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Migraines: Causes and Non-Pharmacological Treatments

Male and female sex hormones in primary headaches

Abstract

Background: The three primary headaches, tension-type headache, migraine and cluster headache, occur in both genders, but all seem to have a sex-specific prevalence. These gender differences suggest that both male and female sex hormones could have an influence on the course of primary headaches. This review aims to summarise the most relevant and recent literature on this topic.

Methods: Two independent reviewers searched PUBMED in a systematic manner. Search strings were composed using the terms LH, FSH, progesteron*, estrogen*, DHEA*, prolactin, testosterone, androgen*, headach*, migrain*, "tension type" or cluster. A timeframe was set limiting the search to articles published in the last 20 years, after January 1st 1997.

Results: Migraine tends to follow a classic temporal pattern throughout a woman's life corresponding to the fluctuation of estrogen in the different reproductive stages. The estrogen withdrawal hypothesis forms the basis for most of the assumptions made on this behalf. The role of other hormones as well as the importance of sex hormones in other primary headaches is far less studied.

Conclusion: The available literature mainly covers the role of sex hormones in migraine in women. Detailed studies especially in the elderly of both sexes and in cluster headache and tension-type headache are warranted to fully elucidate the role of these hormones in all primary headaches.

Keywords: Primary headache, Migraine, Tension-type headache, Cluster headache, Sex hormones, Estrogen, Testosterone, Gender

Introduction

The primary headaches covered in this review are tension-type headache (TTH), migraine and cluster headache (CH). All three entities occur in both men and women, yet display a sex-specific prevalence. These gender differences suggest that both male and female sex hormones could have an influence on the course of primary headaches.

TTH has a female preponderance, and is 1.5 times more frequent in women than in men [1]. CH, on the other hand, appears to have a higher incidence in men, specifically during young adulthood and middle age. Later in life the prevalence of CH evens out between the sexes [2]. Within the group of primary headaches the role of sex hormones has been studied most profoundly in migraine. Prepubertal children have a 3-10% prevalence of migraine without any gender difference [3, 4]. With onset of puberty and its associated hormonal changes, migraine becomes 2–3 times more common in women than in men, suggesting that migraine is influenced by the fluctuating hormonal status through menarche, menstruation, pregnancy, menopause, as well as the use of oral contraceptives and hormonal replacement therapy (HRT) [1, 3, 5–8].

In contrast, the course of migraine throughout the lifespan of men appears relatively stable, further pointing to the unique role of female sex hormones in the migraine phenotype [1]. Here, we summarise relevant literature of the last 20 years covering the influence of female and male sex hormones on primary headaches.

Search strategy and selection criteria

Two independent reviewers conducted a search on PubMed, using their own search string, composed of terms like LH, FSH, Progesteron*, estrogen*, DHEA*, Prolactin, Testosterone, androgen*AND Headach* OR Migrain* OR "Tension type" OR Cluster. This general search was performed on December 7th, 2017. In light of the large amount of published work on the topic and considering the evolution of the diagnostic criteria over time, the first search was conducted respecting a timeframe of 20 years, covering articles published after January 1st 1997. The initial screening was performed based on eligibility of title and abstract. Exclusion criteria included non-availability of abstract, animal studies, and articles in any language other than English. Original studies, published in full, constitute the core of this review. Other quoted references include systematic reviews, case reports, meta-analysis, Cochrane reviews, letters, lectures and comments. Any relevant publications cited in the eligible articles were also included. Differences between reviewers were resolved by careful discussion.

Results

Women

Childhood and adolescence

Almost 60% of girls and 50% of boys suffer from headache at some time during childhood and adolescence, with the prevalence increasing significantly during adolescence in girls, whereas it remains stable for boys [9]. The incidence of migraine is similar in both sexes until the age of 9 (2.5% of girls and 2.4% of boys) and then diverges to the disadvantage of girls [6]. Teenagers who suffer from headache are at greater risk of having headache in adulthood [9].

It is known that during puberty, sexual steroid hormones affect neural circuits and cause permanent changes in important brain areas such as the hypothalamus and the insula [4]. Onset of migraine frequently occurs around the time of menarche, as cyclic hormonal changes begin. Early menarche appears to be a risk factor for the development of migraine [6, 10]. Notably, the first menstrual cycles are often anovulatory and in general ovulation occurs one or two years later. In the USA, the average age of menarche is 12.8 years, but this may vary geographically. Migraine with aura has an incidence peak between ages 12 to 13, while migraine without aura typically presents a few years later. Thus, migraine without aura may be associated with the establishment of a regular ovulatory menstrual cycle [7]. Headaches are reported in 53% of adolescent girls at the onset of menses. Pubertal development and age seem to modulate the effect of ovarian hormones on migraine. In fact, high urinary levels of pregnandiol glucuronide, a metabolite of progesterone, are associated with a higher migraine frequency in girls before menarche, but with a lower frequency after menarche [11]. Hershey et al. identified specific genomic patterns in girls suffering from menstrual migraine, suggesting a genetic predisposition for the development of this condition during adolescence [12].

TTH shows a similar, increasing trend in girls by the time of menarche. The incidence ratio between boys and girls changes from 1.3:1 during childhood to 1:1.2 after menarche [13].

It is noteworthy to mention, that pathological changes in sexual hormones can cause a secondary headache. For instance, hyperprolactinemia manifests in up to 45% of childhood cases with headache as a first symptom [14–16].

Adulthood

Migraine Women have a 3.25-fold higher risk of suffering from migraine than men [17]. A prevalence peak is reached in women between the ages of 35 and 45, with 25-30% of the general female population being affected, in comparison to only 8% of the general male population [18]. Female migraine patients also report a significant higher burden of disease and greater use of analgesic compared to men [6, 13].

In terms of deciphering the pathophysiological mechanism of the preponderance of migraine in women, neuroimaging studies have revealed sex-specific activation patterns, with an increased activation of the insula and precuneus in women. These regions are involved in pain, sensation and affective processing [19]. Sex hormones can cross the blood-brain barrier passively and are at least partially responsible for these sex differences [18]. Most available literature focuses on the effects of estrogen, while the role of progesterone has been less thoroughly investigated.

The relationship between estrogen and migraine is complex, involving modulation by genomic and non-genomic effects [20, 21]. Obese women appear to have more than a twofold risk of episodic and chronic migraine, probably due to the pathological estrogen production in adipose tissue [22, 23]. Substantial evidence points to the serotonergic system as a key player in migraine pathogenesis [7]. Estrogen modulates serotonergic neurotransmission, by increasing the expression of the tryptophan hydroxylase and decreasing the expression of the serotonin reuptake transporter [7, 24, 25]. Estrogen also activates the endogenous opioidergic system, which has an analgesic effect on persistent, inflammatory pain [26]. Furthermore, estrogen induces vascular changes by modulating vasodilation and suppressing vascular inflammatory responses [6, 27, 28].

The levels of calcitonin gene-related peptide (CGRP), a neuropeptide with a key role in migraine pathophysiology, are higher in women of reproductive age than in

men. Cyclic hormonal fluctuations influence CGRP release and consequently the trigeminovascular system [29]. While studies have reported a positive relationship between CGRP and estrogen levels, newer studies suggest an inverse relationship between the two [24].

Experimental studies suggest progesterone to play a protective role, by reducing nociception in the trigeminovascular system, inhibiting neurogenic edema, and histamine secretion from mast cells and decreasing prostaglandin production [7, 24, 30, 31].

Multiple studies have examined the association between polymorphisms in estrogen or progesterone receptor genes and migraine risk, with inconclusive findings [32–37]. In their meta-analysis, Schürks et al. and Li et al. concluded that exon 4 325C > G and exon 8 594G > A polymorphisms are risk factors for migraine, while the often examined PROGINS variant in the progesterone receptor gene did not seem to play a significant role in the Caucasian population [38, 39]. On the contrary, Joshi et al. found a protective role of the PROGINS polymorphism in an Indian population [40].

Prolactin could also play a modulatory role in migraine. Parashar et al. found higher prolactin levels in migraineurs compared to controls [41]. An association between high prolactin levels and migraine chronification has been proposed by Cavestro et al. [42], where Peres et al. detected decreased nocturnal prolactin peaks in chronic migraine patients [43].

There are a few reports suggesting that testosterone can play a role in migraine in women [44, 45]. In one case report, the 5α reductase inhibitor finasteride was administered to a young woman with migraine and led to an almost complete remission [45]. The mechanism of action of testosterone on migraine pathophysiology is still unknown, but may involve modulation of cerebral blood flow, serotonergic tone, and susceptibility to cortical spreading depression [44].

Menstrual migraine The probability of migraine to occur during the perimenstrual period is twice as high compared to any other moment of the menstrual cycle [46]. Almost half of female migraine patients report an association between headache and their menstrual cycle [17]. Depending on whether migraine occurs exclusively during the perimenstrual period or also at other times, the International Headache Society (IHS) distinguishes a pure menstrual migraine from a menstrually-related migraine (Table 1). Migraine associated with menstruation is mostly of the type without aura [21].

Pure menstrual migraine and menstrually-related migraine have an overall prevalence of respectively 1% and 7% in the general population [47]. Data from specialized headache clinics suggest that perimenstrual attacks are more severe, long-lasting and difficult to treat with abortive anti-migraine medication [48]. However, these results could not be confirmed in the general population [49]. Menstrual migraine appears to limit work and social activities more frequently than common migraine and is often associated with a dysphoric mood [17].

The "Estrogen withdrawal hypothesis", developed by Somerville and colleagues in 1972, postulates that attacks of menstrual migraine are triggered by the decrease in estrogen levels preceding menstruation [21]. A drop in estrogen may cause an increased sensitivity to prostaglandins and a release of neuropeptides such as CGRP, substance P and neurokinins which could result in neurogenic inflammation [17]. This physiological response provokes alterations in the microvasculature of the dura mater, changes in calcium and magnesium concentrations, and an imbalance in serotonin and dopamine concentrations [17, 21, 50]. Estrogen withdrawal might lead to an increased oxidative stress in the cells [51]. To confirm this hypothesis, intramuscular injections of estrogen were administered before menstruation and thereby postponing migraine attacks [52, 53]. On the contrary, progesterone injections only led to postpone menses, but not migraine [52, 54].

More recent studies confirm that an estrogen drop can trigger migraine, especially if this drop is preceded by a phase of high estrogen levels, as in the luteal phase of the menstrual cycle, and if the magnitude of the decrease is greater than 10 μg [55, 56]. Interestingly, women with migraine seem to have a faster drop in estrogen levels than non-migraineurs [57].

Welch et al. tried to explain estrogen effects on menstrual migraine with a "mismatch theory". Under normal circumstances, genomic effects of estrogen can counterbalance non-genomic mediated membrane excitability.

Table 1 IHS classification (ICHD-3) for pure menstrual and menstrually-related migraine

Pure menstrual migraine	Menstrually-related migraine
A. Attacks, in a menstruating woman, fulfilling criteria for migraine without aura	A. Attacks, in a menstruating woman, fulfilling criteria for migraine without aura
B. Attacks occur exclusively on day 1 ± 2 (i.e, days − 2 to + 3)[a] of menstruation[b] in at least two out of three menstrual cycles and at no other times of the cycle	B. Attacks occur on day 1 ± 2 (i.e, days − 2 to + 3)[a] of menstruation[b] in at least two out of three menstrual cycles and additionally at other times of the cycle

[a]The first day of menstruation is day 1 and the preceding day is day − 1; there is no day 0
[b]For the purposes of this classification, menstruation is considered to be endometrial bleeding resulting from either the normal menstrual cycle or from the withdrawal of exogenous progestogens, as in the case of combined oral contraceptives and cyclical hormone replacement therapy

In low estrogen states, this inhibiting genomic effect does not suffice, and migraine attacks occur more frequently [58, 59].

In one retrospective study with 85 female patients with menstrual migraine, 35.3% reported migraine headache onset by the end of menstruation, which is days after the estrogen drop. The authors hypothesize that this type of migraine headache is not related to hormonal changes but most probably to transient anemia due to blood loss [56].

Hormonal treatment of menstrual migraine, like perimenstrual application of estrogen gel or a transdermal estradiol patch, can lead to less frequent, shorter and less intensive attacks [46, 47, 52, 60]. Attacks may recur after discontinuation of hormonal treatment [17]. Following the estrogen withdrawal hypothesis, eliminating estrogen cycling appears to be a useful strategy for long-term prophylaxis of menstrual migraine. Therefore, continuous combined contraceptive therapy regimes, containing both estrogen and progesterone, can be considered. However, there is currently no evidence that hormonal therapy is more effective than non-hormonal pharmacological treatment strategies. Hormonal therapy is particularly recommended if other indications like acne or hirsutism exist. Contraindications should be ruled out [17, 53]. Alternatively, progesterone-only contraceptives can be considered. A significant reduction in migraine intensity and frequency is reported [17, 61–63]. As progesterone has no experimental effect on cortical spreading depression, progesterone-only contraception is hypothesized to be a safer choice for women with aura [62, 64], but no clinical evidence has confirmed this theory. The selective estrogen receptor modulator Tamoxifen might also be beneficial in women with menstrual migraine. However, its use is not generally recommended due to possible and in part serious side effects [65]. Some studies suggest that phytoestrogens like soy isoflavone, dong quai or black cohosh could have a beneficial effect on migraine [17]. Martin et al. examined the efficacy of the gonadotropin-releasing hormone antagonist goserelin as a prophylactic therapy. Goserelin alone did not affect migraine headache frequency. Some benefit was obtained when combined with 100 μg estradiol [66]. Glaser et al. demonstrated that continuous testosterone therapy through a subcutaneous implant for 3 months led to headache improvement in 92% of migraine patients [44].

Migraine with aura The female dominance is also seen in migraine with aura. In prevalence studies performed after 1988 it reaches a prevalence of 1.2-3.7% in men and 2.6-10.8% in women [67]. In contrast to menstrual migraine, migraine with aura occurs more frequently with high estrogen levels [68]. Estrogen seems to change cortical susceptibility and contributes to the development of cortical spreading depression. The amplitude of the spreading depression depends on the estrogen level [69]. The threshold for cortical excitability and subsequent cortical spreading depression is lowered through several genomic and non-genomic mechanisms, including upregulation of NMDA receptors, downregulation of GABA neurons and modulation of axonal plasticity [4, 69, 70].

Exogenous hormone-induced headache In the Western world, almost one third of women of reproductive age use oral contraception [55]. The IHS identifies two headache entities related to the use of hormonal contraceptives: exogenous hormone-induced headache and estrogen-withdrawal headache (Table 2).

Headache is one of the most common side effects of hormonal therapies [71]. For instance Tamoxifen, mentioned above as a possible treatment for menstrual migraine, can also cause headache. The onset of hormone-induced headache is typically within the first months of use [72]. Combined contraception remedies (oral pill, transdermal patch, vaginal ring) appear to be associated with both migraine and non-migraine headaches [73]. The effect in migraine patients is variable. One out of two female migraine patients report no change of the headache pattern, 15% experience an improvement, while 28% report worsening [74]. A negative effect occurs more often in migraine with aura [72]. Headaches most frequently occur in the “pill-free” week [53]. The neuronal nociceptive sensitivity is increased in this week and the probability of getting a headache is 20% higher [74, 75]. Higher age (> 35 years) and a positive family history for migraine are risk factors [76, 77].

Possible contraceptive strategies to reduce headache include extended-cycle combined hormonal contraception,

Table 2 IHS classification (ICHD-3) for exogenous hormone-induced headache and estrogen-withdrawal headache

Exogenous hormone-induced headache	Estrogen-withdrawal headache
A. Headache or migraine fulfilling criteria C and D	A. Headache or migraine fulfilling criteria C and D
B. Regular use of exogenous hormones	B. Daily use of exogenous estrogen for ≥3 weeks, which has been interrupted
C. Headache or migraine develops or markedly worsens within 3 months of commencing exogenous hormones	C. Headache or migraine develops within 5 days after last use of estrogen
D. Headache or migraine resolves or reverts to its previous pattern within 3 months after total discontinuation of exogenous hormones	D. Headache or migraine resolves within 3 days of its onset

progesterone-only contraception or new generation hormones like estradiol valerate/dienogest [17, 62, 78, 79]. Eliminating the pill-free week is associated with improvement of headache, pelvic pain and quality of life [55].

In progestin-only methods (oral pill, subdermal implant, depot-injection, levonorgestrel-releasing intrauterine system) headache is a common complaint at the beginning of therapy but classically improves after a few months. There is no known association between progestin-only methods and the worsening of migraine [74]. On the contrary, frequency and intensity of migraine can significantly improve with this type of contraception. Ten percent of patients discontinue treatment due to side effects, particularly spotting [80, 81].

Migraine with aura is associated with a twofold risk of major cardiovascular events, like ischemic stroke. This risk is directly proportional to aura frequency [55]. In the meta-analysis of Schürks et al. a relative stroke risk of 1.73 (95% CI 1.31-2.29) was found for any type of migraine. The relative risk of stroke in women suffering from migraine with aura is 2.08 (95% CI 1.3-3.31). The relative risk of cardiovascular deaths in women with migraine is 1.60 (95% CI 1.72-2.43) [82]. Older combined hormonal therapies with high dosed estrogen (50–150 μg) are associated with a 4.4-fold risk of stroke in migraine patients, in particular in migraine with aura and should not be used anymore. The modern low-estrogen contraceptives (< 25 μg) seem much safer [55, 56]. The 2017 consensus statement from the European Headache Federation and the European Society of Contraception and Reproductive Health recommends against the use of combined hormonal contraceptives in women with migraine with aura seeking hormonal contraception. They postulate a strong recommendation to prefer non-hormonal (condoms, copper-bearing intrauterine device, permanent methods) or progestogen-only alternatives. The same strategy is preferred in women with migraine without aura who have additional cardiovascular risk factors, like smoking, arterial hypertension, previous history of a trombo-embolic event. When there are no such risk factors, combined hormonal contraceptives are considered a possible contraceptive option with monitoring of migraine frequency and characteristics in women without aura. Other medical conditions like polycystic ovary syndrome or endometriosis can influence the risk/benefit profile and have an impact on the preferred type of contraception [83].

Tension-type headache The impact of hormones on TTH is less frequently studied. Like migraine, TTH occurs more often in women than in men and some studies have suggested an increase during hormonal changes such as menses or pregnancy. Menstruation can be an aggravating factor in 40-60% of patients [13]. There is no evidence that TTH is influenced by hormonal contraception [77].

Cluster headache The hypothalamus is thought to be involved in CH pathophysiology based on its periodic time locked occurrence. Sex hormones appear to modulate hypothalamic activity and could be effective as a treatment for therapy refractory CH [84]. Both male and female cluster patients show low testosterone levels and testosterone supplementation could have a positive effect on headache attacks [2]. In the first studies from the early 1990s, testosteron supplementation did not prove effective, but more recent data show a good response in a subgroup of cluster patients [84]. Clomifen is a selective estrogen modulator, primarily used for ovulatory stimulation in women. In men, it leads to an increase in luteinizing and follicle stimulating hormones (LH, FSH) and subsequently to higher testosterone levels. Furthermore, in animal model, it reduces prostaglandine production [85]. In a case-series of 7 patients with chronic cluster headache and 8 patients with episodic cluster headache, Clomifen led to pain freedom after 15 days on average [84].

Evidence of dysregulation of the hypothalamus-hypophysial axis in trigeminal autonomic cephalgias could be derived from a case with high nocturnal prolactin levels in a female patient suffering from short, unilateral, neuralgiform headache with conjunctival injection and tearing (SUNCT) [86].

Other headache types Pituitary diseases are often associated with secondary headaches. Especially in female patients with prolactinoma, migraine-like headaches or worsening of a known migraine are reported. Mainly mechanic aspects such as compression of pain sensitive structures play a role in the development of headache, but probably increased hormonal secretion has an impact as well [87]. Prolactin is involved in regulation of neuronal excitability and neurotransmission efficacy [88]. Headache is commonly localized on the same side of the tumor and gets better after treatment with dopamine agonists [89, 90].

Perimenopause

Perimenopause is a period of decrease in reproductive capability in middle-aged women. During this period the growth and development of ovarian follicles stops and the pattern of estrogen and progesterone production changes. Signs of perimenopause include irregular menses and periodic amenorrhea starting several years before menopause, also called the menopausal transition. The average age of onset is 40 to 55 years and the average duration is 4 years, but in some women perimenopause can last from several months up to 10 years [91].

