



Article

Reduced Endocannabinoid Tone in Saliva of Chronic Orofacial Pain Patients

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Abstract: Background: the endocannabinoid system (ECS) participates in many physiological and pathological processes including pain generation, modulation, and sensation. Its involvement in chronic orofacial pain (OFP) in general, and the reflection of its involvement in OFP in salivary endocannabinoid (eCBs) levels in particular, has not been examined. Objectives: to evaluate the association between salivary (eCBs) levels and chronic OFP. Methods: salivary levels of 2 eCBs, anandamide (AEA), 2-arachidonoylglycerol (2-AG), 2 endocannabinoid-like compounds *N*-palmitoylethanolamine (PEA), *N*-oleoylethanolamine (OEA), and their endogenous precursor and breakdown product, arachidonic acid (AA), were analyzed using liquid chromatography/tandem mass spectrometry in 83 chronic OFP patients and 43 pain-free controls. The chronic OFP patients were divided according to diagnosis into musculoskeletal, neurovascular/migraine, and neuropathic pain types. Results: chronic OFP patients had lower levels of OEA ($p = 0.02$) and 2-AG ($p = 0.01$). Analyzing specific pain types revealed lower levels of AEA and OEA in the neurovascular group ($p = 0.04, 0.02$, respectively), and 2-AG in the neuropathic group compared to controls ($p = 0.05$). No significant differences were found between the musculoskeletal pain group and controls. Higher pain intensity was accompanied by lower levels of AA ($p = 0.028$), in neuropathic group. Conclusions: lower levels of eCBs were found in the saliva of chronic OFP patients compared to controls, specifically those with neurovascular/migraine, and neuropathic pain. The detection of changes in salivary endocannabinoids levels related to OFP adds a new dimension to our understanding of OFP mechanisms, and may have diagnostic as well as therapeutic implications for pain.

Keywords: endocannabinoids; anandamide; 2-AG; chronic pain; orofacial pain; neuropathic pain; migraine; saliva



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1. Introduction

Endocannabinoids (eCBs) are major constituents of the endocannabinoid system (ECS), and include fatty acid derivatives such as *N*-arachidonylethanolamine [anandamide (AEA)] and 2-arachidonoylglycerol (2-AG). Both ligands have a high affinity to cannabinoid-1 and 2 receptors (CB₁R and CB₂R, respectively), which are also activated by Δ^9 -tetrahydrocannabinol (Δ^9 -THC). This psychoactive component found in marijuana has pain reducing effects [1]. CB₁Rs are primarily located at presynaptic sites in the nervous systems. CB₂Rs are found predominantly (but not exclusively) on immune cells [2].

The ECS is important in many physiological and pathological pathways as well as acting as a homeostatic system, including pain regulation in general [3–6], and specifically neuropathic and inflammatory pain [7].

The role of the ECS in pain has been extensively studied. Cannabinoid receptors and ligands are found at most levels of the pain pathway from peripheral sites (peripheral nerves and immune cells) to central integration sites (spinal cord), and higher brain regions (periaqueductal grey and rostral ventrolateral medulla associated with descending control of pain) [8]. CB₁Rs are also found in brain areas associated with pain sensation (thalamus and amygdala) and body sites associated with pain processing and transfer [9]. The peripheral location CB₂Rs enables involvement in pain mechanisms by preventing pro-inflammatory cytokine secretion near nerve endings [10]. Furthermore, eCBs may inhibit pain processes related neurotransmitter secretion via activation of cannabinoid receptors [11] and may also stimulate thermosensitive transient receptor potential (TRP) channels, such as TRPV1, known to be involved in pain stimulation.

In addition to the main eCBs, several eCB-like molecules have been identified and characterized such as *N*-palmitoylethanolamide (PEA) and *N*-oleoylethanolamide (OEA), which do not bind to the cannabinoid receptors, but have the same degradation pathway as AEA involving the membrane-associated fatty-acid amide hydrolase (FAAH), which catabolizes these ligands into free arachidonic acid (for AEA), palmitic (for PEA), and oleic (for OEA) acids and ethanolamine. The endogenous levels of these ligands were found to be modulated in pain and inflammatory murine models, suggesting eCBs play a role in the tonic inhibition of pain responses and the setting of nociceptive thresholds [12]. In this study (for simplicity reasons) these components also will be termed eCBs as AEA and 2-AG. Interestingly, changes in eCB ‘tone’, represented by the expression of cannabinoid receptors, their functional activity (upregulated or downregulated), and the relative amount of eCBs, may render a subject susceptible to disease. In fact, reduced eCB ‘tone’, also termed ‘clinical eCB deficiency syndrome’, is associated with migraine, fibromyalgia, irritable bowel syndrome, schizophrenia, multiple sclerosis, Huntington’s, Parkinson’s, anorexia, chronic motion sickness, and autism [13–15]. Specifically, AEA levels were lower in the spinal fluid of patients with chronic headache such as migraine [5], and FAAH was suggested as a target for treatment in cases of AEA deficiency migraine [10].

Over the last decade saliva has been recognized as a “diagnostic window to the body” [16], because its gene product distribution across Gene Ontological categories, such as molecular function, biological processes, and cellular components, are very similar to plasma [17]. In most studies regarding pain biomarkers, plasma has been the analytical medium and only a handful, have assessed the saliva concentration of these pain markers [18–21]; one of them has been recently published by our group [22]. Saliva has many advantages over serum as a diagnostic fluid, it is easy to collect, store and ship and can be obtained at a low cost in sufficient quantities for analysis. For patients, the non-invasive collecting techniques dramatically reduce anxiety and discomfort and simplify procurement of repeated samples for longitudinal monitoring. Saliva collection is safer than venipuncture, which exposes health care providers to infectious diseases. Saliva is also easier to handle for diagnostic procedures since it does not clot, reducing the manipulations required [23].

In fact, components of the ECS were found in saliva in conditions such as stress, fear, obesity, and malnutrition [24–27]. However, as far as we know, no data regarding salivary eCB levels in chronic pain patients in general and those with craniofacial pain in particular, have been collected or examined. Importantly, by evaluating salivary eCBs levels we may achieve a breakthrough in diagnosis and treatment individualization, such as fine-tuning when medical cannabis, a valid therapeutic agent for treating pain, would be beneficial.

