary to direct effects on the sympathetic adrenal system, rather than due to pain. This may explain why a consistent relationship does not exist between the cortisol rise and pain severity.

Future Developments

Improvements in bioengineering will enable researchers to measure the levels of many salivary molecules with minimal delay and at lower cost. Such measurement technology would allow researchers to obtain a more comprehensive picture of biomolecule variations with greater ease. Many biomolecules that are involved in nociceptive pathways and signal modulation such as SP, glutamate or the neurotrophic factors (for example NGF and BDNF) are measurable in the saliva [Jasim 2020] and many other candidates will undoubtedly follow in the future. At least some of these have been shown to have circadian and gender variability [Jasim 2020, Cain 2017]. Future studies should consider these diurnal changes as well as the effects of the heterogeneity factors that we have identified.

Limitations

The use of the sign test is an important limitation with respect to our quantitative data synthesis of the effect of cold pain on salivary cortisol. We recognize that the number of experiments included in the analysis is relatively low although, on a more encouraging note, in the 10 minute and 10-20 minute after cold pain time domains most of the experiments had effect directions that could be included in the sign test, meaning that the use of this test is

appropriate and does not misrepresent the data. On the whole, the conclusion with respect to the pattern of cortisol change in cold pain is therefore at best modest in its level of certainty.

A further limitation of the conclusions in this review relate to the participants' demographics. All studies took place in countries with developed economies (CHECK). More importantly, the mean age of participants is to years for all the 35 studies included in the review and between and for the 10 studies included in the cortisol - cold analysis. This is a relatively young age group. Current evidence suggests that daily cortisol output increases with age [Gaffey 2016] though the effect of age on the cortisol response is unknown.

Conclusions

Salivary biomarkers have been studied in relation to acute pain in different settings with considerable variation in the populations studied, the noxious stimulus and pain induction methodology. This has resulted in a highly heterogeneous and complex landscape for salivary biomarkers associated with pain. In this review, we have summarised the current literature on this topic.

In order to be useful in clinical practice as a guide to acute pain treatment, the ideal salivary acute pain biomarker would be one (or a panel of biomarkers) that shows reliable change after acute noxious stimuli, within a short time interval of at most a few minutes, in both healthy people and those with acute or chronic health conditions. It should be either minimally or predictably affected by change in the organism's internal or external environment.

Considering these criteria, it is clear that there remain considerable challenges in identifying a single biomarker or even a panel of biomarkers in the saliva that can be used in guiding the treatment of people with acute pain conditions. Furthermore, none of the biomarkers that have been studied in relation to acute pain can be considered specific to the stimulation of nociceptive pathways.

Cortisol is the most commonly studied biomarker in saliva. Change in salivary cortisol and salivary inflammatory biomarkers in response to acute cold pain consistently occurs in healthy pain free individuals, probably 10-20 minutes after the onset of cold pain. There is evidence

that this cortisol response can be altered with psychological intervention and also that it is modified in people with chronic pain conditions.

Although released as a result of the acute stimulation of nociceptive pathways, cortisol levels are predominantly affected by acute stress. At least in part this explains the absence of consistent correlation between reported pain intensity and the salivary concentrations of the associated biomarkers.

Cortisol secretion is also affected by a complex multitude of factors in both healthy individuals and those with physical and mental health disorders or chronic stress. Therefore absolute levels are unlikely to be useful as a measure of HPA activation after acute pain, trauma or chronic psychological stress. This difficulty is augmented by the lack of consistency between different assays for cortisol.

There are differences in the response to different modalities of pain in both cortisol and other salivary biomarkers.

In order to advance this area of research, it is essential to standardise the methods used in both salivary sample collection and pain induction methods. Additionally researchers should be aware of the wider factors that can affect salivary biomolecule concentration such as salivary flow, the effect of commonly used pharmacologically active substances, exercise, background stress, diurnal variations and participant age and gender so that a more cohesive and clinically relevant research literature can emerge.

Authors' contributions

AV, RZ and SG developed the concept, the research question, and designed the study. AV and QL searched and selected the studies for inclusion in the review and collected the data for the review. All authors contributed to the analysis and interpretation of the data, and also contributed to revising the manuscript. All authors approved the final version of the manuscript.

Declaration of Conflicting Interests

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Supplementary Material

Supplementary Material 1. The file combines the following Appendixes:

Appendix A.1. Detailed search strategy in MEDLINE.

Appendix A.2. Detailed search strategy in EMBASE.

Appendix A.3. Detailed search strategy in Web of Science.

Appendix A.4. Detailed search strategy in CENTRAL.

Appendix A.5. Detailed search strategy in PubMed.

Appendix B. Review protocol developed a priori and registered with the Open Science Framework (OSF). [Available at: osf.io/wk8emI].

Appendix C. Full citations of the included studies (in alphabetical order).

Appendix D. Table of experimental tests that were performed from the participants in the included studies in addition to pain tasks.

Appendix E. List of abbreviations used in the review and supplementary material.

Supplementary Material 2. Data items extracted from the included studies of the review (Table 5 in XLSX format).