The Stages of Reproductive Aging Workshop developed a classification for staging reproductive aging dividing a women's life into three stages based on the menstrual cycle: premenopausal (or reproductive), perimenopausal (or menopausal transition) and menopausal (or postmenopause) phase. There are two phases in the menopausal transition: the early phase, characterized by a variable cycle length (≥ 7 days), and a late amenorrhea phase. Postmenopause can also be divided into two stages. An early stage that lasts 5 to 8 years, characterized by amenorrhea length more than 1 year, low estrogen levels and high FSH level. The late stage is characterized by stable low levels of ovarian hormones [92].

Perimenopause is characterized by fluctuations in both estrogen and progesterone levels. Due to these constant rapid changes in concentrations of ovarian hormones 60-70% of perimenopausal women experience symptoms such as headaches, flushing, mood swings, depression, decreased libido and sleep disturbance [91]. The decrease of estrogen in the late luteal phase leads to low blood serum estrogen and progesterone levels and promotes prostaglandins release by the uterus influencing the menstrual cycle. This estrogen withdrawal becomes more frequent and longer and can have a secondary impact on headache patterns [46, 93].

Migraine Studies show that migraine prevalence in menopause is lower compared to the perimenopausal period. Menopausal transition seems to negatively impact migraine frequency [94, 95]. As perimenopause and menopause consist of several phases, each with a unique hormonal pattern, they all have a different effect on migraine. Another important factor is whether the menopause is naturally or artificially induced and whether HRT is used [92].

Fluctuation in the estrogen level is a known migraine trigger. The hormonal alterations during perimenopause can provoke migraine attacks in 50% of women with menstrual migraine and menstrual related migraine. Rather stable levels of estrogen are replaced by a more fluctuating pattern with periods of rapid decline in estrogen concentration, the so called estrogen withdrawal [95–97]. The amount of estrogen withdrawal episodes is correlated to headache attack frequency in women with menstrual migraine in "early" perimenopause. Likewise women can experience an increase in menstruation frequency and in some cases an increase in vaginal bleeding duration and severity [98]. This is related to an increase in uterine prostaglandins, which also influences central pain mechanisms and the trigeminovascular system provoking menstrual migraine attacks [99, 100]. Another potential mechanism that can increase menstrual migraine attack frequency is iron deficiency caused by menstrual bleeding [101]. Depression, chronic pain syndrome and sleep disturbance can be other symptoms related to perimenopause, which in turn can lead to a secondary increase in migraine [102].

Women suffering from the premenstrual syndrome were shown to experience more migraine attacks in late perimenopause. The attack frequency declines in the menopausal period. The premenstrual syndrome seems a predictor of migraine attack frequency increase for women entering menopause. These women are considered to have high sensitivity to hormonal fluctuations and liability to moderately severe climacteric symptoms, which in turn can have an impact on migraine [92].

Migraine and hormonal replacement therapy (HRT) HRT is used to ease climax symptoms during menopausal transition. It seems to have a significant influence on migraine course. Studies confirm the correlation between the use of HRT, both oral and topical, and migraine [103, 104]. Oral high dosed estrogen can provoke new onset migraine with aura or worsening of pre-existent migraine with aura. Nappi et al. concluded that migraine deteriorated in women using oral estradiol plus medroxyprogesterone acetate. The course of the disease did not change with a transdermal patch [105]. A few years later MacGregor et al. showed that transdermal patches with estrogen can be effective in decreasing migraine attack frequency in perimenopausal and postmenopausal women, supposedly more effectively than oral contraceptives [106]. Gels and patches based on estradiol seem preferable over oral variants as constant blood hormones levels are maintained stable. They should be taken continuously without omission to prevent rapid changes in estrogen blood levels, a known trigger for migraine [105, 107]. These fluctuations in estrogen concentration have a more significant impact on migraine than progesterone levels. Nand et al. studied three groups of patients treated with different doses of progesterone combined with estrogen and revealed that changes in progesterone levels have no influence on migraine course [92].

HRT containing low doses of natural estrogens are linked to an insignificant risk of thromboembolism, in contrast to the above mentioned combined oral contraception. Nevertheless HRT should be stopped immediately in case of a new onset migraine with aura, a clear increase in frequency or worsening of migraine with aura, transitory ischemic attack or other vascular pathology [108].

Migraine and surgical menopause Natural menopause seems to reduce migraine frequency, in contrast to surgically induced menopause [5]. Neri et al. studied a group of postmenopausal women [109]. Improvement of migraine was seen in two thirds of cases compared to

the premenopausal period. At the same time no reduction in days with TTH was observed. In women, who underwent ovariectomy the course of migraine worsened in the majority of women (67%). Thirtythree percent reported migraine improvement. In women with natural menopause 67% reported improvement in migraine course, in 24% of patients no change was observed and 9% reported worsening [109]. There is still a debate on possible migraine worsening in women who undergo procedures such as hysterectomy, dilation and curettage or cesarean section. Arumugam and Parthasarathy found a positive correlation between these procedures and the prevalence of migraine in women [110]. Oldenhave et al. compared a group of 986 hysterectomized women and 5636 women without hysterectomy with one or both ovaries preserved. The amount of days without migraine in the group without hysterectomy was less compared to the hysterectomy group. This data confirms the importance of presence or absence of the uterus on migraine frequency in menopausal women [92].

Tension-type headache The most common risk factors for TTH are considered to be stress, fatigue and sleep disturbance. During perimenopause these symptoms can exacerbate and trigger TTH. But TTH also seems to have a correlation with reproductive hormone levels [111]. In some women menstruation can trigger TTH and also pregnancy and menopause can influence the course of TTH [93, 111]. In retrospective evaluations 38% to 46% of women reported an increase of headache rate during menstruation [112, 113]. Arjona et al. even tried to identify "menstrual TTH" and "menstrual related TTH" based on ICHD-2 criteria for pure menstrual migraine and menstrually-related migraine. These terms were not included into the ICHD [114]. Women in the perimenopause reported their headaches to have new characteristics and prevalence of TTH seems rather high [115]. The prevalence of TTH in postmenopausal women is reported to be higher than in premenopausal women [116].

Cluster headache According to the literature the course of CH in women is biphasic. The first peak of onset is seen around the age of 20 and the second at age 50 to 60. The majority of female cluster patients experience their first attack during menopause [116, 117]. The role of estrogen in CH and the reason for CH onset in these women remain unclear. Estrogen receptors are seen in the trigeminal ganglion and in sensory neurons which makes them susceptible to rapid changes in estrogen level [118]. In menopause the reduced level of estrogen is assumed to provoke CH, while the higher estrogen level in the premenopausal phase can have a protective effect [119]. However, based on the available literature, there is no clear evidence on the relationship between CH and hormonal changes in women [120, 121].

In 2006 van Vliet et al. published a large retrospective study in which data from more than 200 women with CH were analyzed using questionnaires. Among women with CH 9% reported more intense CH attacks during menstruation, while frequency didn't change. Eighty-six percent of women were using lifelong oral contraceptives in this trial. Initiation of oral contraceptives was associated with an increase of days with headache in 12% of participants. In 4% of the cases headache frequency was reduced. Out of 111 pregnant women with episodic CH 26 (23%) women reported "expected" CH attacks not to occur. After childbirth 8 of them experienced CH attacks in the first month. Nineteen patients (17%) had attacks during pregnancy and 11 of them did not report any changes in attack frequency or intensity [120].

Elderly

In the elderly, headache is less frequent compared to younger patients. Headache disorders are mostly primary, but the relative frequency of secondary headache is higher in the elderly [122]. In a random population sample, the prevalence of headache in women and men aged 55 to 74 years is approximately 66% and 53%, respectively, compared to 92% and 74%, respectively, in their younger counterparts between the ages of 21 to 34 years. The prevalence further declines in patients aged over 75 to 55% for women and 22% in men [123]. In a population survey, the prevalence of frequent headache in elderly women was 20% and 10% in elderly men [124]. Another survey showed a 3-month prevalence of headache among patients aged more than 66 years of 40.6% in men and 49.7% in women [125]. In summary, all studies show that headache is more prevalent in women compared to men at all ages, even among the elderly. Hormonal factors take account for the sex-specific difference in headache prevalence. However, literature data about the relationship between headache and hormonal activity in elderly women are scarce. Only the relationship between migraine and estrogen has been extensively studied in older women, possibly because of the high prevalence of migraine and its sensitivity to hormonal fluctuations.

Up to 51.9% of elderly patients referred for specialist consultation report onset of headache after 65 years of age [126]. Some primary headache disorders, and mostly hypnic headache, have the tendency to start after the age of 50, in contrast to most primary headache disorders, which usually start at a younger age. However, migraine still accounts for 0.5% of all new-onset headache disorders after the age of 65 [127, 128]. The low estrogen level in elderly women may explain why onset of migraine in this age group is uncommon. Migraine with

onset at older age affects women and men equally, while in younger age groups women outnumber men [129].

Migraine As mentioned above, the "estrogen withdrawal hypothesis" attributes migraine episodes to the fluctuation of estrogen levels throughout women's reproductive events. After menopause, women's serum levels of estradiol drop. A lower frequency and severity of migraine episodes is expected because of the stable low serum levels of estrogen. Migraine prevalence declines after menopause compared to the fertile period. However, the prevalence of migraine after the menopause is still 10 to 29% across studies [5].

Interestingly, the decreased burden of migraine after the menopause is more evident in population-based studies when compared with those performed in headache clinics or menopause clinics [94, 109, 115, 130–134]. This can be explained by a possible selection bias towards more severe forms of migraine in clinic-based studies as compared to population-based studies [5]. Menopause has a different and variable effect on migraine with or without aura [8]. In a population-based study, the burden of migraine without aura decreased after menopause while that of the variant with aura remained stable [130]. In a headache clinic-based study migraine without aura remained unchanged or even worsened in the majority of patients possibly because of the above mentioned selection bias of clinic-based studies [135]. Collectively, these data suggest that migraine without aura improves more frequently after menopause compared to migraine with aura. This can be a possible consequence of migraine without aura being more sensitive to female sex hormones [5]. However, the available studies might have failed to show any change in the frequency of migraine with aura after the menopause because of low statistical power [136]. When migraine with aura does not subside with age, characteristics may change, with increasing occurrence of aura without headache. These auras constitute a difficult differential diagnosis with transient ischemic attacks [137, 138]. An aura is generated by cortical spreading depression while migraine pain has been linked to the neurovascular system. Elderly subjects may exhibit an intact cortical spreading depression phenomenon, while the propensity to neurovascular inflammation declines [139]. It is likely that those changes can be a consequence of the postmenopausal estrogen drop. However, to the best of our knowledge, this has not been proven yet.

Together with female sex hormones, male sex hormones might have an influence on the course of headache disorders among elderly women. Only one case-control study assessed the levels of androstenedione and testosterone in the serum of postmenopausal women with and without migraine and found no differences in the levels of these hormones when comparing women with and without migraine [140].

In conclusion, the postmenopausal drop of estrogen might be beneficial for elderly women with migraine. However, the proportion of women experiencing migraine in menopause is still relevant.

Tension-type headache The effect of menopause on TTH is less clear than the corresponding effect on migraine. One population-based study addressing the topic found that the frequency of TTH decreased less than that of migraine after menopause. However, that same study pointed out that fluctuations of sex hormone levels during the life cycle might influence TTH as well as migraine [131].

Hormonal therapy Hormonal manipulation in elderly women cannot be considered for migraine prevention at this time. HRT is contraindicated from 10 years after menopause or in women aged 60 years or older due to its potential cardiovascular side effects [141]. No other hormonal therapy has been attempted in the prevention of migraine in elderly women. Clomiphene citrate has been used to treat chronic cluster headache and refractory primary SUNCT in single cases of elderly males [142, 143]. Clomiphene has a direct effect on hypothalamic estrogen receptors and estrogen modulates hypothalamic orexin expression. Hypothalamic estrogen receptors co-localize to orexin neurons. Therefore, clomiphene might upregulate orexin A levels, which in turn inhibits the trigeminal nucleus caudalis activity and secondarily suppresses the trigemino-autonomic reflex, preventing hypothalamic-driven headache [142]. These results are promising in considering hormonal therapies as prevention for headache disorders in elderly women. However, there are no studies to date.

Males

Migraine

Migraine is notoriously known to be two to three times more prevalent in women than in men. Migraine is characterized by its fluctuating nature, where periods of remission are interspersed by relapse, with men more likely to have longer periods of remission compared to women. This female dominance of migraine suggests that factors increasing female vulnerability and/or protecting males deserve greater focus in migraine pathophysiology [144]. Interestingly, a study has shown that male-to-female transsexuals who use antiandrogens to suppress male sex characteristics and estrogens to induce female sex characteristics have migraine rates similar to genetic females, further adding to the notion that gender-specific hormones play a role in migraine prevalence. The authors suggest that this similarity in

migraine prevalence could include structural differences in the transsexual brain or that migraine headache is part of the female gender role [145].

Animal models of migraine have attempted to investigate the gender specific difference in migraine prevalence. In an animal model of familial hemiplegic migraine type 1 (FHM1), it has been shown that orchiectomy increases susceptibility to cortical spreading depression, a response partially reversed with testosterone replacement [146]. Also, female FHM1 mutant mice were more susceptible to cortical spreading depression than males [146–148].

Another explanation for increased prevalence of migraine in women could be attributed to inherent differences in pain perception and processing. The fundamental subjectivity of pain perception complicates quantification of pain, yet it is generally accepted that women and men experience pain differently due to both biological and psychosocial traits [144]. Clinical studies are often not designed to decipher gender-specific difference [149].

Cluster headache

In contrast to migraine, cluster headache has traditionally been considered a male disease [150]. While the characteristic physical attributes of cluster headache patients could point to high testosterone levels, the exact opposite has been shown to be true [151]. Low testosterone levels in patients with episodic and chronic cluster headaches were first noted in the 1970ies and later reproduced [152–154]. Another study found low testosterone levels in the episodic but not chronic cluster headache, a difference attributed by the authors to the disruption of REM sleep [154].

The role of testosterone in cluster headache was further studied by Stillman et al. in their investigation of laboratory findings of 7 male and 2 female patients with treatment refractory cluster headache. Results of all 9 patients demonstrated low serum testosterone levels. After supplementation with either pure testosterone in the male patients or combination testosterone/estrogen therapy in the female patients, pain freedom was achieved for the first 24 h. Four male chronic cluster patients achieved headache remission. The authors concluded that abnormal testosterone levels in patients with episodic or chronic cluster headaches refractory to maximal medical management may be predictive of therapeutic response to testosterone replacement therapy [2].

Discussion

Reviewing recent literature, it becomes evident that most experimental data on the causal relationship between sex hormones and primary headaches covers women suffering from migraine in the reproductive or perimenopausal phase of their life. Particularly the effect of estrogen has been studied and has been found to be of considerable value in the pathogenesis of migraine. The estrogen withdrawal hypothesis plays a central role here, but it is assumed that this is only part of the mechanism. Some therapeutic strategies have been developed based on this knowledge. Continuous combined contraceptive therapy regimes can be considered as a treatment for menstrual migraine. However, there is currently no evidence to support the superiority of hormonal therapy over non-hormonal pharmacological treatment strategies. When using hormonal therapies in migraine patients, whether it is as a contraceptive or as a treatment, potential cardiovascular risks should be considered when deciding which type of hormones to use.

For the other primary headaches and more so ever for headaches in male patients, the role of sex hormones is vague. Is there more to know? It seems plausible that trying to uncover the effects of sex hormones on the other primary headaches may offer new insights in pathophysiological mechanisms. The more we know on this matter, the more targeted possible new therapies can be.

Conclusion

All three primary headaches, migraine, TTH, and CH, occur in both genders, but with a sex-specific prevalence. Also, headache patterns display a temporal evolution that correlates to the hormonal shifts of a life cycle. Collectively, these findings suggest that both male and female sex hormones could play an important role in the pathophysiology of primary headaches. Reviewing the available literature on this matter, we can conclude that especially the role of estrogen in female migraine patients has been well-studied. Detailed studies especially in the elderly of both sexes, in CH, and TTH are warranted in order to clearly elucidate the role of sex hormones in not just migraine, but all primary headaches.

Abbreviations

CGRP: Calcitonin gene-related peptide; CH: Cluster headache; FHM1: Familial hemiplegic migraine type 1; FSH: Follicle stimulating hormone; GABA: Gamma-aminobutyric acid; HRT: Hormonal replacement therapy; ICHD: International Classification of Headache Disorders; IHS: International Headache Society; LH: Luteinizing hormone; NMDA: N-methyl-D-aspartate; SUNCT: Short, unilateral, neuralgiform headache with conjunctival injection and tearing; TTH: Tension-type headache

Competing interests

All the authors declared no competing interests related to the contents of this review. Furthermore, all authors declare that they have received no direct or indirect payment in preparation of this manuscript.

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Novel hypotheses emerging from GWAS in migraine?

Abstract

Recent technical advances in genetics made large-scale genome-wide association studies (GWAS) in migraine feasible and have identified over 40 common DNA sequence variants that affect risk for migraine types. Most of the variants, which are all single nucleotide polymorphisms (SNPs), show robust association with migraine as evidenced by the fact that the vast majority replicate in subsequent independent studies. However, despite thorough bioinformatic efforts aimed at linking the migraine risk SNPs with genes and their molecular pathways, there remains quite some discussion as to how successful this endeavour has been, and their current practical use for the diagnosis and treatment of migraine patients. Although existing genetic information seems to favour involvement of vascular mechanisms, but also neuronal and other mechanisms such as metal ion homeostasis and neuronal migration, the complexity of the underlying genetic pathophysiology presents challenges to advancing genetic knowledge to clinical use. A major issue is to what extent one can rely on bioinformatics to pinpoint the actual disease genes, and from this the linked pathways. In this Commentary, we will provide an overview of findings from GWAS in migraine, current hypotheses of the disease pathways that emerged from these findings, and some of the major drawbacks of the approaches used to identify the genes and pathways. We argue that more functional research is urgently needed to turn the hypotheses that emerge from GWAS in migraine to clinically useful information.

Keywords: Genetics, Genome-wide association study, Disease pathway, Single-nucleotide polymorphism

Background

It has long been recognised that migraine is a disease with a strong genetic component [1–3]. Migraine runs in families, and epidemiological studies in twins and families have indicated that risk for migraine is conferred by a combination of genetic and environmental factors, both contributing equally [2, 3]. These studies also indicated that the genetic contribution seems stronger in migraine with aura than the more common migraine without aura subtype. Considerable progress has been made with elucidating the pathophysiological mechanisms in migraine. Evidence is accumulating that cortical spreading depolarisation (CSD) is the electrophysiological substrate of the migraine aura [4, 5]. Activation of the trigeminovascular system that consists of meningeal perivascular nerves, the trigeminal ganglion and brainstem centres reaches thalamus and ultimately the cortex to give the sensation of head pain in migraineurs during attacks [6]. Several animal studies showed that CSD can activate the headache mechanisms [7, 8], but proof that this also occurs in humans is essentially lacking. Knowledge on the underlying molecular mechanisms, to large extent, comes from genetic studies of very rare monogenic forms of migraine — i.e., hemiplegic migraine and syndromes in which migraine is prominent (for review see [9]). In brief, genes in familial hemiplegic migraine (FHM) (*CACNA1A*, *ATP1A2* and *SCN1A)* encode subunits of ion transporters (neuronal voltage-gated $Ca_V2.1$ Ca^{2+}, $Na_V1.1$ Na^+ channels, and glial Na^+K^+ ATPases, respectively) and functional studies in cellular and animal models suggest neuronal hyperexcitability as a common theme. Genes with vascular and/or glial cell function emerged from investigating syndromes in which migraine is prevalent,

such as *NOTCH3* in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and *CSNK1D* in familial advanced sleep phase syndrome (FASPS). Since one such high impact mutation is causative for disease, the identification of these genetic factors directly benefits the clinical diagnosis of patients with these rare disorders and may lead to the development of better treatment.