2. Methods

2.1. Participants

The study followed the STROBE guidelines and met the requirements of the Hadassah Medical Organization’s (HMO) Institutional Review Board (IRB) (Approval No. 0662-

HMO-17). All data were fully anonymized; Informed consents were waived according to the instructions of the Ethics Committee. The medical records of 83 (56 female, 27 male) orofacial pain (OFP) patients attending the Orofacial Pain Clinic, at the Hebrew University-Hadassah Faculty of Dental Medicine, between 2017–2018 were reviewed. 43 age-matched (28 female, 15 male) pain-free participants acted as a control group. Inclusion criteria: over 18 years of age; diagnosis of chronic OFP for at least 3 months; able to provide saliva sample of at least 200 μ L per 10 min. Exclusion criteria: cannabis use, refusal or inability to consent, medical conditions affecting salivary gland function such as autoimmune disease primarily Sjogren's syndrome and rheumatoid arthritis, history of head and neck cancer treated using radiotherapy, chemotherapy and/or biological targeted therapies, graft versus host disease, fibromyalgia.

2.2. Orofacial Pain Diagnosis

Based on OFP diagnosis, etiology and pain characteristics the patients were divided into 3 groups as described by Sharav and Benoliel [28]:

Musculoskeletal group: Pain from temporomandibular disorders (TMD) including masticatory muscle pain (MMP), temporomandibular joint (TMJ) pain, and combined muscle and joint pain. Diagnosed according to the Diagnostic Criteria for Temporomandibular Disorders (DC/TMD) [29].

Neurovascular group: Migraine and Tension type headache, diagnosed according to the ICHD-3 [30] and "neurovascular orofacial pain" (NVOP) for facial pain with migrainous features in the second and/or third divisions of the trigeminal nerve [31].

Neuropathic group: Trigeminal neuralgia (TN) [32], Painful post-traumatic trigeminal neuropathy (PTN) [33], Burning mouth syndrome (BMS), post herpetic neuralgia (PHN), and persistent idiopathic facial pain (PIFP) [34].

The thorough extra oral examination included: cranial nerve examination and masticatory apparatus palpation as previously described [35,36]. Intra-oral examination was performed to exclude dental, periodontal and mucosal pathology. Brain and brainstem imaging were performed for TN to exclude intracranial pathology.

2.3. Collection of Medical Records

Demographic data including gender, age, BMI, medications, and relevant medical history was collected. Pain characteristics including onset (months), intensity, and quality were recorded on the intake form routinely used in our clinic [32,36,37]. Pain intensity was rated using a verbal pain scale (VPS) in the saliva collection day, where 0 represents no pain and 10 the worst imaginable pain.

2.4. Saliva Collection

Unstimulated saliva was collected for 10 min as described previously [22,38] into pre-calibrated tubes. Saliva was collected between 9:00 AM and 12:00 PM. All participants refrained from exercising, eating, drinking and brushing their teeth in the 2 h before saliva collection. Patients did not take their medications, including sialagogues, before saliva collection. Volunteers rested for 10 min before saliva collection, sitting in an upright position in a quiet room and were asked not to speak or leave the room until after the saliva was collected.

Saliva samples were immediately stored at -80°C , until further processing. After defrosting, the samples were centrifuged at $3500\times g$ for 10 min at 2°C to remove insoluble materials, cell debris and food remnants. The supernatant was aliquoted into polypropylene tubes and then stored at -80°C until further processing.

The samples were run in the following order, 2 OFP samples from the same group and then a gender- and BMI-matched control sample.

Endocannabinoid extraction, purification, and measurement:

The extraction, purification, and quantification of saliva eCBs was performed by stable isotope dilution liquid chromatography/tandem mass spectrometry (LC-MS/MS)

as previously described by us [14,39–42]. Briefly, AEA, 2-AG, their breakdown product arachidonic acid (AA), PEA, and OEA were extracted, purified, and quantified as follows. Total salivary protein was precipitated using ice-cold acetone and Tris buffer (50 mM, pH 8.0). Samples were then homogenized using a mixture of 0.5 mL ice-cold methanol/Tris buffer (50 mM, pH 8.0), 1:1, and 7 μ L internal standard (22.4 ng d₄-AEA). The homogenates were then extracted using ice-cold CHCl₃:MeOH (2:1, v/v), and immediately washed with ice-cold chloroform three times. The samples were dried under a thin stream of nitrogen and reconstituted in MeOH. Analysis by LC-MS was conducted using an AB Sciex (Framingham, MA, USA) Triple Quad 5500 Mass Spectrometer and a Shimadzu (Kyoto, Japan) UHPLC System. Liquid chromatographic separation was acquired using a Kinetex (Phenomenex) column (C18, 2.6 mm particle size, 100 * 2.1 mm). The levels of each compound were analyzed by multiple reaction monitoring. The molecular ion and fragment for each compound were measured as follows: m/z 348.3 → 62.1 (quantifier) and 91.1 (qualifier) for AEA, m/z 379.3 → 91.1 (quantifier) and 287.3 (qualifier) for 2-AG, m/z 305.2 → 91.1 (quantifier) and 77.1 (qualifier) for AA, m/z 326.2 → 62.1 (quantifier) and 55.1 (qualifier) for OEA, m/z 300.3 → 283.2 (quantifier) and 62.0 (qualifier) for PEA, and m/z 352.3 → 66.1 (quantifier) and 91.1 (qualifier) for [²H₄] AEA. Sample levels of AEA, 2-AG, AA, PEA, and OEA were measured against a standard curve and then expressed as fmol/mg proteins.

2.5. Statistics: The Statistical Analysis Was Performed Using SPSS Version 25

To examine the differences in eCB levels for nominal and categorical background variables, *t*-tests and one-way analysis of variance were performed, additional post-hoc Scheffe tests were performed when differences were significant. Spearman coordinator was used to examine the specific categories that made up the differences. The differences between eCBs types and specific background variables were examined for each diagnosis separately by using the non-parametric Mann Whitney and Kruskal–Wallis test.

3. Results

Of the 126 participants in the study, 83 had chronic OFP (Figure 1), 21 (25.4%) in the musculoskeletal group, 31 (37.3%) in the neurovascular group, and 31 (37.3%) in the neuropathic group (Figure 1A). The remaining 43 were the pain-free control group. The specific pain diagnoses and incidence of each diagnosis within general pain group are summarized in Figure 1C. Figure 2, summarizes gender distribution according to the different groups.

64 chronic OFP patients used pain medication including: Tricyclic antidepressants—Amitriptyline, Nortriptyline (27 patients), Pregabain (10 patients in the neuropathic group), Carbamazepine (10 in the neuropathic group), Topiramate (10 patients in the neurovascular group), Duloxetine (3 patients), Clonazepam (10 patients in the neuropathic group), Valporic Acid (1 patient in the neurovascular group), Tripan (1 patient, as abortive treatment in the neurovascular group). Some of the patients used more than one medication (for details see: Supplementary Table S1).