Parallel research aimed to identify genetic factors for the common forms of migraine, foremost migraine with aura and migraine without aura, suggests that these migraine types are brought about by a combination of multiple genetic variants, each with low impact, and environmental factors [10]. The most effective approach thus far to identify genetic factors for these forms of migraine are genome-wide association studies (GWAS), which test for differences in allele frequencies of several million single nucleotide polymorphisms (SNPs) spread over the genome in large groups of cases and controls [11]. Allelic differences at SNPs with a p-value $< 5 \times 10^{-8}$ are taken as proof that a migraine risk factor is located at that position. Due to their small effect size (allelic odds ratio of 1.03–1.28), no single SNP can have clinical use in migraine risk prediction; however, one can envisage that combined knowledge from many variants will highlight which genes and pathways are involved in migraine pathophysiology, as well as more direct applications through approaches like polygenic risk scoring, where additive effects of multiple migraine risk SNPs can be used to score patients and then analyse those scores against clinical variables. Spearheaded by the International Headache Genetics Consortium (IHGC; www.headachegenetics.org/), which brought together headache geneticists and clinicians from around the globe, various large-scale association studies were conducted. Here we will review the main findings of their studies: the DNA variants that were identified, what efforts were made to link these to genes and molecular pathways, and whether any hypotheses emerged that may guide the development of migraine treatments.

Main text

Genome-wide association studies in migraine

In the past decade IHGC researchers have conducted several GWAS for migraine (for review see [10, 12]). With increasing samples sizes available for investigation, the number of associated gene variants also increased. Initial sample sizes consisted of a few thousand patients with migraine with aura (in the 2010 GWAS [13]) or migraine without aura (in the 2012 GWAS [14]) that had been recruited in specialised headache clinics and yielded one and six associated SNPs, respectively. A SNP refers to a specific location on the genome with two alleles, indicated by an rs-number (e.g., rs9349379, the Reference SNP cluster ID number for that SNP that is searchable in the SNP database [dbSNP: www.ncbi.nlm.nih.gov/SNP/]). In the 2011 GWAS [15], 5122 women with migraine from the Women's Genome Health Study were investigated and three associated SNPs were identified, two of which surfaced also in the 2012 GWAS. In more recent efforts, meta-analyses were performed on genotypic data of the previous cohorts that was combined with data of other cohorts to yield much larger groups of migraine patients; i.e., 23,285 cases in the 2013 GWAS [16] and 59,674 cases in the 2016 GWAS) [17], which led to 13 and 44 associated SNPs, respectively. In GWAS, genotypic information of migraine cases is compared with data of ever-increasing numbers (in the latest study 316,078) of control subjects. Notably, it is customary to not screen for (and remove) cases (~ 15% in the case of migraine) from the control sets that typically are from large population-based cohorts. An important message from these GWAS is that migraine-associated SNPs are generally very robust findings due to their stringent statistical methodology and consequently most of them have been replicated in subsequent studies. Secondly, all associated SNPs have a small genetic effect with allelic odds ratio of 1.03–1.28 (for the disease-increasing risk allele) [13–17], which resonates earlier claims that no single genetic factor is sufficient to cause migraine, which is no different for any other disorder studied with GWAS [18].

The difficult road from associated SNPs to genes and mechanisms

Whereas there is little doubt that the identified variants (indicated by their rs ID number) are genuine findings, robustly linking those variants to genes and pathways is difficult due to the complexity of local genomic effects. Firstly, most attention in literature goes to reporting the index SNP (i.e., the SNP with the lowest p-value in a genomic region), but there can be multiple independent association signals at the same locus, called secondary SNPs, which may affect, for example, other regulatory features of the same (or neighbouring) gene. The 2016 GWAS, with its 44 migraine-associated SNPs (associations were with the subtype migraine without aura), implicated 38 distinct genomic loci, of which six contained an independent secondary signal (Table 1).

Secondly, traditionally the most straightforward approach in interpreting a GWAS signal was to link the index SNP to the *nearest* gene, under evidence that regulatory effects tend to largely act on short distances [19, 20]. The strength of this inference depends on a number of factors, such as the size and gene density of the identified locus; while long-range *trans*-eQTL ('expression quantitative trait loci' which explain small fractions of the genetic variance of a gene expression phenotype) effects exist in the genome, the preponderance towards

Table 1 Migraine-associated single nucleotide polymorphisms and the molecular pathways they are linked to

Genomic region[a]	Index SNP[b]	Secondary SNP[c]	Gene nearest index SNP	Genes overlapping credible SNPs	Genes prioritised with DEPICT	Pathways identified with g:GOSt tool
1	rs10218452		*PRDM16*	*PRDM16*	*PRDM16*	Vascular function; Metal ion homeostasis
		rs12135062				
2	rs1572668		*LRRIQ3*			
3	rs2078371		*TSPAN2*		*NGF*	
		rs7544256				
4	rs6693567		*ADAMTSL4*	*RPRD2*	*ECM1*	Vascular function
5	rs1925950		*MEF2D*	*MEF2D*		Vascular function
6	rs138556413		*CARF*	*CARF*	*NBEAL1*	
7	rs10166942		*TRPM8*	*TRPM8*		Ion channel activity
		rs566529				
8	rs6791480		*TGFBR2*		*TGFBR2*	Vascular function; Metal ion homeostasis
9	rs13078967		*GPR149*		*ARHGEF26*	Vascular function
10	rs7684253		*SPINK2*		*REST*	Vascular function; Metal ion homeostasis; Ion channel activity
11	rs9349379		*PHACTR1*	*PHACTR1*		Vascular function
12	rs140002913		*NOTCH4*			
13	rs10456100		*KCNK5*	*KCNK5*		Ion channel activity
14	rs67338227		*FHL5*	*FHL5*		Vascular function
		rs4839827				
15	rs28455731		*GJA1*		*GJA1*	Vascular function
16	rs1268083		*HEY2*	*HEY2, NCOA7*	*HEY2*	Vascular function
17	rs186166891		*SUGCT*	*SUGCT*		
18	rs10155855		*DOCK4*			
19	rs6478241		*ASTN2*	*ASTN2*		
20	rs2506142		*NRP1*	*NRP1*	*NRP1*	Vascular function; Metal ion homeostasis
21	rs10786156		*PLCE1*	*PLCE1*	*PLCE1*	Vascular function
		rs75473620				
22	rs12260159		*HPSE2*	*HPSE2*	*HPSE2*	
23	rs2223089		*ARMS2*	*PLEKHA1, HTRA1*	*HTRA1*	Vascular function
24	rs4910165		*MRVI1*	*MRVI1*	*MRVI1*	
25	rs11031122		*MPPED2*	*MPPED2*		
26	rs10895275		*YAP1*	*YAP1*	*YAP1*	Vascular function
27	rs561561		*IGSF9B*	*IGSF9B*		
28	rs1024905		*FGF6*		*FGF6*	Vascular function
29	rs11172113		*LRP1*	*LRP1*	*LRP1*	Vascular function; Metal ion homeostasis
		rs11172055				
30	rs11624776		*ITPK1*			
31	rs77505915		*CFDP1*	*CFDP1, TMEM170A*		
32	rs4081947		*ZCCHC14*		*ZCCHC14*	Vascular function; Metal ion homeostasis
33	rs75213074		*WSCD1*			
34	rs17857135		*RNF213*	*RNF213*		Metal ion homeostasis
35	rs111404218		*JAG1*		*JAG1*	Vascular function; Metal ion homeostasis

Table 1 Migraine-associated single nucleotide polymorphisms and the molecular pathways they are linked to *(Continued)*

Genomic region[a]	Index SNP[b]	Secondary SNP[c]	Gene nearest index SNP	Genes overlapping credible SNPs	Genes prioritised with DEPICT	Pathways identified with g:GOSt tool
36	rs4814864		*SLC24A3*	*SLC24A3*		Ion channel activity
37	rs144017103		*CCM2L*	*CCM2L*	*CCM2L*	Vascular function
38	rs12845494		*MED14*			

[a]Genomic region is an independent genomic region (> 250 kb apart) that harbours at least one migraine risk SNP; [b]Index SNP is the SNP with the lowest *p*-value at a genomic region. [c]Secondary SNP is a genome-wide significant SNP that is not in linkage disequilibrium with the index SNP. Associations were identified for the migraine without aura subtype. DEPICT, data-driven expression-prioritized integration for complex traits; g:GOSt tool refers to web-based gene functional profiling software g:Profiler128 (http://biit.cs.ut.ee/gprofiler/) (depicted are only the more prominent pathways vascular function, metal ion homeostasis, ion channel activity pathways) (Compiled and adapted from [17, 33])

short distance *cis*-eQTLs suggest we can be fairly confident in linking a locus where only a single gene resides within the associated SNPs to hypothesise about function. One way to combat this is to combine the evidence from association-test statistics with linkage disequilibrium information (i.e., prior information on how haplotype patterns [alleles of close by SNPs] behave at each locus) in a Bayesian approach [21], to define what is called a *credible set of SNPs* (i.e., the set of SNPs that with a 99% chance contain the causal SNP at a locus). Using the genomic location of the credible SNPs the most likely gene(s) associated with migraine were identified (Table 1). Since causal variants are often located in intronic or intergenic regions in gene-dense areas, inferring which of the many genes within the credible set is involved based on SNP data alone can be tricky. In practice, this means that all the genes at such loci need to be taken forward to post-hoc analyses, which imposes power challenges to such analyses. Information on the gene's function and participation in known biological pathways can be used to prioritise causative genes using methods such as DEPICT [22], which prioritises genes if their predicted function is shared with that of genes at other associated loci more often than expected. Together, analysis of credible SNPs and DEPICT analysis identified 37 genes that are likely to be causal (Table 1).

Thirdly, even in cases where only a single gene is implicated, the complexity of gene regulation can provide additional challenges; this was clearly demonstrated by the fact that intronic SNP rs9349379 that had been linked in this way to *PHACTR1* in multiple migraine GWAS (as well as coronary artery disease, cervical artery dissection, fibro-muscular dysplasia, and hypertension) and where the credible set comprises only rs9349379, detailed functional follow-up analyses revealed that this SNP influences the expression of *EDN1* (coding for endothelin-1 (ET-1), 600,000 base pairs [bp] upstream) [23]. ET-1 is a potent vasoconstrictor that acts on smooth muscle cells and has previously been implicated in migraine [24]. Although hypothesis-free methods such as GWAS and sequencing studies are meant to provide the roadmap towards core pathophysiology of a disease, they rely on direct and well-designed 'wet-lab' functional follow-ups to nail down the key molecular mechanisms. As the type of assay, whether animal- or cell-based, needed for a functional follow-up very much depends on the actual variant and the gene it affects, it is not possible to give specific directions on how to go about for a particular variant (for recent reviews on technical possibilities see [25, 26]).

Emerging molecular pathways from GWAS hits?

The most profound hypothesis that emerged from the 2016 IHGC GWAS publication [17] (with only a minute fraction of the genes/loci identified thus far) was the enrichment of genes involved in the vascular system among the identified genetic risk factors for migraine. Briefly, tissue expression enrichment analysis was performed, where the expression of genes (from GTEx data) within 50,000 bp of credible-set SNPs was assessed in 42 different human tissue types. These analyses identified that arterial and gastrointestinal tissues were significantly enriched for expression of migraine-associated genes. Indeed, no less than 15 of the implicated genes are related to vascular function of which four (*MEF2D, YAP1, LRP1, JAG1*) were significantly enriched in vascular tissues, as shown by in silico tissue expression enrichment analysis [17].

The 2016 gene expression enrichment results suggested that vascular dysfunction is important in migraine susceptibility and fuelled the long-running debate whether migraine is a disease of vascular dysfunction, or of neuronal dysfunction with vascular changes playing a secondary role. However, the 2016 finding by no means suggests that a neuronal origin of migraine is now excluded, already because at least five genes (*PRDM16, MEF2D, FHL5, ASTN2, LRP1*) (also) have a neuronal function. Another, rather unexpected, hypothesis that emerges is that metal ion homeostasis might contribute to migraine susceptibility, as 11 genes (*PRDM16, TGFBR2, REST, FHL5, NRP1, MMPED2, LRP1, ZCCHC14, RNF213, JAG1, SLC24A3*) with such function are among the 37 genes. Of note, ion channel activity (*TRPM8, REST, KCNK5, SLC24A3*), which emerged from genetic studies in monogenic FHM, and pain signalling (*TRPM8*) were much less prominent signals [27].

A more recent [28] tissue enrichment analysis of the 2016 IHGC GWAS summary statistics — utilising two gene expression datasets (GTEx and 'Franke lab') and chromatin data (highlighting active regulatory regions) from the Roadmap Epigenomics and ENCODE (EN-TEx) projects — reported enrichment of both vascular and neurological enrichment. More specifically, cardiovascular enrichments were found for migraine without aura with gene expression data, and for migraine without aura and 'all' migraine with EN-TEx data. Whereas, analysis using Roadmap data found the strongest enrichment for migraine (all subtypes) was neurological (neurospheres and fetal brain, neither of which were present in GTEx and EN-TEx). These results highlight the importance utilising multiple tissues, cell types and regulatory measures in such enrichment analyses aimed at interpreting GWAS risk loci.

It is useful to keep in mind that a GWAS SNP only 'tags' the disease locus, implying that the identified SNP is only correlated — because of linkage disequilibrium — with the disease-causing variant, which is not 'the end of the road' as far as understanding the functional consequences. Efforts at combining information across phenotypes either directly at the summary statistic phase [29] or by comparative analysis of correlated phenotypes [30] as well as increasing the size of the migraine GWAS itself (leading to more implicated loci) will yield improvements on the locus side of the analysis; concurrently, considerable efforts are being focused on improving the quality of the next layer of information, which links SNPs to function, through various -omics studies assaying the genome in general [26, 31], and the improvement of these resources and better methodology will increase the statistical power on the post-hoc side of the analysis. However, it is crucial to realise that rapid progress can also be made in migraine specifically by targeted follow-ups (such as for the rs9349379/EDN1/ET-1 study) [23], given that we now have a set of well-characterised loci waiting for such detailed characterisation. For example, several of the mechanisms implied by the two latest GWAS (such as regulation of vascular tone, ion homeostasis) may present directly testable hypotheses.

What lies ahead?

GWAS in migraine have been fruitful in the sense that they yielded several dozens of robustly identified loci in the genome that harbour genetic risk factors. Despite clear challenges how to link associated SNPs to actual genes and pathways, the likelihood that the correct genes are identified is increased by bioinformatics tools. Emerging hypotheses suggest that vascular function and metal ion homeostasis are among the pathways involved in migraine pathophysiology. Other pathways such as neuronal function and ion channel activity are less prominent among the genes identified thus far. Current initiatives of IHGC to conduct even larger GWAS (close to 100 K cases) appear to identify many more risk loci (> 100) [32] that may support current hypotheses and likely generate new ones. Over time, the genetic landscape of migraine will be more complete so one may predict migraine risk using approaches like polygenic risk scores, which is not yet sufficiently accurate [33–35]. One major challenge will be to elucidate the functional consequences of the associated SNPs and identify how they may affect migraine risk at the individual level. Efforts to functionally characterise GWAS signals, for other diseases than migraine, have been considering high-throughput cell-based (e.g., induced pluripotent stem cells [iPSCs]) and animal models (e.g., Drosophila) [26]. Considerable amount of research is needed before migraine GWAS findings will show diagnostic or prognostic value and lead to the development of (personalised) treatment options.

Abbreviations

CADASIL: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; CSD: Cortical spreading depolarisation; DEPICT: Data-driven expression-prioritized integration for complex traits; FASPS: Familial advanced sleep phase syndrome; FHM: Familial hemiplegic migraine; GWAS: Genome-wide association study; IHGC: International Headache Genetics Consortium; SNP: Single nucleotide polymorphism

Competing interests

The authors declare that they have no competing interests.

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Migraine-provoking substances evoke periorbital allodynia in mice

Abstract

Background: Administration of endogenous mediators or exogenous chemicals in migraine patients provoke early headaches and delayed migraine-like attacks. Although migraine provoking substances are normally vasodilators, dilation of arterial vessels does not seem to be the sole contributing factor, and the underlying mechanisms of the delayed migraine pain are mostly unknown. Sustained mechanical allodynia is a common response associated with the local administration of various proalgesic substances in experimental animals and humans. Here, we investigated the ability of a series of endogenous mediators which provoke or do not provoke migraine in patients, to cause or not cause mechanical allodynia upon their injection in the mouse periorbital area.

Methods: Mechanical allodynia was assessed with the von Frey filament assay. Stimuli were given by subcutaneous injection in the periorbital area of C57BL/6J mice; antagonists were administered by local and systemic injections.

Results: Calcitonin gene related peptide (CGRP), but not adrenomedullin and amylin, pituitary adenylyl cyclase activating peptide (PACAP), but not vasoactive intestinal polypeptide (VIP), histamine, prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2), but not $PGF_{2\alpha}$, evoked a dose-dependent periorbital mechanical allodynia. The painful responses were attenuated by systemic or local (periorbital) administration of antagonists for CGRP (CLR/RAMP1), PACAP (PAC-1), histamine H_1, PGE_2 (EP_4), and PGI_2 (IP) receptors, respectively.

Conclusions: The correspondence between substances that provoke (CGRP; PACAP, histamine, PGE_2, PGI_2), or do not provoke (VIP and $PGF_{2\alpha}$), migraine-like attacks in patients and periorbital allodynia in mice suggests that the study of allodynia in mice may provide information on the proalgesic mechanisms of migraine-provoking agents in humans. Results underline the ability of migraine-provoking substances to initiate mechanical allodynia by acting on peripheral terminals of trigeminal afferents.

Keywords: Migraine, calcitonin gene related peptide, pituitary adenylyl cyclase activating peptide, prostaglandin, histamine, vasoactive intestinal polypeptide, allodynia

Background

Migraine is a pain disorder that affects about 15% of the adult population worldwide. Thus, the burden of migraine is enormous in terms of suffering, disability, healthcare, and social and economic costs [1]. For these reasons, migraine is ranked among the most disabling medical conditions [2]. Although considerable progress has been made in the development of new treatment options [3, 4], our current understanding of the mechanisms underlying migraine pain is incomplete. Migraine attacks are elicited by a variety of provoking agents [5], and this peculiar feature provides an opportunity to explore disease mechanisms by endogenous mediators or exogenous chemicals that provoke migraine-like attacks in patients [6].

A prototypical example of a migraine-provoking agent is glyceryl trinitrate (GTN). Occupational exposure to, or treatment with, organic nitrates has long been known to provoke headaches [7–10]. Typically, GTN causes an early, mild and short-lived headache minutes after administration, followed by a remarkably delayed migraine-like

attack hours later [9, 10]. The ability of GTN to provoke the mild/early headache is temporally associated with the short-lived (<10 min) release of nitric oxide (NO) [11] and ensuing vasodilation [12]. However, the prolonged migraine-like attacks typically begin with a remarkable delay of hours, underlying the temporal dissociation between the early vasomotor response and the delayed proalgesic effect [6, 13, 14]. Thus, the vascular response can hardly explain the delayed migraine-like attack, which, therefore, implicates additional mechanisms. Recently, we reported that GTN administration in mice evokes an early and short-lived (10 minutes) vasodilatation due to a direct vascular action of NO, and a delayed and prolonged (8 hours) periorbital mechanical allodynia (PMA) that is independent from vascular changes and is due to the activation of an oxidative stress-mediated pathway in the soma of trigeminal primary sensory neurons [15]. We also observed that GTN-evoked PMA in mice exhibits a temporal pattern [15] similar to the migraine-like attacks in patients, which are characterized by delayed onset and prolonged duration [6].

In the last three decades, rigorous studies with randomized, double blind and crossover designs have been undertaken, resulting in a systematic investigation of the ability of a series of endogenous mediators or exogenous chemicals to provoke early headaches and delayed migraine-like attacks [6]. Vasodilatation has been proposed as the underlying mechanism of migraine headaches [16]. Notably, both intra and extracranial artery vasodilatation or only intracranial artery vasodilatation have been reported in association with spontaneous migraine attacks [17–19]. Although vasodilatation is elicited by a majority of the migraine provoking agents [6, 14, 20], the vascular response does not seem essential for generating delayed migraine attacks, as robust vasodilators, such as the vasoactive intestinal polypeptide (VIP) or adrenomedullin, do not induce migraine [21, 22]. Thus, an experimental animal model that explores the correspondence between the pain-producing ability of mediators that provoke migraine might be useful for a better understanding of the pro-migraine action of such mediators.

Here, we have investigated whether a series of endogenous mediators, which have been found to provoke or not provoke migraine-like attacks in patients, elicit or do not elicit delayed and prolonged PMA after their injection in the periorbital skin of mice. Provocation tests in humans are usually performed by systemic administration of the stimulus [6]. However, in the present study in mice the local administration was purposively chosen to investigate the interaction between the various mediators and the peripheral terminals of trigeminal nociceptors. These mediators include calcitonin gene-related peptide (CGRP), adrenomedullin, amylin, pituitary adenylyl cyclase activating peptide (PACAP), VIP, histamine, prostaglandin E_2 (PGE_2), prostacyclin (PGI_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The receptor type implicated in the PMA evoked by each mediator was also studied. A close correspondence was found between agents that provoke/not provoke delayed migraine in patients and PMA in mice. Thus, the study of PMA in mice may provide information on the proalgesic mechanisms that, in humans, result in the development of migraine-like attacks provoked by endogenous mediators and exogenous chemicals.

Methods

Animals

In vivo experiments were carried out according to the European Union (EU) guidelines and Italian legislation (DLgs 26/2014, EU Directive application 2010/63/EU) for animal care (research permit #194/2015-PR). C57BL/6J mice (male, 20-22 g, 6-7 weeks old; Envigo, Milan, Italy) were used. Animals were housed in a temperature (20-24°C)- and relative humidity (45-65%) -controlled *vivarium*, maintained on a 12-hour dark/light cycle (light off from 7.00 PM to 7.00 AM), and with free access to food and water. Animal studies were reported in compliance with the ARRIVE guidelines [23]. The total number of C57BL/6J mice used was 486. Group size of n=6 animals for behavioural experiments were determined using G*Power (v3.1) [24] to detect a minimum difference between paired means of 1.4 standard deviations (or 1.8 standard deviations between groups) in post-hoc tests with type 1 and 2 error rates of 5 and 20%, respectively [15]. Allocation concealment was performed using a randomization procedure (http://www.randomizer.org/). Experiments were done in a quiet, temperature-controlled (20-24°C) room between 9.00 AM and 5.00 PM and were performed by an operator blinded to drug treatment. At the end of each experiment, mice were euthanized with inhaled CO_2 plus 10-50% O_2.

Reagents

CGRP, amylin, PACAP, VIP, $PGF_{2\alpha}$, olcegepant, astemizole, ER819762 and Ro1138452 were from Tocris Bioscience (Bristol, UK); adrenomedullin, PACAP6-38, PGE_2, PGI_2 and histamine were from Sigma Aldrich (Milan, Italy); the mouse monoclonal anti-CGRP antibody (clone [4901]) and the inactive immunoglobulin (mouse monoclonal IgG2a) were from Abcam (Cambridge, UK)

Behavioural experiments

Treatment protocols

C57BL/6J mice were injected subcutaneously in the periorbital area (p.orb., 10 µl/site) with CGRP, adrenomedullin, amylin, PACAP, VIP, histamine, PGE_2, PGI_2 and $PGF_{2\alpha}$ (0.15, 1.5 and 15 nmol) or their vehicles (0.9%

NaCl). The subcutaneous injection was performed unilaterally on the right side of the periorbital area. The mouse was restrained by the double handed manual restraint method [25]. Briefly, the mouse was lifted by the base of the tail and placed on a solid surface with one hand and the tail was pulled back. Then, it was quickly and firmly picked up by the scruff of the neck behind the ears with the thumb and index finger of the other hand. In this way, the mouse face was constrained, and the operator was able to inject the tested compound. Injection was performed as quickly as possible by a single operator, with only minimal animal restraint.

Some mice were pre-treated (30 minutes before the stimuli) with intraperitoneal (i.p., 10 ml/kg) olcegepant (1 μmol/kg corresponding to 0.869 mg/kg), astemizole (4 μmol/kg corresponding to 1.8 mg/kg), ER819762 (60 μmol/kg corresponding to 29.4 mg/kg) or their vehicle (4% dimethyl sulfoxide, DMSO, and 4% tween 80 in 0.9% NaCl) and intravenous (i.v., 1 ml/kg) PACAP6-38 (12 nmol/kg corresponding to 48 μg/kg) and Ro1138452 (30 μmol/kg corresponding to 10.4 mg/kg), or their vehicle (0.9% NaCl). Other mice received locally (p.orb., 10 μl, 30 minutes before the stimuli) olcegepant (4 nmol/site), astemizole (10 nmol/site), ER819762 (10 nmol/site), or their vehicle (4% DMSO and 4% tween 80 in 0.9% NaCl) and PACAP6-38 (240 pmol/site) and Ro1138452 (10 nmol/site), or their vehicle (0.9% NaCl), or a mouse monoclonal anti-CGRP antibody or, as a control, a mouse monoclonal IgG2a (both, 60 pmol/site). In another set of experiments, C57BL/6J mice received intraplantar (i.pl., 20 μl, 30 minutes before the stimuli) injections of olcegepant (4 nmol/site), astemizole (10 nmol/site), ER819762 (10 nmol/site), or their vehicle (4% DMSO and 4% tween 80 in 0.9% NaCl), or PACAP6-38 (240 pmol/site) and Ro1138452 (10 nmol/site), or their vehicle (0.9% NaCl).

Acute nociceptive test

Immediately after the p.orb. injections, mice were placed inside a plexiglass chamber and spontaneous nociception was assessed by measuring the time (seconds) that the animal spent face rubbing the injected area with its paws [26] over the next 10 minutes. The p.orb. injection with vehicles produced nociceptive behaviour for a maximum of 3 seconds.

Periorbital mechanical allodynia

The measurement of PMA was performed by using the up-and-down paradigm as described previously [27, 28] in the same mice in which acute nociceptive responses were monitored for 10 minutes after the stimulus. Animals were allocated in a restraint apparatus designed for the evaluation of periorbital mechanical thresholds. The apparatus consists in an individual clear three-walled plexiglass box (4 H × 4 W x 10 L cm) with an opening for the tail and one for the head and front paws, located on a platform to allow the operator to access to the periorbital area. The box size allowed for head and forepaw movements but prevented the animal from turning around inside it (Fig 1A). One day before the first behavioural observations, mice were habituated to the apparatus. PMA was evaluated in the periorbital region over the rostral portion of the eye (*i.e.*, the area of the periorbital region facing the sphenoidal rostrum) of the mice [29] (Fig 1a), before (basal threshold) and after (0.5, 1, 2, 4, 6, 8 hours) treatment.

The day of the experiment, after 20 minutes of adaptation inside the chamber, a series of 7 Von Frey filaments in logarithmic increments of force (0.02, 0.04, 0.07, 0.16, 0.4, 1.0 and 1.4 g) were applied to the periorbital area perpendicular to the skin, with sufficient force to cause slight buckling, and held for approximately 5 seconds to elicit a positive response. The response was considered positive by the following criteria: mouse vigorously stroked its face with the forepaw, head withdrawal from the stimulus, or head shaking. The stimulation initiated with the 0.16 g filament. Absence of response after 5 seconds led to the use of a filament with increased weight, whereas a positive response led to the use of a weaker (*i.e.* lighter) filament. Six measurements were collected for each mouse or until four consecutive positive or negative responses occurred. The 50% mechanical withdrawal threshold (expressed in g) was then calculated from these scores by using a δ value of 0.205, previously determined.

Statistical Analysis

All data were expressed as mean ± s.e.m. Statistical analysis was performed by the unpaired two-tailed Student's t-test for comparisons between two groups. Group means for single factor experiments were analysed with a one-way ANOVA, while behavioural experiments with repeated measures employed a two-way mixed model ANOVA, first to determine the presence of an interaction effect, and then to compare the control and treated groups of mice at each time point tested. In both cases, post-hoc comparisons employed the Bonferroni criterion to maintain the experiment-wise error rate at 5%. To avoid uncertainties that would follow from the use of these parametric methods on data that may not attain an interval level of measurement, as well as the potential violation of other ANOVA assumptions, including that of normal sampling distribution, analyses were repeated using non-parametric methods. Both methods led to similar conclusions, and we presented only the parametric analyses, which maintain the original, and more intuitive, units of measure. Statistical analyses were performed on raw data using Prism 5

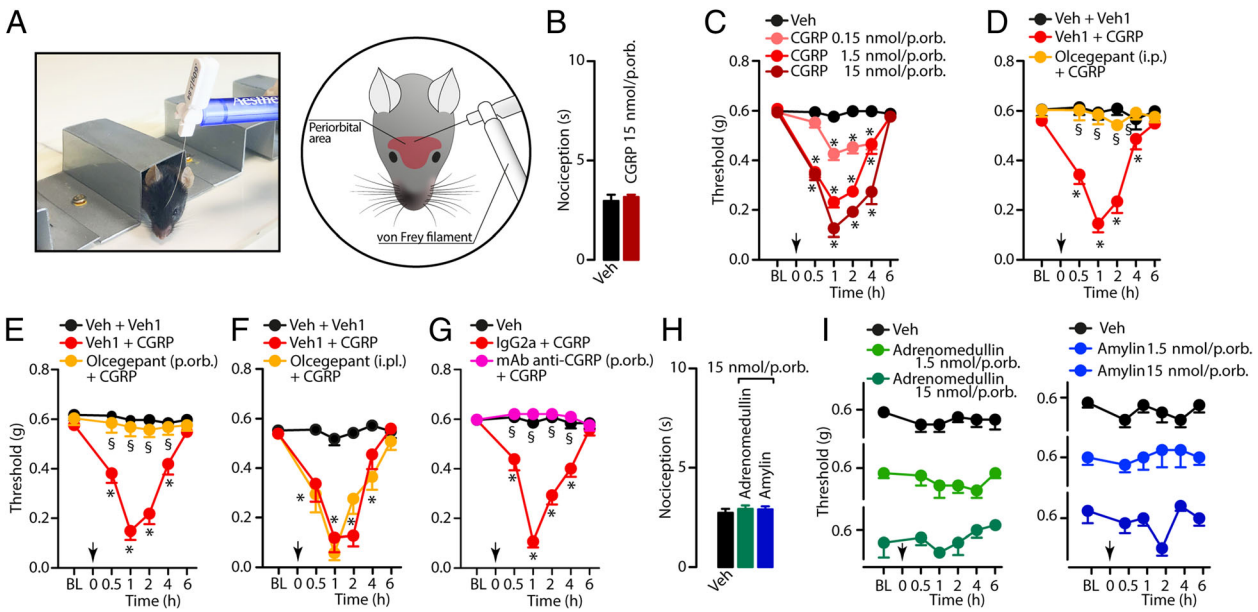

Fig. 1 a Photograph of restraint apparatus and von Frey monofilament used for evaluating periorbital mechanical allodynia (PMA) and drawing indicating the region where periorbital von Frey stimulus was applied during testing. **b** Subcutaneous injection in the periorbital area (p.orb., 10 μl/15 nmol/site) of CGRP does not cause spontaneous nociceptive behaviour. **c** Dose- and time-dependent PMA evoked by p.orb. Injection of CGRP (10 μl/0.15–15 nmol/site). **d**-**f** Effect of pretreatment with intraperitoneal (i.p. 1 μmol/kg), p.orb. (10 μl/4 nmol/site) and intraplantar (i.pl., 20 μl/4 nmol/site) olcegepant on PMA evoked by CGRP (p.orb., 10 μl/1.5 nmol/site). **g** Effect of pretreatment with a monoclonal anti-CGRP antibody (mAb anti-CGRP) or mouse monoclonal IgG2a (IgG2a) (both p.orb., 10 μl/60 pmol/site) on PMA evoked by CGRP (p.orb., 10 μl/1.5 nmol/site). Adrenomedullin or amylin injection (p.orb., 10 μl/1.5–15 nmol/site) does not evoke spontaneous nociceptive behaviour (**h**) or PMA (**i**). C57BL/6J mice were used. Veh is the vehicle of CGRP (**b**-**g**), adrenomedullin or amylin (**h**, **i**), and veh1 is the vehicle of olcegepant (**d**-**f**). Arrows indicate time of CGRP, adrenomedullin or amylin administration. Olcecepant, mAb anti-CGRP or IgG2a were given 30 min before the stimulus. Error bars indicate mean ± s.e.m., $n = 6$ mice *per* group. *$P < 0.05$ vs. Veh or Veh + Veh1, §$P < 0.05$ vs. Veh1 + CGRP; two-way ANOVA with Bonferroni post hoc correction

GraphPad software (GraphPad Software Inc., San Diego, CA, USA), as well as IBM SPSS (v.25, IBM Corp., Armonk, NY, USA). P<0.05 was considered statistically significant.

Results

CGRP, adrenomedullin, amylin

CGRP, amylin and adrenomedullin belong to the larger calcitonin family of peptides, which activate, with different potencies, a series of receptors resulting from the multiple combinations of the 3 forms of the calcitonin (CT, further divided into the a, b and δ(1-47)b subtypes) receptor and the CT receptor-like receptor (CLR) with the 3 forms of receptor-activity-modifying proteins (RAMPs) [30]. Although CGRP can bind to all these receptor complexes, it exhibits a higher affinity for the RAMP1/CLR [30]. Adrenomedullin binds with higher potency to the RAMP2-3/CLR and amylin to the RAMP1/CT(a) and the RAMP1-2/CT(b) [30]. Whereas periorbital (p.orb., 10 μl/site) injection of CGRP (0.15, 1.5 and 15 nmol/site), even at the highest dose, did not evoke an acute spontaneous nociceptive response (Fig. 1b), it did cause a robust, dose-dependent and sustained PMA (Fig. 1c). The prolonged PMA was present at 0.5 hour, peaked at 2 hours and declined, to return to baseline values, 6 hours after CGRP injection. Systemic intraperitoneal (i.p., 1 μmol/kg) or local (p.orb., 4 nmol/site), but not intraplantar (i.pl., 20 μl, 4 nmol/site) injection (30 minutes before CGRP) of the CGRP receptor antagonist, olcegepant, prevented PMA (Fig. 1d-f). Furthermore, p.orb. (10 μl) pretreatment (30 min before) with a monoclonal anti-CGRP antibody (60 pmol/site), but not with the inactive mouse monoclonal IgG2a, also prevented the development of PMA induced by p.orb. CGRP (Fig. 1g).

Local (p.orb., 10 μl) administration of adrenomedullin or amylin at the same pro-allodynic dose of CGRP (1.5 or 15 nmol/site), was unable to produce any measurable acute nociceptive response, even at the highest dose. Adrenomedullin or amylin also failed to produce PMA over the entire period of observation (6 hours) (Fig. 1h, i).

PACAP and VIP

The members of the family of the PACAP and VIP vasoactive peptides act on VPAC-1 and VPAC-2 receptors with comparable affinity, whereas the PAC-1 receptor isoform has 100-fold higher affinity for PACAP [31, 32]. Local (p.orb., 10 μl) injection of PACAP (0.15, 1.5 and 15 nmol/

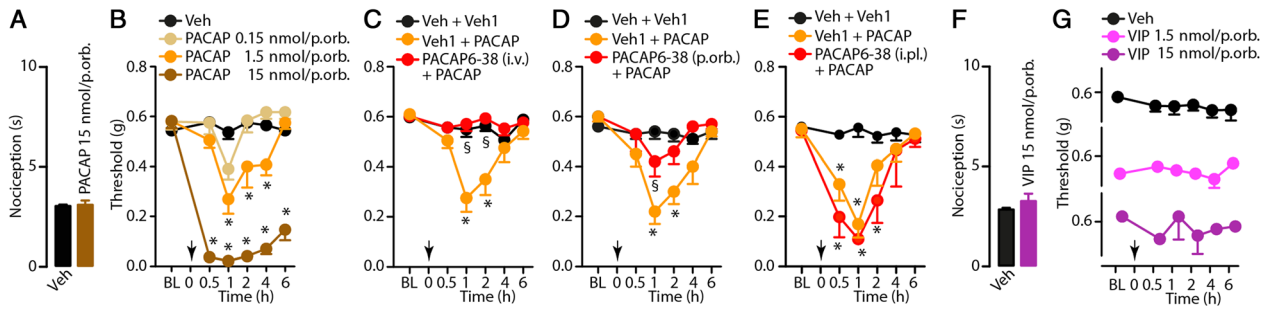

Fig. 2 a PACAP periorbital (p.orb., 10 μl/15 nmol/site) injection does not evoke spontaneous nociceptive behaviour. **b** Dose- and time-dependent periorbital mechanical allodynia (PMA) evoked by p.orb. Injection of PACAP (10 μl/0.15–15 nmol/site). **c**-**e** Effect of pretreatment with intravenous (i.v., 12 nmol/kg), p.orb. (10 μl/240 pmol/site) or intraplantar (i.pl., 20 μl/240 pmol/site pmol) PACAP6–38 on PMA evoked by PACAP (p.orb., 10 μl/1.5 nmol/site). VIP injection (p.orb., 10 μl/1.5 and 15 nmol/site) does not evoke spontaneous nociceptive behaviour (**f**) or PMA (**g**). C57BL/6J mice were used. Veh is the vehicle of PACAP (**a**-**e**) or VIP (**f**, **g**) and veh1 is the vehicle of PACAP6–38 (**c**-**e**). Arrows indicate time of PACAP or VIP administration. PACAP6–38 was given 30 min before the stimulus. Error bars indicate mean ± s.e.m., $n = 6$ mice *per* group. $^{*}P < 0.05$ vs. Veh or Veh + Veh1, $^{§}P < 0.05$ vs. Veh1 + PACAP; two-way ANOVA with Bonferroni post hoc correction

site), which did not provoke any detectable spontaneous nociceptive behaviour even at the highest dose, induced a marked, dose-dependent and sustained (1-6 hours) PMA (Fig. 2a, b). Intravenous (i.v., 1 ml/kg, 12 nmol/kg) or p.orb. (10 μl, 240 pmol/site), but not i.pl. (20 μl, 240 pmol/site), pretreatment with the selective PACAP receptor antagonist, PACAP6-38, prevented PACAP-induced PMA (Fig. 2c, e). VIP (1.5 or 15 nmol/site, p.orb.) was unable to produce either acute nociception or PMA (Fig. 2f, g).

Histamine

Histamine is a ubiquitous mediator released from mast cells, enterochromaffin-like cells and neurons, implicated in pathophysiological responses such as arousal state, allergy, inflammation, itch and pain [33–35]. Its actions are mediated by four distinct receptors, the H_1, H_2, H_3 and H_4 receptors [36]. Local injection (p.orb., 10μl) of histamine (0.15, 1.5 and 15 nmol/site) was unable to produce any spontaneous acute nociception, even at the highest dose administered, but induced a dose-dependent and sustained (4-6 hours) PMA (Fig. 3a, b). Systemic (i.p., 10 ml/kg, 4 μmol/kg) or p.orb. (10 μl, 10 nmol/site), but not i.pl. (20 μl, 10 nmol/site) pretreatment with the histamine H_1 receptor antagonist, astemizole, prevented histamine-induced PMA (Fig. 3c-e).

PGE_2, PGI_2, $PGF_{2\alpha}$

Prostanoids are ubiquitous mediators which play a major role in a large variety of physiological responses and pathological process, including inflammation and pain [37]. Cyclooxygenase inhibition by non-steroidal anti-inflammatory drugs (NSAIDs), which prevents the transformation of arachidonic acid into the inactive prostaglandin precursor, prostaglandin H_2 (PGH_2), is a mainstay of the acute migraine attack, thus implicating prostaglandins in migraine pain [38, 39]. PGE_2 administration in the mouse paw is known to evoke spontaneous acute nociception [40]. Accordingly, we found that PGE_2 (0.15, 1.5 and 15 nmol/site), but not PGI_2 (0.15, 1.5 and 15 nmol/site) or $PGF_{2\alpha}$ (1.5-15 nmol/site) injection into the mouse periorbital skin elicited a marked spontaneous nociceptive response (Fig. 4a, f, k). Furthermore, injection of both PGE_2 and PGI_2, but not $PGF_{2\alpha}$, evoked a dose-dependent sustained (0.5-6 hours) PMA (Fig. 4b, g, k). Pretreatment with i.p. (10 ml/kg, 60 μmol/kg) and p.orb. (10 μl, 10 nmol/site), but not i.pl. (20 μl, 10 nmol/site) prostaglandin receptor 4 (EP_4) antagonist, ER819762, prevented PGE_2-induced spontaneous nociception and PMA (Fig. 4a, c-e). Pretreatment with i.v. (1 ml/kg, 30 μmol/kg) and p.orb., (10 μl, 10 nmol/site), but not i.pl. (20 μl, 10 nmol/site) antagonist for the prostacyclin receptor (IP), Ro1138452, prevented PGI_2-induced PMA (Fig. 4h-j). Conversely, Ro1138452 (i.v., 30 μmol/kg) did not affect spontaneous nociception and PMA evoked by PGE_2 and ER819762 (i.p., 60 μmol/kg) did not affect PMA evoked by PGI_2 (Fig. 4a, c, h).