Mean salivary levels (fmol/mg protein) of eCBs: AEA, 2-AG, OEA, PEA, and AA are summarized in Table 1. Significantly higher levels of AA, OEA and PEA were found in men compared to women ($p < 0.001$) (Table 1). Specifically—OEA, PEA and AA levels were lower in women than men in the neurovascular group ($p = 0.002$, $p < 0.001$, $p = 0.048$, respectively), women had lower levels of 2-AG and AA than men in the neuropathic group ($p = 0.061$, $p = 0.037$, respectively)—data not shown.

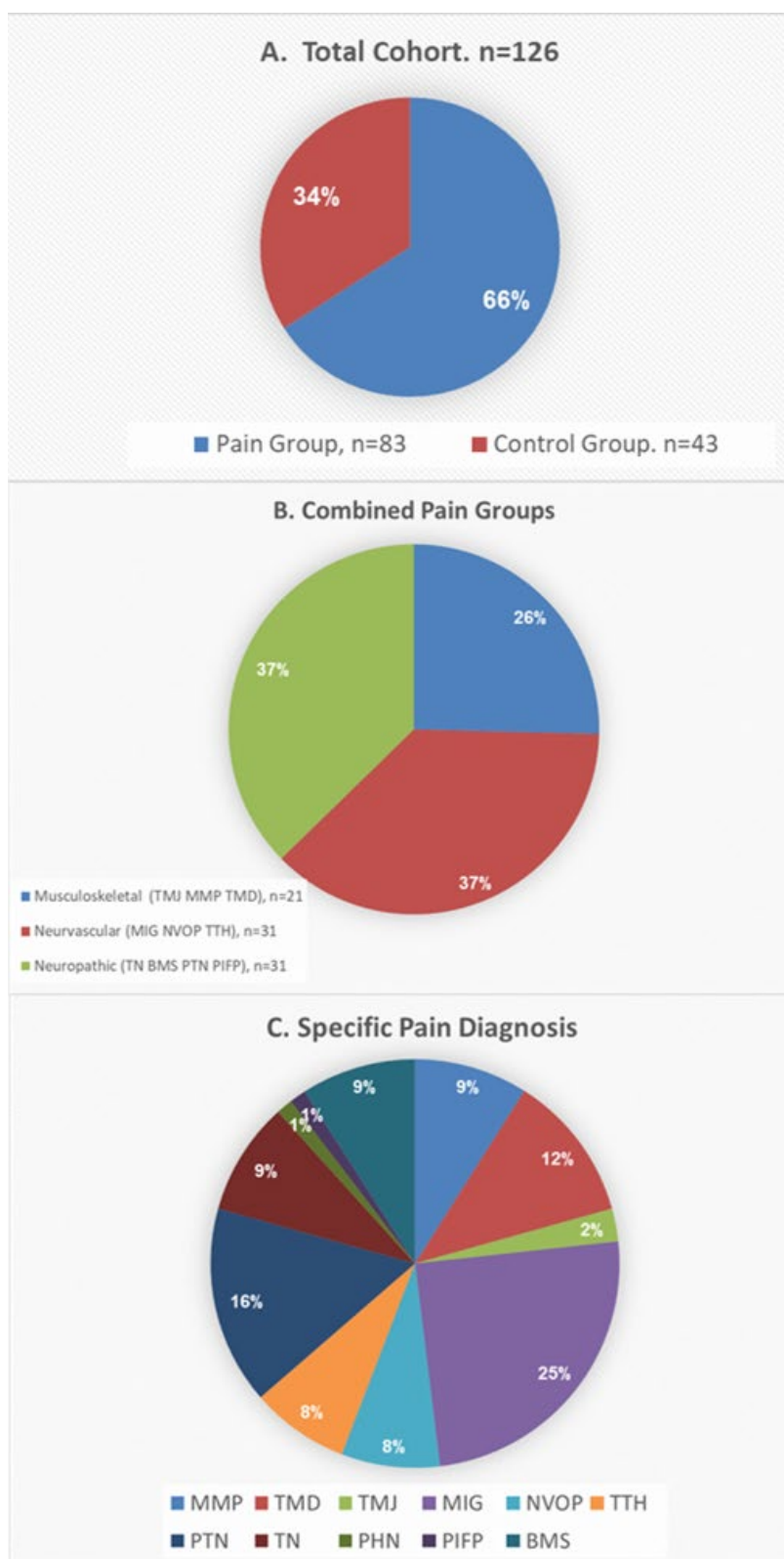


Figure 1. Cohort distribution according to diagnosis. Diagnoses are grouped according to etiology and characteristics, see Sharav and Benoliel [28]: MMP—Masticatory Myofascial Pain, TMD—Temporomandibular disorder, TMJ—Solely Temporomandibular joint origin, Mig—Migraine, NVOP—Neurovascular orofacial pain (orofacial migraine), TTH—Tension Type Headache (primary headache), PTN—Post Traumatic Neuropathy, TN—Trigeminal neuralgia, PHN—Post Herpetic Neuralgia, PIFP—Persistent idiopathic facial pain, BMS—Burning Mouth Syndrome.

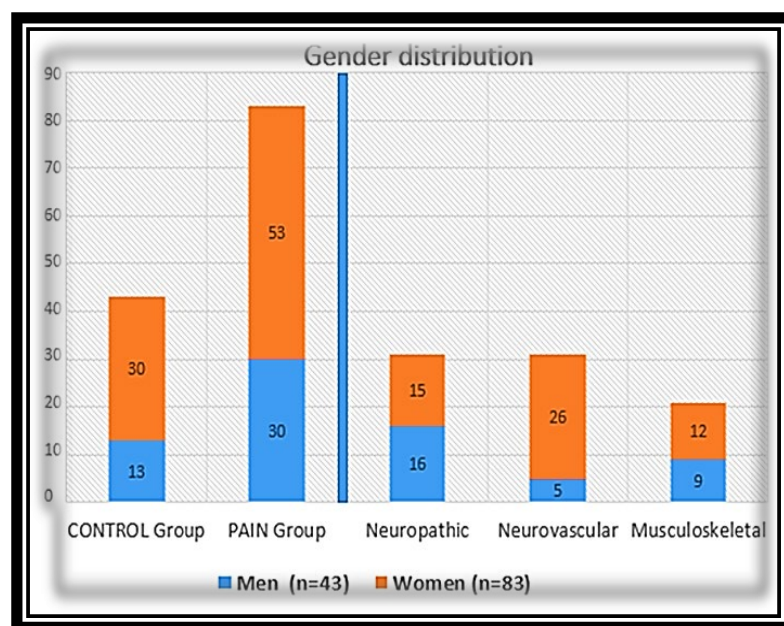


Figure 2. Cohort distribution according to gender.

Table 1. Mean salivary eCBs levels in the total cohort (126 patients) and in men and women (fmol/mg protein).

Endocannabinoids (eCBs)	fmol/mg (Mean ± SD)	Women (Mean ± SD)	Men (Mean ± SD)	p Value
AEA	0.17 ± 0.3	0.15 ± 0.17	0.21 ± 0.69	0.28
2-AG	41.3 ± 40.6	44.15 ± 45.32	37.71 ± 29.25	0.40
OEA	49.1 ± 59.8	37.93 ± 50.35	73.16 ± 70.33	<0.001
PEA	11.5 ± 11.8	8.34 ± 7.96	17.73 ± 15.30	<0.001
AA	7659.1 ± 1876.1	12112.25 ± 1154	3262.18 ± 2452	<0.001

2-AG—2-arachidonoylglycerol, PEA—N-palmitoylethanolamine, AEA—N-arachidonylethanolamine, AA—arachidonic acid, OEA—N-oleoylethanolamine. -t-test.

Table 2 presents significant higher levels of OEA ($p = 0.02$), and 2-AG ($p = 0.01$) in the control group compared to the pain groups. The lower part of the table summarizes the differences in salivary eCBs in the three pain groups (musculoskeletal, neurovascular and neuropathic) compared to controls.

Table 2. Salivary eCBS levels in controls compared to total pain group and specific pain groups (fmol/mg protein).

* Group	AA (Mean ± SD)	PEA (Mean ± SD)	OEA (Mean ± SD)	2-AG (Mean ± SD)	AEA (Mean ± SD)
Control	2327.1 ± 2539	12.9 ± 11.75	67.7 ± 77.09	54.71 ± 36.47	0.2 ± 0.18
Pain	1710.2 ± 1552	10.7 ± 11.93	41.2 ± 47.04	35.88 ± 41.29	0.17 ± 0.2
p-value	0.09	0.34	0.02	0.01	0.94
Musculoskeletal	2177.3 ± 1868	9.9 ± 9.30	40.4 ± 37.06	39.6 ± 40.91	0.1 ± 0.14
Neurovascular	1354.3 ± 1354	8.8 ± 10.21	35.6 ± 40.31	33.2 ± 41.45	0.0 ± 0.09
Neuropathic	1749.80 ± 143	13.2 ± 14.71	47.3 ± 58.63	35.9 ± 42.56	0.2 ± 0.45
p-value	0.17	0.36	0.11	0.09	0.07

* Neuropathic group: BMS, TN, PHN, PIFP. Neurovascular group: primary headaches—MIG, NVOP, TTH. Musculoskeletal group: MMP, solely TMJ, TMD (combined MMP and TMJ). The data was subjected to t-test for Control vs. Pain and One way AVOVA for Control vs. specific pain groups.

Table 3 summarizes significant differences between each pain group and controls, specifically AEA and OEA were lower in neurovascular group ($p = 0.02$, 0.04 , respectively), 2-AG was lower in the neuropathic group ($p = 0.05$). No significant difference was found between the musculoskeletal group and controls.

Table 3. Salivary eCBs in specific pain groups compared to controls (*fmol/mg protein*).

Group	eCBs	Pain (mean ± SD)	Control (mean ± SD)	<i>p</i> -Value
Neurovascular	AEA	0.09 ± 0.09	0.17 ± 0.18	0.02
	OEA	35.65 ± 40.31	67.7 ± 77.09	0.04
	AA	1354.3 ± 1386.94	2327.15 ± 2539.27	0.06
Neuropathic	2-AG	35.97 ± 42.56	54.71 ± 36.47	0.05
Musculoskeletal	None	-	-	NS

- The data was subjected to non-parametric Mann-Whitney *U* test.

Table 4 summarizes salivary eCBs levels in relation to pain severity within neuropathic and neurovascular groups: In the neuropathic group, significant differences were found in AA levels ($p = 0.028$) between those with mild versus moderate and severe pain.

Table 4. eCBs in relation to pain severity levels in neuropathic and neurovascular pain groups (*fmol/mg protein*).

Group	eCBs	Pain Level *	<i>n</i>	fmol/mg Protein (Mean ± SD)	<i>p</i> -Value
Neurovascular	OEA	Mild	3	85.42 ± 97.27	0.097
		Moderate	7	30.83 ± 24.87	
		Severe	19	31.65 ± 30.98	
	PEA	Mild	2	22.44 ± 27.85	0.062
		Moderate	7	7.97 ± 6.86	
		Severe	14	7.64 ± 5.86	
Neuropathic	PEA	Mild	3	17.26 ± 9.96	0.067
		Moderate	7	5.57 ± 4.44	
		Severe	14	9.02 ± 7.14	
	AA	Mild	2	4027.43 ± 596	0.028
		Moderate	7	1214.79 ± 1336	
		Severe	14	1514.9 ± 1202	

* Pain level, according to VPS (Verbal Pain Score). Mild = VPS:1–3, Moderate = VPS:4–6, Severe = VPS:7–10. /Nonparametric Kruskal–Wallis test.

4. Discussion

In this study, we highlight, for the first time, the significant reduction in salivary eCB levels in chronic OFP patients. Consequently, we suggest salivary eCB levels may be utilized as biomarkers for OFP. The ECS is involved in a number of physiological and pathological processes. Its anti-nociceptive effects appear to be mediated by CB₁Rs within the CNS [43,44] and via both CB₁Rs and CB₂Rs peripherally [45,46]. Pain sensation can be modulated by the ECS at receptor and ligand levels. At the receptor level, CB₁R expression was lower in mice with neuropathic diabetes compared to controls [47]. Similarly, CB₂R levels are lower in chronic versus acute pain patients [48]. In contrast, FAAH mRNA levels were higher in chronic pain patients, implying enhanced AEA degradation. At the ligand level, Stensson et al., demonstrated higher AEA and 2-AG levels in patients with fibromyalgia following 15 weeks of strength-based exercise which decreased pain and depression. The authors hypothesized that this was due to the effect of eCBs on CB₂R. The effect was unrelated to the CNS level, as sensation and acute pain threshold were unchanged [49].