Discussion

The members of the calcitonin family of peptides activate a variety of receptors deriving from the dimerization of CLR or CL with RAMP proteins. Adrenomedullin, which stimulates the combinations of the CLR with RAMP2 or RAMP3 with a potency higher than CGRP (AM_1 and AM_2 receptor, respectively), and amylin, which is equipotent to CGRP on the receptor combinations formed by the three CT subtypes with RAMP1, RAMP2 or RAMP3, failed to evoke allodynia. A possible effect of amylin and adrenomedullin on the RAMP1/CLR, or of CGRP on the different combinations of receptors for amylin and adrenomedullin has been claimed to contribute to the pro-migraine action of

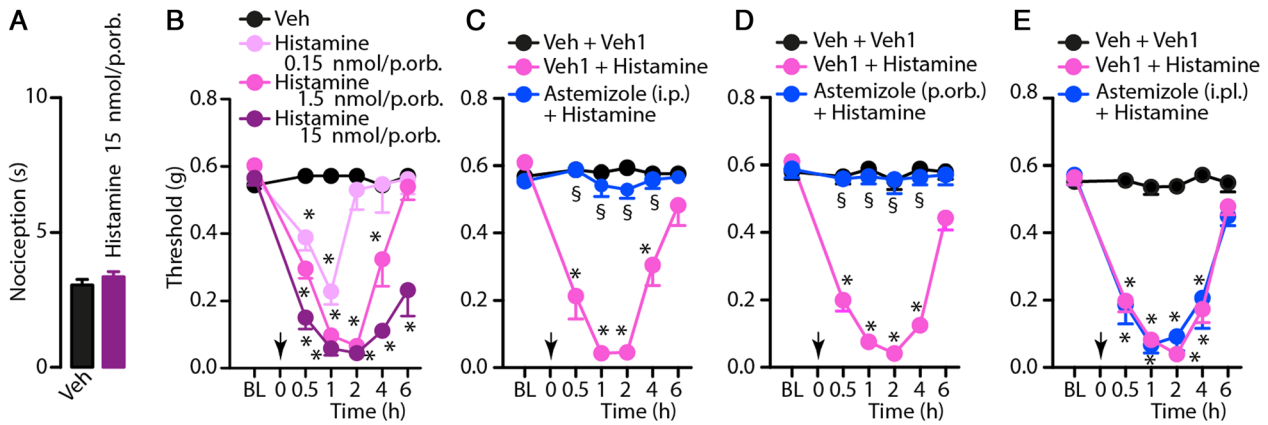

Fig. 3 a Histamine periorbital (p.orb., 10 μl/15 nmol/site) injection does not evoke spontaneous nociceptive behaviour. **b** Dose- and time-dependent periorbital mechanical allodynia (PMA) evoked by p.orb. Injection of histamine (10 μl/0.15–15 nmol/site). **c**-**e** Effect of pretreatment with intraperitoneal (i.p., 4 μmol/kg), p.orb. (10 μl/10 nmol/site) or intraplantar (i.pl., 20 μl/10 nmol/site) astemizole on PMA evoked by histamine (p.orb., 10 μl/1.5 nmol/site). C57BL/6J mice were used. Veh is the vehicle of histamine and veh1 is the vehicle of astemizole (**c**-**e**). Arrows indicate time of histamine administration. Astemizole was given 30 min before histamine. Error bars indicate mean ± s.e.m., $n = 6$ mice *per* group. $^{*}P < 0.05$ vs. Veh or Veh + Veh1, $^{§}P < 0.05$ vs. Veh1 + histamine; two-way ANOVA with Bonferroni post hoc correction

CGRP or its receptor [30]. As previously reported in the mouse hindpaw [41] and periorbital area [15], we confirm that CGRP causes a robust and sustained mechanical allodynia, which is attenuated by both systemic and local administration of the selective RAMP1/CLR (CGRP receptor) antagonist, olcegepant. The observation that, under the same experimental conditions neither adrenomedullin nor amylin evoked PMA negates the implication of their preferred receptors in CGRP-mediated pain-like responses. Furthermore, the present results do not support the hypothesis that amylin or adrenomedullin act on RAMP1/CLR to evoke pain-like responses. Previous results that CGRP administration to migraineurs induced delayed migraine-like attacks [42], whereas adrenomedullin was found to be inactive [22], strengthened and excluded the role in migraine mechanism of CGRP and adrenomedullin, respectively. The present findings on the calcitonin related peptides, recapitulating human results, support the predictive value of mouse PMA in investigating pain mechanisms of migraine.

Clinical trials with anti CGRP or anti RAMP1/CLR monoclonal antibodies, while showing excellent efficacy and safety profile, also indicate that a subset of migraine patients either do not respond or have a partial benefit [4, 43, 44]. This observation suggests that additional mechanisms and mediators contribute to migraine pain, thus prompting the study of substances other than CGRP. PGE_2 and PGI_2, two prostaglandins that induce headaches and migraine-like attacks in humans [45–48], elicited PMA in mice. In contrast, $PGF_{2\alpha}$, a prostaglandin, which was unable to evoke migraine-like attack in patients [49], failed to elicit PMA in mice. The use of selective antagonists for the EP_4 and IP receptors showed that PGE_2 and PGI_2 caused allodynia exclusively by activating the respective preferred receptor. This conclusion suggests that in humans PGE_2 and PGI_2 elicit migraine-like attacks by acting on EP_4 and IP receptors, respectively. As reported previously, in the mouse hindpaw [40], PGE_2 was the sole compound among all the presently investigated substances that evoked an early spontaneous nociceptive response, which, similarly to allodynia, was abated by EP4 receptor antagonism. However, given that only one of the migraine-provoking substances elicited spontaneous nociception, the significance of such early non-evoked pain-like responses for migraine pain mechanism remains unclear.

Histamine, a key proinflammatory and allergic mediator with a proalgesic role provokes migraine-like attacks in patients [50–52]. Furthermore, anecdotical reports and clinical investigations [53] have proposed the use of increasing doses of histamine to desensitize the pain-producing mechanism in migraine patients. Present data show that, by targeting the H_1 receptor subtype, histamine evokes PMA in mice and provides indirect support to the contribution of the H_1 receptor, rather than H_2 receptors [51], in provoking migraine [52], and to the desensitization process that is supposed to ameliorate migraine [53]. It should be underlined that, despite the ability of histamine to sensitise nociceptors *via* H_1 receptor activation, the H_1-antagonists were not always effective in reducing migraine [54].

VIP and PACAP, which belong to the glucagon/secretin family of regulatory peptides, stimulate three distinct receptors: the PAC-1, selectively activated by PACAP, and the VPAC-1 and VPCA-2, which are equipotently activated by both PACAP and VIP. The observation that PACAP, but not VIP, elicited allodynia, suggests that the

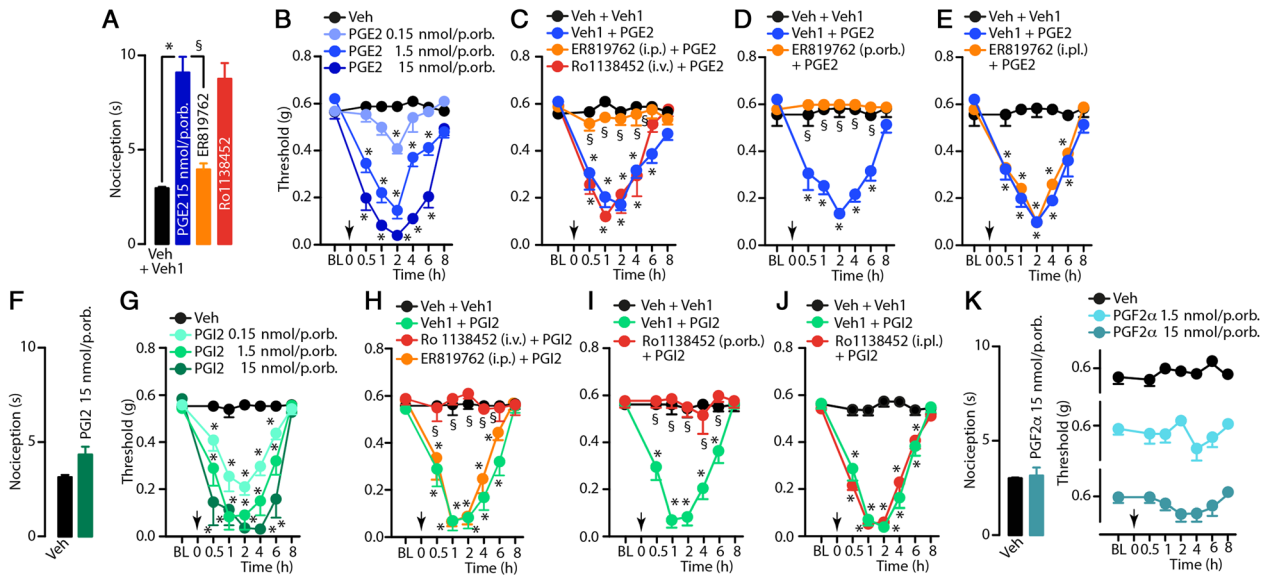

Fig. 4 a Effect of pretreatment with intraperitoneal (i.p., 60 µmol/kg) ER819762 or intravenous (i.v., 30 µmol/kg) Ro1138452 on the spontaneous nociceptive behaviour evoked by periorbital (p.orb., 10 µl/15 nmol/site) injection of PGE_2. **b** Dose- and time-dependent periorbital mechanical allodynia (PMA) evoked by p.orb. Injection of PGE_2 (10 µl/0.15–15 nmol/site). **c** Effect of pretreatment with ER819762 (i.p., 60 µmol/kg) and Ro1138452 (i.v., 30 µmol/kg) on PMA evoked by PGE_2 (p.orb., 10 µl/1.5 nmol/site). **d**, **e** Effect of pretreatment with p.orb. (10 µl/10 nmol/site) or intraplantar (i.pl., 20 µl/10 nmol/site) ER819762 on PMA evoked by PGE_2 (p.orb., 10 µl/1.5 nmol/site). **f** PGI_2 (p.orb., 10 µl/15 nmol/site) does not evoke spontaneous nociceptive behaviour. **g** Dose- and time-dependent PMA evoked by PGI_2 (p.orb., 10 µl/0.15–15 nmol/site). **h** Effect of pretreatment with Ro1138452 (i.v., 30 µmol/kg), and ER819762 (i.p., 60 µmol/kg) on PMA evoked by PGI_2 (p.orb., 10 µl/1.5 nmol/site). **i**, **j** Effect of pretreatment with p.orb. (10 µl/10 nmol/site) or i.pl. (20 µl/10 nmol/site) Ro1138452 on PMA evoked by PGI_2 (p.orb., 10 µl/1.5 nmol/site). **k** $PGF_{2\alpha}$ (p.orb., 10 µl/1.5–15 nmol/site) does not evoke spontaneous nociceptive behaviour or PMA. C57BL/6J mice were used. Veh is the vehicle of PGE_2 (**a**-**e**), PGI_2 (**f**-**j**) or $PGF_{2\alpha}$ (**k**) and veh1 is the vehicle of ER819762 (**d**, **e**), Ro1138452 (**i, j**) or a combination of vehicles of ER819762 and Ro1138452 (**a**, **c**, **h**). Arrows indicate time of PGE_2, PGI_2 or $PGF_{2\alpha}$ administration. ER819762 and Ro1138452 were given 30 min before PGE_2 or PGI_2. Error bars indicate mean ± s.e.m., $n = 6$ mice *per* group. $^{*}P < 0.05$ vs. Veh or Veh + Veh1, $^{§}P < 0.05$ vs. Veh1 + PGE_2 or Veh1 + PGI_2; one- or two-way ANOVA with Bonferroni post hoc correction

PACAP/PAC-1 is the sole pathway implicated in generating pain-like responses. PACAP and VIP are both vasodilator substances [55, 56]. The ability of PACAP, and not VIP, to cause allodynia in mice and migraine-like attacks in humans [21, 57], supports the hypothesis that vasodilatation is not *per se* a major factor contributing to allodynia in mice and migraine pain in humans. These findings are in line with previous observations that PACAP, but not VIP, causes delayed activation and sensitization of central trigeminovascular neurons *via* activation of the PAC1 receptor [58]. The implication of mast cells has been proposed in the pathway activated by PACAP to elicit pain. Mast cells may release PACAP [59], and PACAP, *via* a hitherto uncharacterized receptor, degranulates mast cells [60]. The present model could be used to further explore local mechanisms that, activated by PACAP and implicating mast cells, result in pain responses.

The underlying mechanism that promotes migraine attacks is unclear. Clinical investigation with small molecule CGRP receptor antagonists underscores the key role of CGRP in the genesis of migraine pain [4, 61]. However, the specific site(s) of the proalgesic action of CGRP in migraine pain remains elusive. Recent clinical trials with monoclonal antibodies that block CGRP or its receptor [43, 44], underline the hypothesis that the pain produced by CGRP during migraine attacks originates at a peripheral site, outside the blood brain barrier. However, the precise location of such a peripheral site is uncertain. The observation that PMA was attenuated only if antagonists were given locally, close to (p.orb.), but not at distance from (i.pl.) the site where the respective agonists were injected, indicates that the anatomical site where pain hypersensitivity initially originates is the terminal region of peripheral trigeminal fibres.

Differences may exist between the trigeminal fibres of the skin and those innervating meningeal blood vessels [62, 63] that are possibly implicated in migraine pathogenesis. Nevertheless, the local subcutaneous injection of stimuli was adopted purposively to selectively investigate the interaction between pro-migraine mediators and peripheral terminals of trigeminal nociceptors and to minimize confounding factors, deriving from the systemic administration or the surgical procedures required for dural application of the stimuli. The old dispute regarding the contribution of the peripheral or central nervous system to allodynia and

hyperalgesia [64–67] has not yet been completely resolved. The present investigation reports a condition of hypersensitivity that originates peripherally in the periorbital area of mice, but by no means implies that central neural pathways do not contribute to sustain allodynia. However, pathways and mechanisms regulating mechanical hypersensitivity in the central nervous system are not the object of the present study. Clinical investigation shows that, while blockade of the CGRP system provides benefit in a large proportion of patients, a subset of migraineurs appears to be resistant [43, 44], thus suggesting that additional mediators and mechanisms contribute to migraine pain. The ability to evoke PMA in mice adds support to the role of additional migraine provoking mediators in spontaneous pain attacks.

Conclusions

The major finding of the present study is the strict correspondence between mediators that provoke migraine in patients and evoke periorbital allodynia in mice. The same correspondence was observed between mediators that do not provoke migraine in patients and do not evoke allodynia in mice. An additional relevant finding is that, although most of the pro-allodynic substances tested in the present study are vasodilators, two robust vasodilators, VIP and adrenomedullin, did not evoke allodynia, thus indicating that vascular activity is not *per se* sufficient to elicit pain. Cutaneous allodynia is frequently reported by migraine patients during the attack [68, 69]. However, it should be considered that migraine-like attacks induced by provoking substances are characterized by delayed and prolonged spontaneous, non-evoked pain. Therefore, mechanical allodynia cannot recapitulate the complete spectrum of the pain modalities experienced by migraineurs during their attacks. Nevertheless, disclosing the mechanisms used by the different mediators, and particularly CGRP, to evoke delayed and sustained mechanical allodynia in mice may provide insights for a better understanding of the mechanisms by which the same substances generate migraine pain in patients.

Abbreviations

ANOVA: Analysis of variance; CGRP: Calcitonin gene related peptide; CLR: CT receptor-like receptor; CT: Calcitonin; DMSO: Dimethyl sulfoxide; EP_4: Prostaglandin receptor 4; GTN: Glyceryl trinitrate; i.p.: Intraperitoneal; i.pl.: Intraplantar; i.v.: Intravenous; IP: Prostacyclin receptor; NSAIDs: Non-steroidal anti-inflammatory drugs; p.orb.: Periorbital; PACAP: Pituitary adenylyl cyclase activating peptide; PG: Prostaglandin; PGI_2: Prostacyclin; PMA: Periorbital mechanical allodynia; RAMPs: Receptor-activity-modifying proteins; VIP: Vasoactive intestinal polypeptide; VPAC: VIP receptor

Competing interests

The authors declare that they have no competing interests.

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Quantitative magnetic resonance imaging of the upper trapezius muscles – assessment of myofascial trigger points in patients with migraine

Abstract

Background: Research in migraine points towards central-peripheral complexity with a widespread pattern of structures involved. Migraine-associated neck and shoulder muscle pain has clinically been conceptualized as myofascial trigger points (mTrPs). However, concepts remain controversial, and the identification of mTrPs is mostly restricted to manual palpation in clinical routine. This study investigates a more objective, quantitative assessment of mTrPs by means of magnetic resonance imaging (MRI) with T2 mapping.

Methods: Ten subjects (nine females, 25.6 ± 5.2 years) with a diagnosis of migraine according to ICHD-3 underwent bilateral manual palpation of the upper trapezius muscles to localize mTrPs. Capsules were attached to the skin adjacent to the palpated mTrPs for marking. MRI of the neck and shoulder region was performed at 3 T, including a T2-prepared, three-dimensional (3D) turbo spin echo (TSE) sequence. The T2-prepared 3D TSE sequence was used to generate T2 maps, followed by manual placement of regions of interest (ROIs) covering the trapezius muscles of both sides and signal alterations attributable to mTrPs.

Results: The trapezius muscles showed an average T2 value of 27.7 ± 1.4 ms for the right and an average T2 value of 28.7 ± 1.0 ms for the left side ($p = 0.1055$). Concerning signal alterations in T2 maps attributed to mTrPs, nine values were obtained for the right (32.3 ± 2.5 ms) and left side (33.0 ± 1.5 ms), respectively ($p = 0.0781$). When comparing the T2 values of the trapezius muscles to the T2 values extracted from the signal alterations attributed to the mTrPs of the ipsilateral side, we observed a statistically significant difference ($p = 0.0039$). T2 hyperintensities according to visual image inspection were only reported in four subjects for the right and in two subjects for the left side.

Conclusions: Our approach enables the identification of mTrPs and their quantification in terms of T2 mapping even in the absence of qualitative signal alterations. Thus, it (1) might potentially challenge the current gold-standard method of physical examination of mTrPs, (2) could allow for more targeted and objectively verifiable interventions, and (3) could add valuable models to understand better central-peripheral mechanisms in migraine.

Keywords: Magnetic resonance imaging, Migraine, Myofascial trigger points, Trapezius muscle, T2 mapping, Trigemino-cervical complex

Background

Migraine belongs to the primary headaches, representing the sixth most disabling disorder worldwide, with number-1-status in the age group between 15 to 49 years of age, and affecting about 16% of the European population [1, 2]. To date, much attention has been paid to central mechanisms of migraine, including, but not limiting research to the activation of the trigemino-vascular system [3, 4]. However, it becomes more and more evident that headache may be linked to nociceptive inputs from peripheral structures that can converge upon the same bipolar neurons, with pain from the pericranial head or the neck and shoulder region being referred to the brainstem and meninges and being experienced as headache [5–8]. Thus, research on the development and maintenance of primary headache increasingly points towards a widespread pattern including structures also beyond the central nervous system, with neck and shoulder muscle pain, clinically often represented by the presence of myofascial trigger points (mTrPs), getting in the focus [5, 6, 9, 10].