Data on cannabinoid treatment for headache and OFP are of poor quality and based on case series, case reports, anecdotal reports or retrospective analysis [50]. We hypothesized that the evaluation of levels of 5 salivary eCBs in chronic OFP patients may enhance our understanding of the diagnosis and treatment of these patients, and therefore we examined their salivary levels in 83 chronic OFP patients with different pain etiologies (musculoskeletal, neurovascular and neuropathic) and compared them to 43 pain-free controls. Overall salivary eCBs levels were lower in the chronic OFP patients than controls (Table 3), and these changes were significant for the 3 eCBs—2-AG, OEA, and AA. 2-AG

influences pain sensation via multiple mechanisms, including its effects on TRPV1, CB₁R, and CB₂R signaling, and by serving as a prostaglandin precursor, thereby modulating inflammation and pain [51]. OEA levels were significantly reduced in our pain patients, however its role in pain modulation is unclear. Lower visceral and inflammatory responses via PPAR α -mediated signaling in mice [52] suggests that OEA may act via TRPV1 [53]. OEA may have several physiological roles, including pain perception, but some studies suggest that it has a lesser impact on pain, and a greater effect on energy balance [54,55].

The neuropathic pain patients in the current study had significant reductions in salivary 2-AG levels. Indeed, decreased plasma 2-AG levels have been measured in neuropathic pain patients with optic neuromyelitis [56]. Donvito and colleagues showed that mice without CB₁R and CB₂R experienced neuropathic pain, while overexpression of CB₂R prevented pain [1]. There were no significant differences in salivary eCB levels in the musculoskeletal pain patients compared to controls, and this may be due to the less severe nature of the pain (especially when considering neurovascular or neuropathic pain) [28].

Consistent with this notion, we found that those with higher pain intensity had lower eCB levels (specifically AA; Table 4). The main and significant differences were found when comparing mild pain (VPS 1–3) to moderate (VPS 4–6) and severe pain (VPS 7–10). No significant difference was found between moderate and severe pain. These data suggest that eCB levels correlate with pain sensation, and that their salivary levels may be used as an aid in the diagnosis and assessment of pain. Additionally, salivary PEA, AA and OEA levels were significantly higher in men than women, as reported by Fanelli et al., regarding 2-AG [57]. These differences may be due to physiological differences in expression and localization of ECS components in males and females [58] and may partially explain the higher incidence of chronic pain in general and craniofacial pain in particular in women [28,59].

eCBs have been detected in the saliva under various circumstances, e.g., stress [25,60], food, taste, and appetite [27,61], as well as obesity [26]. Whereas our results do not imply that salivary eCBs are directly related to central eCBs, they rather suggest that they may be associated with orofacial pain (OFP). One may hypothesize that central eCB levels could be reflected by changes in eCB ‘tone’ in the blood and then may influence salivary content of eCBs, since salivary components originate from the salivary glands or may be derived from passive diffusion or active transport from blood that is filtered and processed from the rich glandular vasculature. However, this assumption should be experimentally validated in future studies.

Our findings also imply the existence of a faulty mechanism of pain modulation in chronic OFP patients, as previously suggested for other modalities, e.g., defective conditioned pain modulation [62–64]. The fundamental mechanism where pain is for survival, and therefore beneficial, is disrupted in chronic pain. This may be due to, or associated with, lower eCBs levels. Indeed, human studies include many factors that may influence the results, some of which are quantifiable, and others that are seemingly impossible to measure. This is even more apparent when studying pain, an experience involving emotions and sensations. For example, prophylactic and abortive pain medications may affect results, and they have diverse influences and side effects for different patients with different diagnoses. Psychological elements can be primary or secondary to chronic pain, and these elements are highly individual, and may be related to medication dosage, absorption, sleep quality, and even to the queue at the entrance to the clinic. In this case, eCBs concentrations are dynamic and depends on circadian rhythm (particularly 2-AG), stress, inflammation and others [65]. Any of these factors can influence pain and eCBs levels directly or indirectly.

Taken together, a more focused, selective study is required to: (i) confirm the accuracy of these results for specific pain diagnoses and at different time points; (ii) investigate the effects of successful pain treatment on eCB levels; (iii) identify high-risk groups in order to enable early diagnosis, screening and therapeutic follow-up; and (iv) determine if salivary eCB levels are indeed related to central eCB ‘tone’.

In conclusion, the findings of the current study, that salivary eCBs levels are lower in chronic pain patients than in pain-free individuals, may help select the best therapeutic interventions for these patients. For instance, specific medications or formulations may alter the enzymes involved in the breakdown/synthesis of eCBs, and indirectly affect eCB ‘tone’. Moreover, salivary eCB levels can be used as a diagnostic tool and predictor of success before medical cannabis is used in this patient population.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules27144662/s1>, Table S1: Pain group: Specific diagnosis, group diagnosis and medications.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ECS	endocannabinoid system
eCBs	endocannabinoids and endocannabinoid-like compounds
AEA	Anandamide
2-AG	2-arachidonoylglycerol
PEA	N-palmitoylethanolamine
OEA	N-oleoylethanolamine
AA	Arachidonic acid
OFP	Orofacial Pain
VPS	Verbal Pain Score
IHS	International Headache Society
TMD	Temporomandibular Disorders (i.e., myofascial pain or joint)
DC/TMD	Diagnostic Criteria for Temporomandibular Disorders
Mig	Migraine
NVOP	Neurovascular Orofacial Pain (orofacial migraine)
TTH	Tension Type Headache
PTN	Post Traumatic Neuropathy
TN	Trigeminal Neuralgia
PHN	Post Herpetic Neuralgia
PIFP	Persistent Idiopathic Facial Pain
BMS	Burning Mouth Syndrome

References

1. Donvito, G.; Nass, S.R.; Wilkerson, J.L.; Curry, Z.A.; Schurman, L.D.; Kinsey, S.G.; Lichtman, A.H. The Endogenous Cannabinoid System: A Budding Source of Targets for Treating Inflammatory and Neuropathic Pain. *Neuropsychopharmacology* **2018**, *43*, 52–79. [[CrossRef](#)]
2. Felder, C.C.; Glass, M. Cannabinoid receptors and their endogenous agonists. *Annu. Rev. Pharmacol. Toxicol.* **1998**, *38*, 179–200. [[CrossRef](#)] [[PubMed](#)]
3. Hossain, M.Z.; Ando, H.; Unno, S.; Kitagawa, J. Targeting Peripherally Restricted Cannabinoid Receptor 1, Cannabinoid Receptor 2, and Endocannabinoid-Degrading Enzymes for the Treatment of Neuropathic Pain Including Neuropathic Orofacial Pain. *Int. J. Mol. Sci.* **2020**, *21*, 41423. [[CrossRef](#)] [[PubMed](#)]
4. Beltramo, M. Cannabinoid type 2 receptor as a target for chronic—Pain. *Mini Rev. Med. Chem.* **2009**, *9*, 11–25. [[CrossRef](#)] [[PubMed](#)]
5. Sarchielli, P.; Pini, L.A.; Coppola, F.; Rossi, C.; Baldi, A.; Mancini, M.L.; Calabresi, P. Endocannabinoids in chronic migraine: CSF findings suggest a system failure. *Neuropsychopharmacology* **2007**, *32*, 1384–1390. [[CrossRef](#)]
6. Greco, R.; Gasperi, V.; Maccarrone, M.; Tassorelli, C. The endocannabinoid system and migraine. *Exp. Neurol.* **2010**, *224*, 85–91. [[CrossRef](#)]

7. Fine, P.G.; Rosenfeld, M.J. The endocannabinoid system, cannabinoids, and pain. *Rambam Maimonides Med. J.* **2013**, *4*, e0022. [[CrossRef](#)]
8. Burston, J.J.; Woodhams, S.G. Endocannabinoid system and pain: An introduction. *Proc. Nutr. Soc.* **2014**, *73*, 106–117. [[CrossRef](#)]
9. Manzanares, J.; Julian, M.; Carrascosa, A. Role of the cannabinoid system in pain control and therapeutic implications for the management of acute and chronic pain episodes. *Curr. Neuropharmacol.* **2006**, *4*, 239–257. [[CrossRef](#)]
10. Vuckovic, S.; Srebro, D.; Vujovic, K.S.; Vucetic, C.; Prostran, M. Cannabinoids and Pain: New Insights from Old Molecules. *Front. Pharmacol.* **2018**, *9*, 1259. [[CrossRef](#)]
11. Pacher, P.; Batkai, S.; Kunos, G. The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol. Rev.* **2006**, *58*, 389–462. [[CrossRef](#)]
12. Sagar, D.R.; Gaw, A.G.; Okine, B.N.; Woodhams, S.G.; Wong, A.; Kendall, D.A.; Chapman, V. Dynamic regulation of the endocannabinoid system: Implications for analgesia. *Mol. Pain* **2009**, *5*, 59. [[CrossRef](#)]
13. McPartland, J.M.; Guy, G.W.; Di Marzo, V. Care and feeding of the endocannabinoid system: A systematic review of potential clinical interventions that upregulate the endocannabinoid system. *PLoS ONE* **2014**, *9*, e89566. [[CrossRef](#)]
14. Aran, A.; Eylon, M.; Harel, M.; Polianski, L.; Nemirovski, A.; Tepper, S.; Schnapp, A.; Cassuto, H.; Wattad, N.; Tam, J. Lower circulating endocannabinoid levels in children with autism spectrum disorder. *Mol. Autism* **2019**, *10*, 2. [[CrossRef](#)]
15. Russo, E.B. Clinical endocannabinoid deficiency (CECD): Can this concept explain therapeutic benefits of cannabis in migraine, fibromyalgia, irritable bowel syndrome and other treatment-resistant conditions? *Neuro. Endocrinol. Lett.* **2004**, *25*, 31–39.
16. Greabu, M.; Battino, M.; Mohora, M.; Totan, A.; Didilescu, A.; Spinu, T.; Totan, C.; Miricescu, D.; Radulescu, R. Saliva—A diagnostic window to the body, both in health and in disease. *J. Med. Life* **2009**, *2*, 124–132.
17. Loo, J.A.; Yan, W.; Ramachandran, P.; Wong, D.T. Comparative human salivary and plasma proteomes. *J. Dent. Res.* **2010**, *89*, 1016–1023. [[CrossRef](#)]
18. Zidverc-Trajkovic, J.; Stanimirovic, D.; Obrenovic, R.; Tajti, J.; Vecsei, L.; Gardi, J.; Nemeth, J.; Mijajlovic, M.; Sternic, N.; Jankovic, L. Calcitonin gene-related peptide levels in saliva of patients with burning mouth syndrome. *J. Oral Pathol. Med.* **2009**, *38*, 29–33. [[CrossRef](#)]
19. Fischer, M.; Wille, G.; Klien, S.; Shanib, H.; Holle, D.; Gaul, C.; Broessner, G. Brain-derived neurotrophic factor in primary headaches. *J. Headache Pain* **2012**, *13*, 469–475. [[CrossRef](#)]
20. Gerdle, B.; Ghafouri, B.; Ernberg, M. Chronic musculoskeletal pain: Review of mechanisms and biochemical biomarkers as assessed by the microdialysis technique. *J. Pain Res.* **2014**, *7*, 313–326. [[CrossRef](#)]
21. Mandel, A.L.; Ozdener, H.; Utermohlen, V. Identification of pro- and mature brain-derived neurotrophic factor in human saliva. *Arch. Oral Biol.* **2009**, *54*, 689–695. [[CrossRef](#)]
22. Krief, G.; Haviv, Y.; Deutsch, O.; Keshet, N.; Almozino, G.; Zacks, B.; Palmon, A.; Aframian, D.J. Proteomic profiling of whole-saliva reveals correlation between Burning Mouth Syndrome and the neurotrophin signaling pathway. *Sci. Rep.* **2019**, *9*, 4794. [[CrossRef](#)]
23. Segal, A.; Wong, D.T. Salivary diagnostics: Enhancing disease detection and making medicine better. *Eur. J. Dent. Educ. Off. J. Assoc. Dent. Educ. Eur.* **2008**, *12*, 22–29.
24. Ney, L.J.; Matthews, A.; Hsu, C.K.; Zuj, D.V.; Nicholson, E.; Steward, T.; Nichols, D.; Graham, B.; Harrison, B.; Bruno, R.; et al. Cannabinoid polymorphisms interact with plasma endocannabinoid levels to predict fear extinction learning. *Depress. Anxiety* **2021**, *38*, 1087–1099. [[CrossRef](#)]
25. Ney, L.; Stone, C.; Nichols, D.; Felmingham, K.; Bruno, R.; Matthews, A. Endocannabinoid reactivity to acute stress: Investigation of the relationship between salivary and plasma levels. *Biol. Psychol.* **2021**, *159*, 108022. [[CrossRef](#)]
26. Matias, I.; Gatta-Cherifi, B.; Tabarin, A.; Clark, S.; Leste-Lasserre, T.; Marsicano, G.; Piazza, P.V.; Cota, D. Endocannabinoids measurement in human saliva as potential biomarker of obesity. *PLoS ONE* **2012**, *7*, e42399. [[CrossRef](#)]
27. Tarragon, E.; Bindila, L.; Zimmer, P.; Lutz, B.; Meyer, J. Salivary endocannabinoids as mediators in the relationship between omega-6 and omega-3 fatty acid ratio intake from highly-processed foods and anthropometric markers of health in women. *Int. J. Food Sci. Nutr.* **2020**, *71*, 235–241. [[CrossRef](#)]
28. Sharav, Y.B.R. *Quintessence 2015. Orofacial Pain and Headache*; Quintessence: Surrey, UK, 2015.
29. Schiffman, E.; Ohrbach, R.; Truelove, E.; Look, J.; Anderson, G.; Goulet, J.P.; List, T.; Svensson, P.; Gonzalez, Y.; Lobbezoo, F.; et al. Diagnostic Criteria for Temporomandibular Disorders (DC/TMD) for Clinical and Research Applications: Recommendations of the International RDC/TMD Consortium Network * and Orofacial Pain Special Interest Groupdagger. *J. Oral Facial Pain Headache* **2014**, *28*, 6–27. [[CrossRef](#)]
30. Arnold, M. Headache Classification Committee of the International Headache Society (IHS) The International Classification of Headache Disorders, 3rd edition. *Cephalalgia Int. J. Headache* **2018**, *38*, 1–211. [[CrossRef](#)]
31. Benoliel, R.; Sharav, Y.; Eliav, E. Neurovascular orofacial pain. *J. Am. Dent. Assoc.* **2010**, *141*, 1094–1096. [[CrossRef](#)]
32. Haviv, Y.; Khan, J.; Zini, A.; Almozino, G.; Sharav, Y.; Benoliel, R. Trigeminal neuralgia (part I): Revisiting the clinical phenotype. *Cephalalgia Int. J. Headache* **2016**, *36*, 730–746. [[CrossRef](#)] [[PubMed](#)]
33. Haviv, Y.; Zadik, Y.; Sharav, Y.; Benoliel, R. Painful traumatic trigeminal neuropathy: An open study on the pharmacotherapeutic response to stepped treatment. *J. Oral Facial Pain Headache* **2014**, *28*, 52–60. [[CrossRef](#)] [[PubMed](#)]
34. Benoliel, R.; Gaul, C. Persistent idiopathic facial pain. *Cephalalgia Int. J. Headache* **2017**, *37*, 680–691. [[CrossRef](#)] [[PubMed](#)]