Such mTrPs are regarded as hyperirritable spots associated with a taut band of skeletal muscle, reacting painful on compression or stretch, and leading to typical referred pain patterns [5–8]. Indeed, muscular pain in the neck and shoulder area has shown to be particularly common in subjects suffering from migraine, with referred pain patterns from mTrPs in neck and shoulder muscles potentially contributing to migraine symptoms [8, 10]. Studies have repeatedly demonstrated a high occurrence of mTrPs in subjects with migraine and provided evidence of associations between such points and neck mobility [11–15]. The link of mTrPs of the neck area to migraine is further suggested by investigations that were successful to provoke migraine attacks by manual palpation delivered specifically to these points [12, 16]. However, despite the interest in mTrPs, reliable detection and characterization by means of verifiable imaging lacks behind. Accordingly, the current gold standard for detection of mTrPs is still represented by manual palpation of muscles, thus being basically unchanged since decades [17, 18]. The approach of such physical examination is questioned with respect to reproducibility and reliability though, with examiners potentially showing considerable disagreement during the diagnosis of mTrPs [19, 20]. Hence, manual palpation is limited due to missing objective verifications, making controlled studies difficult or even impossible.

Efforts have been undertaken to come up with more reliable methods for the identification of mTrPs, including infrared thermography (IT), needle electromyography (EMG), ultrasound (US) including elastography, and also magnetic resonance imaging (MRI) [21–30]. Studies using IT have shown to not agree on skin temperature patterns in the presence of mTrPs [31]; furthermore, the technique seems not widely available. EMG and US have shown more promising results, but needle EMG is invasive and does not directly visualize mTrPs whilst US has demonstrated inconclusive results and commonly provides mere qualitative, descriptive data in the sense that mTrPs might be registered as hypoechoic regions during US examinations [23–26, 29, 30]. To date, MRI has only been applied in few studies for the purpose of identifying mTrPs, with the focus on qualitative image assessments and evidence being limited due to poor agreement between physicians and raters [21, 22]. However, MRI is characterized by superior soft tissue contrast and principally allows for discrimination of even small soft tissue changes when applied with high resolution, thus suggesting high potential for the evaluation of mTrPs in general. Furthermore, there are now MRI-based techniques at hand that enable sensitive quantitative, thus more objective assessments of the body's musculature even in geometrically complex areas such as the trapezius muscles [32].

Given this background, the present study applies high-resolution, quantitative MRI by means of T2 mapping for the identification of mTrPs in subjects with migraine. Specifically, we aim to assess whether there are quantitatively assessable signal alterations in the muscle area of clinically detectable mTrPs, which are not registered by the eye of a radiologist during mere qualitative image interpretation.

Methods

Ethics

The study was approved by our local ethics committee (registration numbers: 154–12 & 5679/13) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Participants and design

Ten subjects with clinically confirmed migraine were enrolled. These subjects represent a subsample derived from another study that included assessments of mTrPs, with all included subjects having a confirmed history of uni- or bilateral mTrPs within the upper trapezius muscles. Subjects were recruited via an official advertisement on the websites of the two Munich universities, which included a short description of the study's setup and goals.

All subjects first underwent physical examination of the trapezius muscles bilaterally to identify and localize mTrPs, followed by high-resolution MRI during the same appointment. The subjects were instructed to present totally recovered to the study appointment (no physical activity, no passive or active physiotherapy or yoga on the same or previous three days). The inclusion criteria for this study were 1) written informed consent, 2)

age above 18 years, 3) diagnosis of migraine (according to ICHD-3), and 4) reported mTrPs within the upper trapezius muscles. Exclusion criteria were 1) any history of neurological disorders (except for migraine), 2) pregnancy, and 3) general contraindications for MRI (e.g., cochlear implants). After the appointment including physical examination and high-resolution MRI, no follow-up examinations were performed in the context of the present study.

Physical examination

A certified physiotherapist performed bilateral manual palpation of the upper trapezius muscles with the aim to localize one active mTrP per side. During physical examination, (1) a tender spot within a palpable taut band of muscle fibers had to be palpable, (2) the palpation of the identified structure had to lead to referred cranial pain in typical location for the individual subject, and (3) palpation of the identified structure had to result in a spontaneous defensive movement of the subject (jump sign) to qualify as an active mTrP [6, 21, 33, 34]. Two nitroglycerin capsules were attached to the skin adjacent to an identified active mTrP, with each mTrP being localized in the theoretical connecting line between the respective capsules.

In case of the presence of more than one active mTrP within the upper trapezius muscle per side, the point with the highest intensity of referred cranial pain in typical location was chosen. In case that the physiotherapist only identified an unilateral active mTrP, two capsules where placed contralaterally at a pressure-dolerant point, with this point strictly not fulfilling the criteria of an active mTrP.

Magnetic resonance imaging

Imaging was performed in supine position using a 3 T whole-body MRI scanner (Ingenia Elition, Philips Healthcare, Best, The Netherlands) in combination with a 16-channel anterior coil, a 12-channel posterior coil, and a 16-channel head coil. The interval between physical examination and MRI was 45 to 60 min on average. Two sequences were acquired after initial survey scanning:

- T2-weighted DIXON turbo spin echo (TSE) sequence: repetition time (TR) / echo time (TE) = 7000 / 100 ms, field of view (FOV) = 474 × 200 × 84 mm^3, acquisition voxel = 1.8 × 1.8 × 1.8 mm^3, reconstruction voxel = 0.9 × 0.9 × 1.8 mm^3, scan time = 5 min 50 s.
- T2-prepared, three-dimensional (3D) TSE sequence [32]: TR / TE = 1500 / 15 ms, FOV = 480 × 200 × 84 mm^3, acquisition voxel = 2.5 × 2.5 × 3.0 mm^3, reconstruction voxel = 1.7 × 1.7 × 3.0 mm^3, echo train length = 55, echo spacing = 2.2 ms, parallel imaging with reduction factor R = 2 × 1.35 (RL × FH), no partial Fourier, fat suppression using spectral inversion recovery, scan time = 7 min 17 s.

During the T2-prepared, 3D TSE sequence, we used a T2 preparation duration of 15-30-45 ms. The flip angle train was determined according to the vendor's routines for 3D TSE flip angle calculation, leading to a constant signal over the entire shot for the relaxation properties of skeletal muscle [32].

Generation of T2 maps and evaluation of imaging data

To generate T2 maps out of the T2-prepared, 3D TSE sequence, a voxel-by-voxel approach with a combination of variable projection and golden section search was applied [32, 35, 36]. The T2 maps and the T2-weighted DIXON TSE sequence of each subject were uploaded to Horos software together (version 1.1.7; https://www.horosproject.org), followed by co-registration of the sequences and color coding of the T2 maps (Fig. 1).

In all subjects, the investigator first identified the attached nitroglycerin capsules and re-angulated the images to be able to visualize the capsules in-plane using multi-planar reconstruction (MPR; Fig. 1). This was done using the T2-weighted DIXON TSE sequence, with the investigator being strictly blinded to the results of physical examination regarding the presence of a mTrP or only pressure-dolerant point at the area of the tags. To extract T2 values (in ms), manual placement of regions of interests (ROIs) was then performed using the co-registered and color-coded T2 maps. The axial slice in MPR mode at the level of the capsules was used to carefully draw a polygonal ROI covering the trapezius muscle of each side, respectively (Fig. 1). A margin of approximately 5 mm to the outer contour of the trapezius muscles was considered during ROI placements to avoid the accidental inclusion of muscular fascia or intermuscular fat (Fig. 1). Then, one further ROI was drawn in the color-coded T2 maps on the theoretical connecting line between the capsules per side only in case that the investigator identified a circumscribed signal alteration, being attributable to a mTrP (Fig. 1). The adjacent slices were carefully checked in the color-coded T2 maps and the T2-weighted DIXON TSE sequence, providing better anatomical resolution, to exclude unwilling inclusion of signal alterations due to vessel structures. In case that no signal alteration attributable to a mTrP was observed, no further ROIs were drawn after definition of the ROIs delineating the trapezius muscles.

Furthermore, the color-coded T2 maps were re-angulated to visualize the entire upper trapezius muscle horizontally and in-plane when considering axial slices using MPR, with the supposed mTrP being centered. This was followed by linear measurement of the length of the muscle from the origin to the insertion, which was achieved separately for both sides. An additional linear measurement was performed for each side between the muscle insertion and the signal alterations

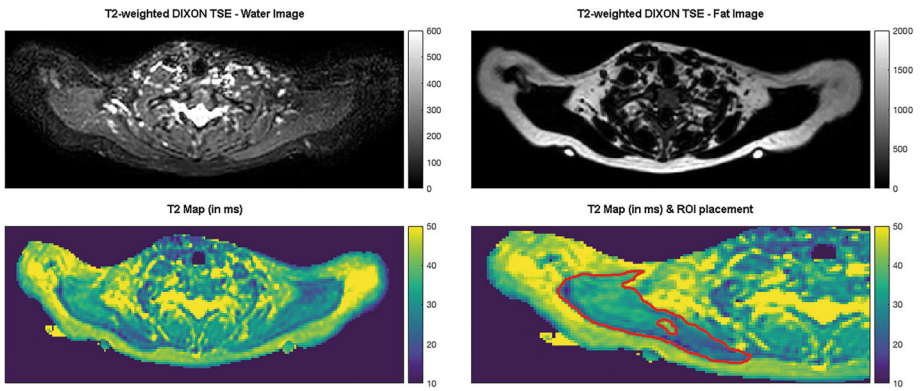

Fig. 1 Magnetic resonance imaging (MRI) including T2 mapping of the upper trapezius muscles. This figure captures an exemplary case by showing representative axial slices of the T2-weighted DIXON turbo spin echo (TSE) sequence (upper row). The left upper corner shows the T2-weighted DIXON TSE water image, the right upper corner captures the T2-weighted DIXON TSE fat image. Furthermore, T2 maps as derived from the T2-prepared TSE sequence are displayed (lower row). The left lower corner pictures the color-coded T2 map, the right lower corner shows the same color-coded T2 map after manual placement of regions of interest (ROIs) in the right upper trapezius muscle and with respect to a signal alteration (T2 elevation) within the muscle. In this exemplary case, the signal alteration was located in the area of a manually defined myofascial trigger point (mTrP), as indicated by the spatial relation to superficially attached nitroglycerine capsules as markers. The signal alteration in terms of the circumscribed T2 elevation shows a T2-hyperintense correlate in the T2-weighted DIXON TSE water image

being attributed to mTrPs, if any. Moreover, the T2-weighted DIXON TSE sequence was qualitatively evaluated to detect any T2 hyperintensities at the area where a signal alteration attributed to a mTrP was identified in the color-coded T2 maps (Fig. 1). This was achieved by careful visual image inspection by the same investigator.

Statistical analyses

All statistical data analyses and generation of graphs were performed using GraphPad Prism (version 6.0, GraphPad Software Inc., La Jolla, CA, USA). A p-value < 0.05 was defined as statistically significant (two-sided).

Descriptive statistics including mean, standard deviation (SD), median, minimum, and maximum values or absolute and relative frequencies were calculated. Regarding the T2 values and length measurements, we separately analyzed the values derived from the left and right side. Shapiro-Wilk normality test indicated non-normally data distribution for the obtained T2 values. We performed Wilcoxon matched-pairs signed rank tests between the T2 values extracted from the left and right trapezius muscles, the left and right signal alterations attributed to mTrPs, the left trapezius muscles and left-sided signal alterations attributed to mTrPs, and the right trapezius muscles and right-sided signal alterations attributed to mTrPs.

Results

Cohort characteristics and physical examination

We included ten right-handed subjects (nine females & one male volunteer, mean age: 25.6 ± 5.2 years, range: 19.4–34.9 years), all diagnosed with migraine according to ICHD-3, who reported on migraine since 13.0 ± 9.1 years on average (range: 1.5–29.2 years). The age of the first manifestation of migraine was 12.6 ± 6.5 years (range: 5.0–24.0 years), and subjects suffered from migraine on 5.5 ± 2.4 days per month (range: 3.0–10.0 days per months) with an average pain rating for migraine attacks of 7.2 ± 0.7 points (range: 6.0–8.0 points) according to a numeric pain rating scale from 0 to 10 points. None of the included subjects had any history of neurological disorders (except for migraine). Registered comorbidities of the study cohort were pollen allergy (one subject), asthma (one subject), and hypothyroidism (one subject). Nine of the included subjects regularly performed endurance sports, seven subjects further stated that they regularly participated in strength sports.

MRI was performed in an inter-ictal period in all subjects, with an interval between the last migraine attack and the study appointment of 0.7 ± 1.5 months (range: 0.1–5.1 months). According to physical examination, a clear mTrP was detected in nine subjects within the right-sided trapezius muscle, whereas seven subjects showed a mTrP in the left-sided trapezius. There were no subjects without mTrPs according to manual palpation.

Imaging of the upper trapezius muscles

Measurement of T2 values

Regarding T2 values of the trapezius muscles, 20 measurements were obtained (ten measurements per side), with an average T2 value of 27.7 ± 1.4 ms (range: 25.5–30.0 ms) for the right-sided and an average T2 value of 28.7 ± 1.0 ms (range: 26.9–30.3 ms) for the left-sided trapezius muscles (Fig. 2). There was no statistically significant difference in these values between sides ($p = 0.1055$).

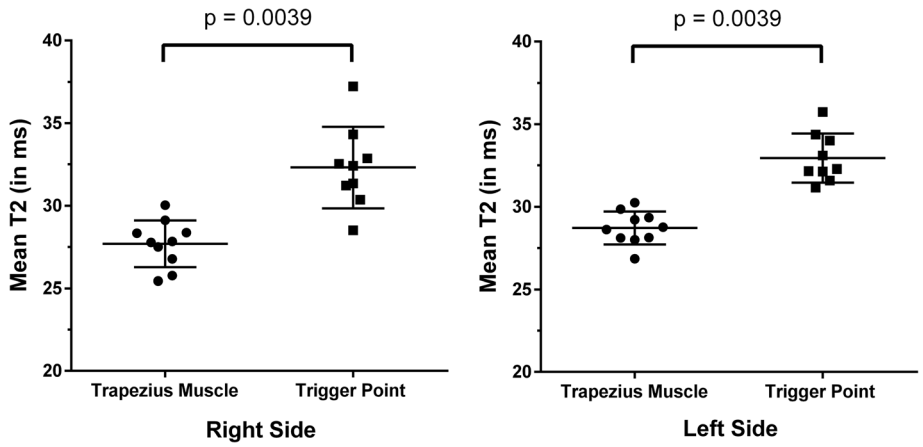

Fig. 2 T2 values of the upper trapezius muscles and myofascial trigger points (mTrPs). The graphs show the T2 values (in ms) of each subject derived from the regions of interest (ROIs) enclosing the trapezius muscles and signal alterations attributed to mTrPs of the right and left side, respectively. Measurements in trapezius muscles were obtained in all subjects bilaterally, whereas measurements of T2 values of signal alterations attributed to mTrPs were achieved in nine subjects per side, respectively (due to missing detectable signal alterations in the remaining subjects). Horizontal lines represent the mean with the standard deviation (SD). A statistically significant difference was observed between measurements for both sides, respectively ($p = 0.0039$)

Concerning T2 values of the signal alterations attributed to mTrPs, nine values were obtained for the right and left side, respectively, with no detected T2 alteration in two different subjects according to evaluation of the color-coded T2 maps. The right-sided signal alterations had an average T2 value of 32.3 ± 2.5 ms (range: 28.5–37.2 ms), the left-sided signal alterations presented a mean T2 value of 33.0 ± 1.5 ms (range: 31.2–35.7 ms; Fig. 2). The difference in T2 values between sides was not statistically significant ($p = 0.0781$).

When comparing the T2 values of the trapezius muscles to the T2 values extracted from the signal alterations attributed to mTrPs of the ipsilateral side, we observed a statistically significant difference for both sides, respectively ($p = 0.0039$ for the right and left side; Fig. 2). Mean T2 values of such signal alterations were higher than the respective T2 values of the trapezius muscles bilaterally in all subjects.

Visual image inspection

According to visual image inspection of the T2-weighted DIXON TSE sequence, T2 hyperintensities at the area where signal alterations attributed to mTrPs were identified in the color-coded T2 maps were reported in four subjects regarding the right side and in two subjects regarding the left side (water images; Fig. 1). No correlates of these T2 hyperintensities on water images were found in the corresponding fat images of the T2-weighted DIXON TSE sequence (Fig. 1).

According to linear measurements between the muscle insertion and the signal alterations being attributable to mTrPs, we observed a distance of 6.0 ± 0.9 cm (range: 3.6–6.8 cm) for the right and a distance of 6.0 ± 1.2 cm (range: 4.3–8.4 cm) for the left side (Fig. 3).

Comparison of physical examination and T2 mapping

When taking both sides together, 15 out of the 16 mTrPs according to physical examination were detected in the color-coded T2 maps by means of corresponding signal alterations and elevated T2 values. The remaining mTrP was not detected in color-coded T2 maps. Furthermore, signal alterations on the theoretical connecting line between the capsules were registered three times without previous detection of mTrPs according to physical examination.

Discussion

This study applied high-resolution MRI with T2 mapping at the level of the upper trapezius muscles for the identification and quantification of active mTrPs in subjects with migraine. When comparing T2 values derived from the trapezius muscles and T2 values of signal alterations attributed to mTrPs, we observed statistically significant differences, with elevated values of such signal alterations when compared to surrounding musculature.

Previous research has shown a high occurrence of mTrPs in subjects with migraine and has linked mTrPs to neck mobility [11–15]. Evidence of an important role of mTrPs of the neck area in the context of migraine is further provided by the finding that migraine attacks can be triggered by manually applied pressure to these points [12, 16]. A widely accepted hypothesis regarding the underlying pathologic mechanism is the concept of the trigemino-cervical complex (TCC) [7, 18, 37]. The TCC is basically characterized by a convergence of

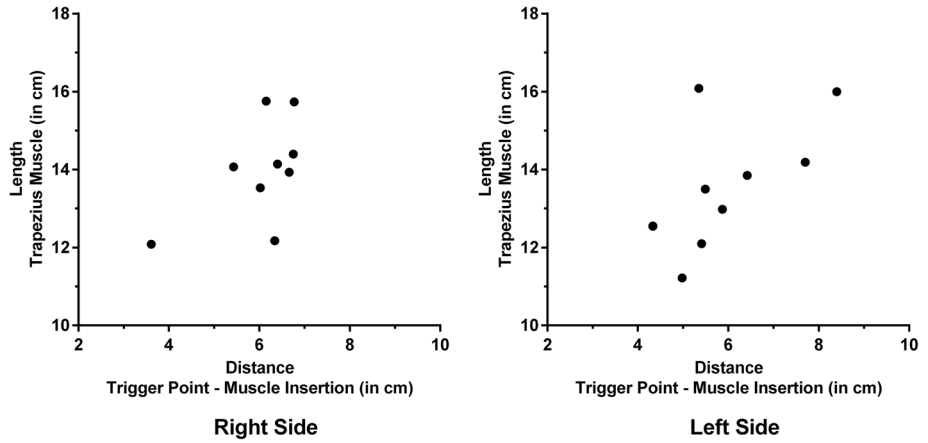

Fig. 3 Localization of myofascial trigger points (mTrPs) within the upper trapezius muscles. The graphs intend to provide information on the localization of mTrPs within trapezius muscles by capturing the linearly measured distances between the signal alteration attributed to the mTrP and the muscle insertion of the trapezius muscle (x-axis) in relation to the entire length of the trapezius muscle (y-axis) for both sides, respectively. Measurements of T2 values of signal alterations attributed to mTrPs were achieved in nine subjects per side

nociceptive inputs originating from the neck and shoulder muscles (C1 to C3) and the first branch of the trigeminal nerve in the trigeminal nuclei; thus, it could represent a connecting loop between the central nervous system and peripheral structures beyond, such as the trapezius muscles and its sensory afferences. In line with this hypothesis, migraine has partially been attributed to nociceptive myofascial inputs that increased cortical neuronal excitability, with reported lower pressure pain thresholds of upper trapezius muscles in subjects with migraine when compared to controls without migraine [38, 39]. This suggests that hyperalgesia perceived in neck and shoulder muscles might indeed contribute to the development and/or maintenance of migraine via cervical-to-trigeminal linking and vice versa, with mTrPs potentially playing a key role as morphologically identifiable correlates.