35. Benoliel, R.; Eliav, E.; Elishoov, H.; Sharav, Y. Diagnosis and treatment of persistent pain after trauma to the head and neck. *J. Oral Maxillofac. Surg. Off. J. Am. Assoc. Oral Maxillofac. Surg.* **1994**, *52*, 1138–1147, discussion 1147–1138. [[CrossRef](#)]
36. Haviv, Y.; Rettman, A.; Aframian, D.; Sharav, Y.; Benoliel, R. Myofascial pain: An open study on the pharmacotherapeutic response to stepped treatment with tricyclic antidepressants and gabapentin. *J. Oral Facial Pain Headache* **2015**, *29*, 144–151. [[CrossRef](#)]
37. Haviv, Y.; Zini, A.; Etzioni, Y.; Klitinich, V.; Dobriyan, A.; Sharav, Y.; Benoliel, R.; Almozino, G. The impact of chronic orofacial pain on daily life: The vulnerable patient and disruptive pain. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol.* **2017**, *123*, 58–66. [[CrossRef](#)]
38. Aframian, D.J.; Helcer, M.; Livni, D.; Markitziu, A. Pilocarpine for the treatment of salivary glands' impairment caused by radioiodine therapy for thyroid cancer. *Oral Dis.* **2006**, *12*, 297–300. [[CrossRef](#)]
39. Azar, S.; Sherf-Dagan, S.; Nemirovski, A.; Webb, M.; Raziell, A.; Keidar, A.; Goitein, D.; Sakran, N.; Shibolet, O.; Tam, J.; et al. Circulating Endocannabinoids Are Reduced Following Bariatric Surgery and Associated with Improved Metabolic Homeostasis in Humans. *Obes. Surg.* **2019**, *29*, 268–276. [[CrossRef](#)]
40. Baraghithy, S.; Smoum, R.; Drori, A.; Hadar, R.; Gammal, A.; Hirsch, S.; Attar-Namdar, M.; Nemirovski, A.; Gabet, Y.; Langer, Y.; et al. Magel2 Modulates Bone Remodeling and Mass in Prader-Willi Syndrome by Affecting Oleoyl Serine Levels and Activity. *J. Bone Miner. Res.* **2019**, *34*, 93–105. [[CrossRef](#)]
41. Knani, I.; Earley, B.J.; Udi, S.; Nemirovski, A.; Hadar, R.; Gammal, A.; Cinar, R.; Hirsch, H.J.; Pollak, Y.; Gross, I.; et al. Targeting the endocannabinoid/CB1 receptor system for treating obesity in Prader-Willi syndrome. *Mol. Metab.* **2016**, *5*, 1187–1199. [[CrossRef](#)]
42. Tartakover Matalon, S.; Azar, S.; Meiri, D.; Hadar, R.; Nemirovski, A.; Abu Jabal, N.; Konikoff, F.M.; Drucker, L.; Tam, J.; Naftali, T. Endocannabinoid Levels in Ulcerative Colitis Patients Correlate With Clinical Parameters and Are Affected by Cannabis Consumption. *Front. Endocrinol.* **2021**, *12*, 685289. [[CrossRef](#)]
43. Walker, J.M.; Hohmann, A.G. Cannabinoid mechanisms of pain suppression. *Handb. Exp. Pharmacol.* **2005**, *168*, 509–554.
44. Woodhams, S.G.; Sagar, D.R.; Burston, J.J.; Chapman, V. The role of the endocannabinoid system in pain. *Handb. Exp. Pharmacol.* **2015**, *227*, 119–143.
45. Agarwal, N.; Pacher, P.; Tegeder, I.; Amaya, F.; Constantin, C.E.; Brenner, G.J.; Rubino, T.; Michalski, C.W.; Marsicano, G.; Monory, K.; et al. Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors. *Nat. Neurosci.* **2007**, *10*, 870–879. [[CrossRef](#)]
46. Richardson, J.D.; Aanonsen, L.; Hargreaves, K.M. Hypoactivity of the spinal cannabinoid system results in NMDA-dependent hyperalgesia. *J. Soc. Neurosci.* **1998**, *18*, 451–457. [[CrossRef](#)]
47. Zhang, F.; Hong, S.; Stone, V.; Smith, P.J. Expression of cannabinoid CB1 receptors in models of diabetic neuropathy. *J. Pharmacol. Exp. Ther.* **2007**, *323*, 508–515. [[CrossRef](#)]
48. Ramesh, D.; D'Agata, A.; Starkweather, A.R.; Young, E.E. Contribution of Endocannabinoid Gene Expression and Genotype on Low Back Pain Susceptibility and Chronicity. *Clin. J. Pain* **2018**, *34*, 8–14. [[CrossRef](#)]
49. Stensson, N.; Gerdle, B.; Ernberg, M.; Mannerkorpi, K.; Kosek, E.; Ghafouri, B. Increased Anandamide and Decreased Pain and Depression after Exercise in Fibromyalgia. *Med. Sci. Sports Exerc.* **2020**, *52*, 1617–1628. [[CrossRef](#)]
50. Fitzcharles, M.A.; Petzke, F.; Tolle, T.R.; Hauser, W. Cannabis-Based Medicines and Medical Cannabis in the Treatment of Nociceptive Pain. *Drugs* **2021**, *81*, 2103–2116. [[CrossRef](#)]
51. Baggelaar, M.P.; Maccarrone, M.; van der Stelt, M. 2-Arachidonoylglycerol: A signaling lipid with manifold actions in the brain. *Prog. Lipid Res.* **2018**, *71*, 1–17. [[CrossRef](#)]
52. Suardiaz, M.; Estivill-Torres, G.; Goicoechea, C.; Bilbao, A.; Rodriguez de Fonseca, F. Analgesic properties of oleoylethanolamide (OEA) in visceral and inflammatory pain. *Pain* **2007**, *133*, 99–110. [[CrossRef](#)]
53. Wang, X.; Miyares, R.L.; Ahern, G.P. Oleoylethanolamide excites vagal sensory neurones, induces visceral pain and reduces short-term food intake in mice via capsaicin receptor TRPV1. *J. Physiol.* **2005**, *564*, 541–547. [[CrossRef](#)]
54. De Fonseca, F.R.; Del Arco, I.; Bermudez-Silva, F.J.; Bilbao, A.; Cippitelli, A.; Navarro, M. The endocannabinoid system: Physiology and pharmacology. *Alcohol Alcohol.* **2005**, *40*, 2–14. [[CrossRef](#)]
55. De Fonseca, F.R.; Navarro, M.; Gomez, R.; Escuredo, L.; Nava, F.; Fu, J.; Murillo-Rodriguez, E.; Giuffrida, A.; LoVerme, J.; Gaetani, S.; et al. An anorexic lipid mediator regulated by feeding. *Nature* **2001**, *414*, 209–212. [[CrossRef](#)]
56. Pellkofer, H.L.; Havla, J.; Hauer, D.; Schelling, G.; Azad, S.C.; Kuempfel, T.; Magerl, W.; Hugel, V. The major brain endocannabinoid 2-AG controls neuropathic pain and mechanical hyperalgesia in patients with neuromyelitis optica. *PLoS ONE* **2013**, *8*, e71500.
57. Fanelli, F.; Mezzullo, M.; Belluomo, I.; Di Lallo, V.D.; Baccini, M.; Ibarra Gasparini, D.; Casadio, E.; Mastroberto, M.; Vicennati, V.; Gambineri, A.; et al. Plasma 2-arachidonoylglycerol is a biomarker of age and menopause related insulin resistance and dyslipidemia in lean but not in obese men and women. *Mol. Metab.* **2017**, *6*, 406–415. [[CrossRef](#)] [[PubMed](#)]
58. Levine, A.; Liktov-Busa, E.; Lipinski, A.A.; Couture, S.; Balasubramanian, S.; Aicher, S.A.; Langlais, P.R.; Vanderah, T.W.; Largent-Milnes, T.M. Sex differences in the expression of the endocannabinoid system within V1M cortex and PAG of Sprague Dawley rats. *Biol. Sex Differ.* **2021**, *12*, 60. [[CrossRef](#)] [[PubMed](#)]
59. Rovner, G.S.; Sunnerhagen, K.S.; Bjorkdahl, A.; Gerdle, B.; Borsbo, B.; Johansson, F.; Gillanders, D. Chronic pain and sex-differences; women accept and move, while men feel blue. *PLoS ONE* **2017**, *12*, e0175737. [[CrossRef](#)]