Despite mTrPs seem to be closely associated with migraine, identification and characterization of such points lacks behind. The gold standard for detection of mTrPs is represented by physical examination of muscles and has basically not changed since decades although other techniques, including in-vivo imaging, have developed in the meantime [17, 18]. It seems evident that mere physical examination has to be questioned with respect to reproducibility and reliability, and investigators have indeed shown considerable disagreement during the diagnosis of mTrPs [19, 20]. Efforts using other techniques than physical examination have been undertaken, providing a mixed and heterogeneous picture regarding results. IT has demonstrated disagreement concerning skin temperature patterns in the presence of mTrPs [31]. EMG revealed that endplate noise was more common in mTrPs than in other areas, and intramuscular activity was higher at rest and during contraction at mTrPs when compared with other sites [29, 30]. US should principally be capable of directly visualizing potential alterations in association with mTrPs; however, results on the detectability and characterization of mTrPs are partially contradictory [23–26]. Nevertheless, advanced combinations of US with texture analysis or elastography were reported to successfully differentiate between mTrPs and asymptomatic muscle tissue and to distinguish the type of mTrPs, with mTrPs typically appearing as hypoechoic regions during US examinations [23, 25, 26]. Nevertheless, US has not found the way to a standardized routine procedure and remains rather experimental. Although MRI principally seems the modality of choice regarding imaging of skeletal musculature due to superior soft tissue contrast, the technique has not been in the focus of research on mTrPs, with previous studies showing poor agreement between physicians and imaging raters or being limited regarding generalizability of findings due to small series [21, 22].

High-resolution MRI-based T2 mapping, enabling quantitative and more objective assessments, has not been applied yet. The results of this first study seem promising, with the key finding of significantly elevated T2 values attributable to mTrPs and an accordance between physical examination and imaging regarding detectability in 15 out of the 16 mTrPs defined by manual palpation. Novel developments in the field of MRI make the application of T2 mapping at the level of the upper trapezius muscle possible now: conventional T2 mapping approaches were primarily based on two-dimensional, multi-slice multi-echo spin echo sequences that showed to suffer from the dependence of the T2 quantification on B1 and B0 errors [40, 41]. In contrast, our T2-prepared TSE sequence offers an

accurate and fast 3D T2 quantification that has shown to be robust to both B1 and B0 errors even at a challenging region such as the neck and shoulder region where large B0 variations can occur [32]. Superiority of the T2 mapping approach to mere structural imaging is suggested by the finding that only a minority of subjects showed T2 hyperintensities on water images derived from the T2-weighted DIXON TSE sequences according to qualitative, visual image evaluation. No clear corresponding signal alterations were observed in the fat images; thus, we can exclude fatty muscle infiltration or other fat-related structures constituting potential mTrPs. At the current stage, edematous changes might best explain the T2 hyperintensities observed; however, the distinct nature of these changes (e.g., due to chronic inflammation or other causes) yet has to be elucidated.

Our approach might already entail clinical implications. Improved and more objective identification of mTrPs within the upper trapezius muscles may help to guide interventions for the treatment of migraine by targeting these points specifically in the context of the TCC. Remarkably, different invasive and non-invasive intervention approaches were applied to target mTrPs in subjects with migraine [18, 42]; yet, guidance is mostly led by physical examinations. The currently rather limited benefit from such interventions might potentially be enhanced by more tailored applications with knowledge about the presence and exact location of mTrPs, information that can be provided by our approach. Specifically, T2 mapping might define the target region for modulation by repetitive peripheral magnetic stimulation (rPMS), which has shown to relieve pain in subjects with migraine when applied to the upper trapezius muscles [43]. Pre- and post-interventional MRI including T2 mapping could allow correlating improved symptoms to quantitative changes within stimulated muscles and mTrPs in the context of longitudinal study designs. Later follow-up MRI with T2 mapping would further allow monitoring such potential quantitative changes over time, thus allowing to determine whether an intervention has caused longer-lasting or rather transient changes within the examined musculature. Furthermore, other conditions such as fibromyalgia, for instance, have also shown associations with mTrPs [44–46]. While this study focused on subjects with migraine, it appears obvious to evaluate our approach in such conditions as well. Thus, applicability of MRI including T2 mapping might not be restricted to the mTrPs of subjects with migraine, but could also be tested as a more objective, quantitative assessment tool in other diseases known to have close links to mTrPs.

When interpreting the results of our study some limitations have to be considered. First, the small size of the cohort and its constitution of predominantly female subjects limit generalizability of findings at the current stage. However, although only ten subjects were enrolled, we were able to analyze bilateral mTrPs in the majority of subjects, thus increasing the number of total measurements. Second, the lack of a control group consisting of non-migraineurs represents a limitation. Inclusion of such a control group might have allowed to more distinctly link signal alterations attributed to mTrPs to the condition of migraine. Third, we strictly focused on the mTrP per side that showed the highest intensity of referred cranial pain, thus not considering potential other mTrPs. It would be interesting to also extract and evaluate T2 values of these and to incorporate also latent mTrPs in future studies. Fourth, we found one mTrP derived from manual palpation that showed no correlate in color-coded T2 maps and three signal alterations on the theoretical connecting line between the markers that were not previously classified as mTrPs by means of manual palpation. In this context, physical examination to detect mTrPs represents the current gold standard, but has shown shortcomings [19, 20]. Currently, it remains unclear whether T2 mapping is superior to manual palpation and whether physical examination or T2 mapping was correct in these cases. Fifth, it has to be acknowledged that MRI is generally more expensive than one-time physical examination, and T2 mapping is not broadly available yet. Thus, our presented approach might not be directly transferred to clinical routine in all centers dealing with subjects suffering from migraine at the current stage. Future studies including larger cohorts, consisting of both males and females and non-migraineurs as controls, are needed to confirm the results of this study and to further evaluate the potential of T2 mapping in the light of physical examination and in comparison to other point-of-care techniques, particularly US.

Conclusions

This study is the first to apply quantitative MRI by means of T2 mapping for the identification of mTrPs within upper trapezius muscles in subjects with migraine. Signal alterations in color-coded T2 maps attributed to mTrPs presented with significantly elevated T2 values in comparison to the surrounding musculature bilaterally, and our approach allowed for the detection of mTrPs even in the absence of qualitatively assessed signal alterations. Our approach might challenge the current gold-standard method of physical examination to detect mTrPs, but our initial results have to be confirmed in larger cohorts and by considering also latent mTrPs. Nevertheless, our approach could already allow to verify the local effect of therapeutic approaches to the muscle (e.g., to mTrPs), enable targeted applications to the mTrPs (e.g., physiotherapy, acupuncture, rPMS, or botulinum toxin), and support studies to elucidate further the role of TCC in migraine.

Abbreviations

3D: Three-dimensional; EMG: Electromyography; FOV: Field of view; IT: Infrared thermography; MPR: Multi-planar reconstruction; MRI: Magnetic resonance imaging; mTrP: Myofascial trigger point; ROI: Region of interest; rPMS: Repetitive peripheral magnetic stimulation; SD: Standard deviation; TCC: Trigemino-cervical complex; TE: Echo time; TR: Repetition time; TSE: Turbo spin echo; US: Ultrasound

Competing interests

The authors declare that they have no competing interests.

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The effect of aerobic exercise on the number of migraine days, duration and pain intensity in migraine: a systematic literature review and meta-analysis

Abstract

Background: In patients with frequent migraine, prophylactic treatments are used. Patients often request non-pharmacological alternatives. One treatment option can be aerobic exercise. The value of aerobic exercise as prophylactic treatment however needs to be determined.

Methods: A systematic review and meta-analysis was performed to investigate the result of aerobic exercise on the number of migraine days, duration and pain intensity in patients with migraine. After screening three online databases, PubMed, Cochrane library and Web of Science, using predefined in- and exclusion criteria, six studies were retained. Pooling of data was performed when possible.

Results: Significant reductions in the number of migraine days after aerobic exercise treatment were found with a mean reduction of 0.6 ± 0.3 migraine days/month. Other outcomes were too variable to pool due to heterogeneity of outcome measurements. Unpooled data revealed small to moderate reductions in attack duration (20–27%) and pain intensity (20–54%) after aerobic exercise intervention. Various exercise intensities are applied.

Conclusion: There is moderate quality evidence that in patients with migraine aerobic exercise therapy can decrease the number of migraine days. No conclusion for pain intensity or duration of attacks can be drawn. Effect sizes are small due to a lack of uniformity. For future studies, we recommend standardized outcome measures and sufficiently intense training programs.

Trial registration: CRD42018091178.

Keywords: Migraine, Headache, Physical therapy, Exercise, Treatment, Headache characteristics

Introduction

Worldwide, migraine is the second most disabling disorder [1]. Additionally, in the age group 15–49 years, migraine is the top cause of years lived with disability [1], magnifying its impact on the working population [1]. On average eighteen days per year per migraine patient are missed from work or household activities. Mean annual costs per-person are €1222 for migraine, which leads to high costs for society [2].

The use of a prophylactic treatment is recommended if headache is present more than 8 days per month, disability is present despite acute medication, headache is present more than three days per month when acute medication is not effective [3–6]. These prophylactic drugs, however, might not be tolerated that well by patients or patients might request non-pharmacological alternatives [4, 7, 8]. In migraine, other non-drug related prophylactic treatments like self-management strategies, manual therapy and aerobic exercise are also being employed [9–14]. In aerobic exercise, a moderate intensity training is performed over a longer period of time, e.g. 30 min.

The rationale for using aerobic exercise in migraine is based on the fact that exercise can play a substantial role in the modulation of pain processing [15–18]. Moreover, the analgesic effects of both short-term [16] and long-term [15, 18] aerobic exercise have been observed at both a central and peripheral level [15, 16, 18].

In 2008, the first narrative review on the effect of aerobic exercise in the treatment of migraine showed promising, though inconclusive results [19]. During the past decade, new studies on the use of exercise as a prophylactic treatment in migraine have been published. The updated version of the International Classification of Headache Disorders (ICHD-III) [20] specifically indicates there is a need for a thorough and systematic overview regarding the effects of aerobic exercise in migraine.

Therefore, the aim of the present study is to summarize the literature published after 2004 on the effectiveness of aerobic exercise in migraine. The research question of this systematic review is: what is the effect of aerobic exercise on the number of migraine days, duration and pain intensity in patients with migraine?

Methods

Search strategy

The format of this systematic review was based on the Preferred Reporting Items for Systematic reviews and Meta-analyses (PRISMA) [21] (Additional file 1). To establish a search strategy, the PICO format was used [22]. Three electronic databases were searched to identify eligible studies: PubMed, the Cochrane library for trials and Web of Science (from January 1, 2004 till February 21, 2018). An additional search for grey literature was not performed. Inclusion and exclusion criteria were determined as depicted in Table 1. The specific search strategy used for PubMed, the Cochrane library for trials and Web of Science is shown in detail in Table 1 and Additional file 2.

Study selection

Based on the predefined inclusion and exclusion criteria the included studies were screened on title and abstract by two investigators (S.M. and J.L.) independently (first screening). Two authors (W.D.H. and J.L.) independently screened the selected full texts (second screening). In case the two authors had diverging opinions, a third author (J.D.P.) was consulted and a decision was made by consensus. Articles were included in the meta-analysis, when data-pooling was feasible based on identical diagnosis (ICHD) and units of outcome measurement.

Data items and collection

Data were manually extracted from the reports by two researchers (S.M. and J.L.). The reports were searched for the following variables: sample size characteristics (migraine diagnosis); experimental intervention characteristics; exercise intensity; control group characteristics and intervention; follow-up period; results of outcome measures (the number of migraine days, duration of attacks and pain intensity) and confounding factors.

To pool data, the random effect model and RevMan software (version 5.3) was used to compute a mean difference between the data of the intervention and control group. For missing standard deviations the *p*-value or confidence intervals were used to calculate the missing value. These calculations are based on the calculations provided in the Cochrane Handbook [23]. Before entering the mean values in the model, the difference was computed between pre-and post-intervention data of the intervention and control group as it demonstrates the mean reduction in migraine days. A PROSPERO record of this systematic review has been registered (ID: CRD42018091178).

Risk of bias in the individual studies

Risk of bias assessment of the selected articles was performed using the Cochrane risk of bias tool (ROB) for randomized controlled trials (RCTs). This checklist can be found in Figs. 1 and 2. Two reviewers (T.V.S. and J.L.) evaluated the included articles independently. The items of the ROB assessment were rated as "1", "0", or "?". An item was rated "1" if sufficient information was available and bias was unlikely. An item was rated "0" if sufficient information was available but the article did not meet a specific criterion. An item was rated "?" if

Table 1 PICOS and eligibility criteria

	Inclusion criteria	Exclusion criteria
Patients (P)	Migraine with or without aura classified by ICHD-II	Non-human subjects (such as models or animals), other types of headache or pregnant women
Intervention (I)	Physical endurance, physical fitness, aerobic exercise, exercise therapy performed during at least 6 weeks	Manual therapy or medication as stand-alone treatment or no intervention such as diagnosing or performing tests on patients
Control (C)	–	–
Outcome (O)	Number of migraine days, attack frequency, pain intensity or duration of migraine attacks	
Study design (S)	Randomized clinical trials, randomized controlled trials or clinical trial	Non-English, non-Dutch or non-French; studies published before January 1, 2004; cohort studies, case control studies, case reports, reviews or meta-analyses

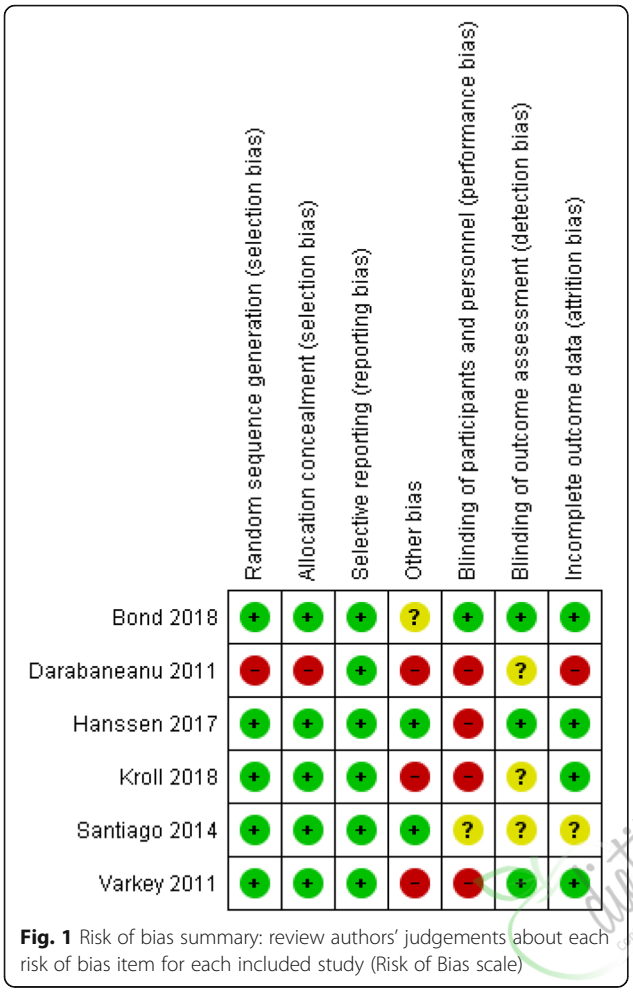

Fig. 1 Risk of bias summary: review authors' judgements about each risk of bias item for each included study (Risk of Bias scale)

unclear information was provided. Disagreement between researchers was solved by consensus.

Six studies [24–29] were scored using the ROB tool for RCTs. In case of doubt in the analysis of the risk of bias the author of the selected study was contacted. Two authors did not provide additional information.

To measure the level of evidence of each study the classification of the Dutch CBO (Centraal BegeleidingsOrgaan-classificatiesysteem) [30] was used (Table 2).

Results

Study selection

The search strategy yielded 83 results in PubMed, 53 in the Cochrane library for trials and 194 in Web of Science. After removal of duplicates, 265 articles were screened on title and abstract. Fifteen studies were retrieved and screened on full text by two researchers (W.D.H. and J.L.). After screening on full text, six studies were found eligible and were included in this review (Fig. 3).

Study characteristics

The included studies were all RCTs, except for one controlled clinical trial (CCT) [25]. All studies included patients with migraine classified by the ICHD-II as mentioned in the inclusion criteria. In three studies, patients were excluded if they performed any kind of regular aerobic training before the start of the study [25] or at least 12 weeks prior to the study [26, 28]. The number of patients enrolled in the different studies ranged from 16 to 110 with a total number of 357 patients with migraine. The mean age of all included patients was 38 years and 88% of them were women. At baseline the mean headache frequency was 9.4 days per month with an average disease duration of 19 years.

Risk of bias and level of evidence

Overall, a moderate risk of bias was present in all of the included studies. This risk of bias was mostly caused by a high dropout rate and a lack of blinding outcome assessors. In all RCTs, subjects in the control group had similar clinical characteristics as compared to the intervention group at baseline [24–29]. A dropout rate of more than 20% is reported in both intervention and control group in four studies [24, 25, 28, 29]. For this reason item 4 scored negatively in these studies. The design of

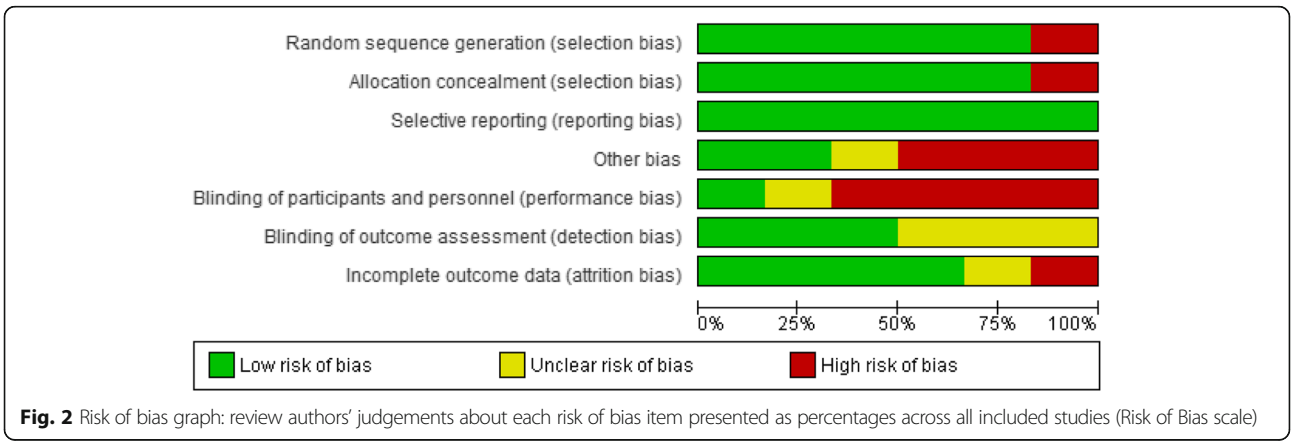

Fig. 2 Risk of bias graph: review authors' judgements about each risk of bias item presented as percentages across all included studies (Risk of Bias scale)

Table 2 Classification of Level of Evidence (Translated from the Dutch classification of CBO)

For articles regarding intervention (prevention or therapy).
A1. Meta-analysis containing at least some trials of level A2 and of which the results of individual trials are consistent.
A2. Randomized comparative clinical trials of good quality (randomized, double-blind controlled trials) of sufficient size and consistency.
B. Randomized controlled trials of moderate (weak) quality or insufficient size or other comparative trials (nonrandomized, cohort studies, patient-control studies)
C. Noncomparative trials
D. Expert opinions

one study [25] is a non-randomized CCT, therefore item 1 was scored as high risk of bias. All comparative studies [24–29] received a score B according to the CBO [30]. An overview of the risk of bias assessment is presented in Figs. 1 and 2.

Synthesis of the results

For each individual study, a summary of the characteristics of the participants, type of intervention and main results is presented in Table 3.

Interventions

Several types of aerobic exercise were used in the studies. One study used a walking program [27], one a combination of cross-training, walking, jogging and cycling [29], two a jogging protocol [25, 26], one a behavioral weight loss program [24] and finally cycling was used in one study [28]. The walking program [27] consisted of 40–45 min of fast walking and was controlled by heartrate and Borg-scale or Rate of Perceived Exertion scale (RPE) [31]. The patients also received 25mg amitriptyline each day [27].