60. Ney, L.J.; Felmingham, K.L.; Bruno, R.; Matthews, A.; Nichols, D.S. Simultaneous quantification of endocannabinoids, oleoylethanolamide and steroid hormones in human plasma and saliva. *J. Chromatogr. B. Anal. Technol. Biomed. Life Sci.* **2020**, *1152*, 122252. [[CrossRef](#)]
61. Mennella, I.; Di Monaco, R.; Balazy, A.; Ferracane, R.; Miele, N.A.; Cavella, S.; Vitaglione, P.S. Alivary endocannabinoids and N-acylethanolamines upon mastication of a semisolid food: Implications in fat taste, appetite and food liking. *Food Funct.* **2018**, *9*, 476–484. [[CrossRef](#)]
62. Staud, R. Abnormal endogenous pain modulation is a shared characteristic of many chronic pain conditions. *Expert Rev. Neurother.* **2012**, *12*, 577–585. [[CrossRef](#)]
63. Khan, J.; Korczeniewska, O.; Benoliel, R.; Kalladka, M.; Eliav, E.; Nasri-Heir, C. Age and gender differences in mechanically induced intraoral temporal summation and conditioned pain modulation in healthy subjects. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol.* **2018**, *126*, 134–141. [[CrossRef](#)]
64. Yarnitsky, D. Conditioned pain modulation (the diffuse noxious inhibitory control-like effect): Its relevance for acute and chronic pain states. *Curr. Opin. Anaesthesiol.* **2010**, *23*, 611–615. [[CrossRef](#)]
65. Hillard, C.J. Circulating Endocannabinoids: From Whence Do They Come and where are They Going? *Neuropsychopharmacology* **2018**, *43*, 155–172. [[CrossRef](#)]