Jogging was performed using [1] an interval program [26] (jogging and walking) or [2] a continuous run of moderate intensity for 30–45 min [25, 26]. To assure patients trained in the aerobic zone (the zone where oxygen is adequately available for the energy production process), heart rate or RPE was measured during warm up, exercise and the cooling-down period.

Indoor cycling training consisted of 15 min warming up, 20 min exercising at moderate intensity and 5 min cooling down using percentages of VO_2peak and Borg-scale or RPE [28].

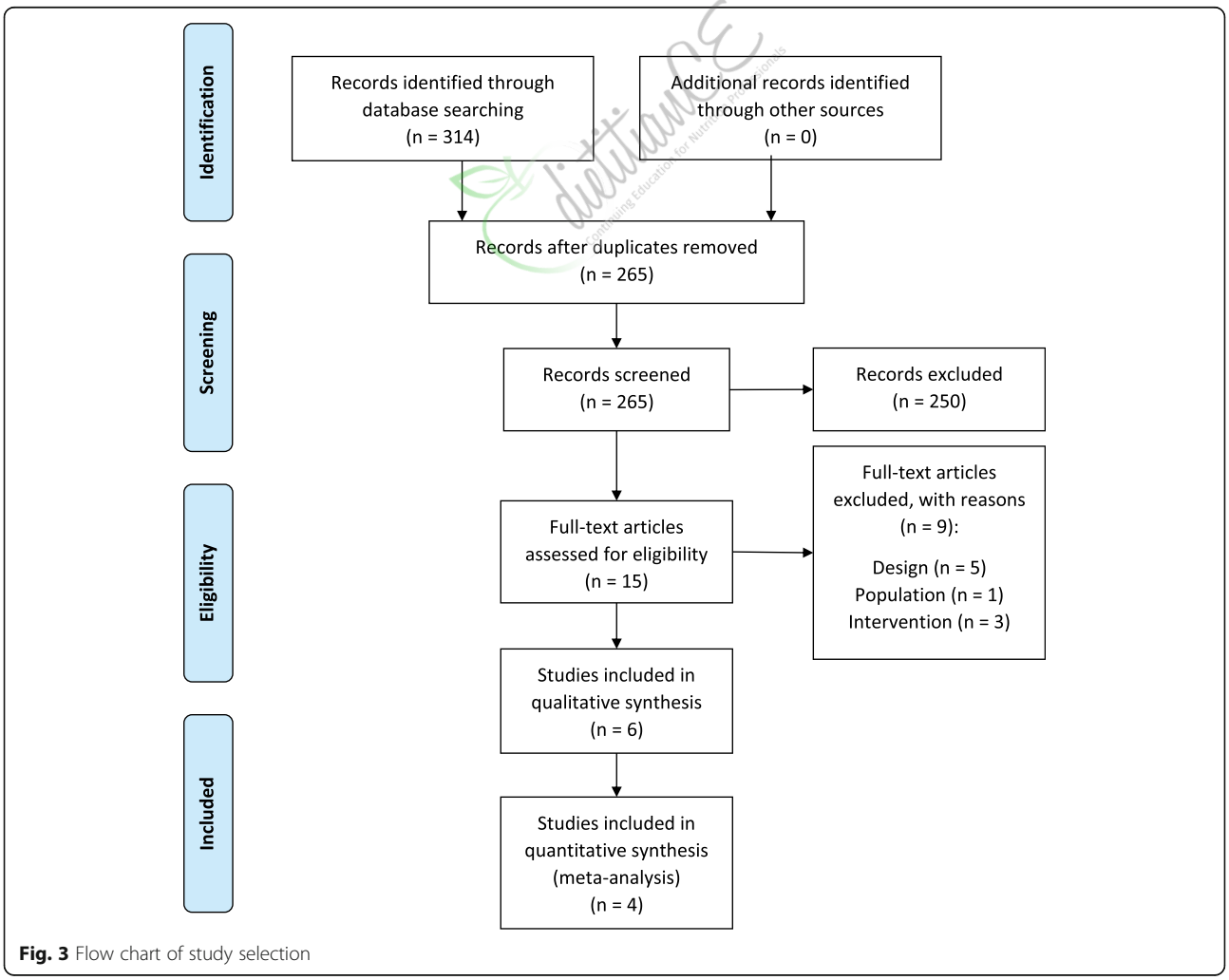

Fig. 3 Flow chart of study selection

Table 3 Synthesis of results

Study ID	Patients	Intervention	Intensity	Control	FU	Results	Confounding
Bond 2018 [24]	*N* = 54 MWA/O, ICHD-III ≥ 3 attacks/m 4–20 migraine d/m (3m)	16w BWL program 250min./w 5x/w home-based	Moderate	*N* = 56 Migraine education Self-management	4m	Number of migraine days: / Pain intensity: + 20% Attack duration: + 23% All results: NS[a]	Overweight or obese (BMI = 25–49.9 kg/m^2) Preventive/abortive pharmacological treatment if stable regimen ≥2m
Darabaneanu 2011 [25]	N = 8 MWA/O, ICHD-II ≥ 2 attacks/m Prior: No aerobic training	10w jogging 50min. 3x/w supervised 1/3 @ home	60–75% VO_2peak	*N* = 8 No intervention	8w	Number of migraine days: − 39% Pain intensity: − 20% Attack duration: − 20%	Dropout 50%
Hanssen 2017 [26]	*N* = 30 I1 = 15 (HIT) I2 = 15 (MCT) EM without aura, ICHD-IIIb Prior: No regular exercise No prophylaxis 8w	12w HIT (4 times) 2x/w 4min. 90% 3min. rest 70% 12w MCT, 2x/w 45min. 2x/w supervised	HIT: 90–95% HR MCT: 70% HR	*N* = 15 Maintain daily physical activity and physical activity recommendations	/	Number of migraine days: −29% (MCT) − 63% (HIT) Pain intensity: / Attack duration: / All results: NS[a]	
Krøll 2018 [29]	*N* = 36 EM and CM combined with NP and TTH, ICHD-IIIb ≥ 2 attacks/m	3m cycling/ cross-training/brisk walking/running 3x/w 45min. 1x/w supervised 2/3 @ home/gym	RPE scale 14–16	N = 36 Maintain daily physical activity	3m	Number of migraine days: −22% Pain intensity: − 20% Attack duration: − 23%	Participants engaged in some form of exercise activity could continue. Preventive and acute medication allowed.
Santiago 2014 [27]	*N* = 24 CM, ICHD-II Prior: No exercise for 3m No prophylaxis	12w fast walking + amitriptyline (25mg/d) 3x/w 40min. supervised weekly by telephone	Aerobic (HR + Borg)	*N* = 26 25mg/d amitriptyline	12w	Number of migraine days: − 78% Pain intensity: − 54% Attack duration: − 27%	Amitriptyline use (TCA)
Varkey 2011 [28]	*N* = 16 MWA/O, ICHD-II 2–8 attacks/m > 1y migraine [b] before age of 50 Prior: < 1x/w exercise 12w	12w indoor cycling 3x/w 40min. supervised ≥ 2/3 @home	RPE scale 14–16	*N* = 31 Relaxation (*N* = 14) 5-20min./w Topiramate (*N* = 17) 25mg/w - 200mg/d	10-12m	Number of migraine days: −28% Pain intensity: − 18% Attack duration: / All results: NS[a]	50% of all ITT patients have 6m FU

Legend: [a] between-group differences, [b] onset, *BWL* behavioral weight loss program, *C* control group, *CM* chronic migraine, *d* day(s), *FU* follow-up, *HIT* high-intensity interval training, *HR* heartrate, *I* intervention, *ICHD* international classification of headache disorders, *ITT* intention-to-treat analysis, *m* month(s), *MCT* moderate continuous aerobic training, *MWA/O* migraine with/without aura, *N* number of, *NP* neck pain, *NS* non-significant, *PP* per-protocol analysis, *RPE* rate of perceived exertion, *TCA* tricyclic antidepressant, *TTH* tension-type headache, *w* week(s)

One study [29] used a combined protocol of cross-training, brisk walking, running or indoor cycling. This training protocol comprised 10 min warming up, 30 min exercising and 5 min cooling down, using RPE to ensure aerobic training [29].

The behavioral weight loss program was designed to accomplish a ≥ 7% weight loss goal in sixteen weeks. In order to achieve this goal, participants performed a gradually progressed exercise protocol to a goal of 250 min per week, a standard calorie- and fat-restricted diet, home-based exercise (50 min, 5 days/week) and were provided instructions in behavioral modification strategies [24].

All participants in the intervention groups trained at least 3 times per week, except in one study [26]. In three studies patients were instructed to train at the local gym, at a maximum frequency of twice per week, if they could not attend the supervised training sessions [25, 28, 29]. To evaluate if patients were training in the aerobic zone, heart rate [25–27], Borg-scale or RPE [26–29] and percentages of VO_2peak [26, 28] were monitored. In one study the training intensity was not monitored [24].

Outcome

Patients kept diaries to report on the the number of migraine days, attack duration, pain intensity and the use of analgesic medication. The reported outcomes were computed from these diaries. Assessments were performed before, during and after the aerobic exercise

treatment. The total follow-up period ranged from 8 weeks to 12 months. In one study no follow-up period was used [26].

Controls

Six studies compared the results of the intervention group with randomized control groups [24–29], only one study had an age-and gender-matched control group [25]. Patients with migraine included in the control groups received either no intervention [25], a treatment based on medication (25mg amitriptyline/day) [27], education [24], advice to maintain a habitual daily activity profile [26, 29], relaxation therapy or topiramate (25mg/week - max. 200mg/day) [28]. In comparison to topiramate treatment, aerobic exercise and relaxation therapy were found to be equally effective regarding the attack frequency and the number of migraine days [28]. Concerning pain intensity, a greater reduction was reported favoring the topiramate group (37%) compared to aerobic exercise (10%) and relaxation therapy (9%) [28]. Moreover, combining amitriptyline and aerobic exercise had a significant effect on the number of migraine days, pain intensity and attack duration compared to amitriptyline treatment alone [27]. In comparison to maintaining daily physical activity and moderate continuous training, high intensity interval training showed larger, although statistically not significant, effect sizes for decreasing the number of migraine days per month [26]. Migraine education and self-management showed an equal effect on pain intensity and attack duration compared to a behavioral weight loss program [24].

Effect of aerobic exercise on the number of migraine days

Three out of six studies reported a significant reduction in the number of migraine days ranging from 22% to 78% [25, 27, 29]. Data-pooling of four studies [25, 26, 28, 29], with a total of 176 patients, show a significant effect of aerobic exercise on the number of migraine days at 10–12 weeks ($p = 0.0006$). A mean reduction of 0.6 ± 0.3 migraine days/month was found favoring the intervention group (Fig. 4). These studies were pooled based on similar diagnosis of migraine, intervention and outcome. Hanssen et al. [26] was mentioned twice as both moderate continuous aerobic training and high-intensity interval training were compared to the control group.

Effect of aerobic exercise on pain intensity and attack duration

Three studies [25, 27, 29] reported a reduction of 20% up to 54% in pain intensity after aerobic exercise combined with a decrease in attack duration of 20–27%. These outcomes (pain intensity and duration) were not pooled due to the heterogeneity of the used units of outcome measurement. For instance duration of attacks was measured in hours per attack [29], hours per month [25] or in different time intervals (6h–12h-18h-24h) [27]. Additionally, Varkey et al. [28] showed a decrease in the use of analgesic medication (71%) in the topiramate group, 6 months after treatment according to the per-protocol analysis. This result was not found in the intention-to-treat analysis. Two other studies [27, 29] measured and reported acute medication use, but no significant between-group differences were found. The first study found no significant differences in acute medication use when comparing a pharmacological treatment to a combined pharmacological and exercise treatment [27]. In the second study, acute medication use decreased non-significantly in the exercise group compared to a group maintaining normal daily activity [29].

Discussion

The aim of this systematic review was to explore the effect of aerobic exercise in patients with migraine on the number of migraine days, attack duration and pain intensity. Moderate quality evidence indicates that in patients with migraine aerobic exercise therapy decreases the number of migraine days. Low quality evidence indicates that aerobic exercise can decrease pain intensity or duration of migraine attacks. To our knowledge, the only other existing review on this topic was published in 2008 [19]. However, Busch et al. [19] acknowledged themselves that none of the included studies in this narrative review met valid criteria of good clinical practice. Therefore, a systematic review was conducted to explore the effects of aerobic exercise using higher quality studies.

Five RCTs [24, 26–29] and one CCT [25] published after 2004, reporting on the effect of aerobic exercise in patients with migraine, were included in this review. The risk of bias of the included trials was low to moderate with a high

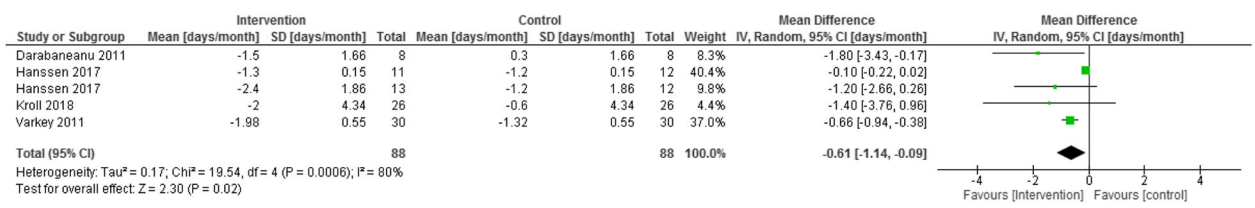

Fig. 4 Pooled data comparing intervention and control group on the number of migraine days (days/month)

risk of performance and detection bias due to a lack of blinding of participants, personnel and outcome assessors.

Based on our meta-analysis, there is moderate evidence that aerobic exercise can lead to a decrease of 0.6 migraine days per month. The clinical relevance of this finding is low. However, it may be of interest if it is added to the value of current usual care. Furthermore, higher training intensities might provide interesting results as the training intensity in the included studies was low. This finding is in line with the findings of Busch et al. [19], who found a decrease of 3.7 migraine days per month. However, this result is based on a single report. In their review two RCTs [32, 33] and six single cohort studies [34–38] were included. However, as mentioned above none of those studies met valid criteria of good clinical practice [19]. In 2015, Luedtke et al. [39] evaluated interventions used by physiotherapists for patients with headache, such as aerobic exercise. Based on six studies, of which the data of one study was not estimable, their meta-analysis indicated a reduction of 2.99 days with migraine, although not significant ($p = 0.23$). In contrast, pooling of data from one CCT [25] and three RCTs [26, 28, 29] in this review shows a significant reduction of migraine days per month. We obtained the mean reduction by using the difference between pre- and post-intervention data. Additionally, all studies provided a long-term exercise protocol for at least ten weeks. This can explain the difference between our results and those in the systematic review of Luedtke et al. [39].

Interestingly, we found that topiramate and tricyclic antidepressants show similar results compared to aerobic exercise in decreasing the number of migraine days per month [28]. Aerobic exercise appears to be a valuable alternative, taking into account that side effects are common with a pharmacological treatment, such as weight changes, memory loss and fatigue [3, 40, 41].

Regarding duration of migraine attacks small to moderate reductions (20–27%) were reported [25, 27, 29], such as a reduction of 20 migraine hours post-treatment in one study [29]. This result is similar to the conclusions of Busch et al. [19]. Due to the heterogeneity of the units of the outcome measurement, interpreting raw data was difficult.

The results of the present review suggest that aerobic exercise can reduce pain intensity (20–54%) in patients with migraine [25, 27, 29], confirming the findings of Busch et al. [19]. The analgesic effects on central and peripheral levels have already been reported [15, 16, 18] but the heterogeneity of the units of the outcome measurement might have biased the results.

Additionally, there is low quality evidence that patients use less analgesic medication as an effect of aerobic exercise [28]. These results contradict the findings of Busch et al. [19], who concluded that analgesic medication intake was not altered by aerobic exercise.

Our review shows low quality evidence for greater treatment effects by combining aerobic exercise with amitriptyline [27].

While our review focuses on the influence of aerobic exercise on clinical parameters of migraine, its underlying mechanisms were beyond the scope of our review. Other reviews provide some hypotheses regarding these mechanisms [9, 11, 42, 43].

This review's patient population consisted of 88% females and 12% males. This is an expected distribution, as a 3:1 female:male ratio is reported in other epidemiologic studies [44]. In the current review, the inclusion criteria were: patients with migraine with and without aura according to the ICHD-II. A similar diagnosis is a major strength of this review as it ensures a homogeneous group and allows pooling of data. Additionally, in all studies patients with and without aura were included. Therefore, patients can easily be compared between studies. However, the control groups consisted of usual care treatments (topiramate and amitriptyline) [27, 28], alternative treatments (relaxation, maintain daily physical activity and migraine education) [24, 26, 28, 29] and no treatment [25]. This may have influenced the comparability, since there might be differences between control groups that received treatment (active controls) and control groups that received no treatment at all (passive controls). Interestingly, no significant difference is found if active controls are compared to aerobic exercise (topiramate, relaxation, migraine education and maintaining habitual function with standard physical activity recommendations) [24, 26, 28]. One can state that these active groups are equally effective compared to aerobic exercise. Significant treatment effects are found, when comparing aerobic exercise with no treatment or maintaining habitual function [25, 29].

Dropout rate in total was high in four of the included studies, respectively 28% [29], 33% [24] and 50% [25, 28]. The most important reason for withdrawal of participants was lack of time to get to and attend three supervised exercise training sessions per week. Since stress is an important trigger for migraine attacks, Varkey et al. [45] suggested home-based training programs to improve compliance and to reduce stress levels [46]. On the other hand, home-based training might be less therapy compliant, which could lead to false interpretation. Positive findings have been suggested for supervised home-based programs [19, 35, 45], although these last two showed a high risk of bias due to the lack of a control group and subjective endpoints.

Our review population is mainly comprised of untrained patients with migraine. This selection of subjects might have biased the results as this does not necessarily

represent a typical migraine population [19]. A moderate intensity level training was chosen to avoid exercise-induced migraine and other negative side effects [28, 29]. Aerobic training was recommended by the American College of Sport Medicine (ACSM) [47] as training 3–5 days a week, 20–60 min, with an intensity of 55/65–90% of maximum heart rate. In this review patients exercised according to the ACSM recommendations of aerobic training for a period of 10 weeks or more with moderate intensity [47]. Positive findings were measured in the intervention group and no negative side effects were registered in any of the trials. Larger exercise volumes, such as high-intensity training or higher exercise duration, seem to be related to larger reductions in the number of migraine days in the intervention group [25, 26, 29].

Recommendations for further research

Major gaps exist in the current knowledge on the effect of aerobic exercise on patients with migraine. Further research to study the effects reported in this systematic review are mandatory to unravel the mechanisms of physical training on migraine [11, 42]. We recommend that future studies use uniform outcome measures of headache characteristics as recommended by the International Headache Society [48], use blinded assessors, provide homogeneous patient samples, design randomized controlled trials comparing aerobic training in patients with migraine with and without supervision to explore the difference between both protocol types, investigate the effect of larger exercise volumes as an intervention protocol and finally investigate the combined effect of pharmacological treatment and aerobic exercise in comparison to a pharmacological treatment alone.

Conclusion

Based on the results of this review, there is a moderate evidence that aerobic exercise decreases the the number of migraine days [25, 26, 28, 29]. Additionally, there is low quality evidence that aerobic exercise decreases the attack duration and pain intensity [25, 27, 29].

Additional files

Additional file 1: PRISMA checklist. (DOCX 17 kb)
Additional file 2: Search string. (DOCX 12 kb)

Competing interests

The authors declare that they have no competing interests.

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CGRP and migraine from a cardiovascular point of view: what do we expect from blocking CGRP?

Abstract

Calcitonin gene-related peptide (CGRP) is a neuropeptide with a pivotal role in the pathophysiology of migraine. Blockade of CGRP is a new therapeutic target for patients with migraine. CGRP and its receptors are distributed not only in the central and peripheral nervous system but also in the cardiovascular system, both in blood vessels and in the heart. We reviewed the current evidence on the role of CGRP in the cardiovascular system in order to understand the possible short- and long-term effect of CGRP blockade with monoclonal antibodies in migraineurs. In physiological conditions, CGRP has important vasodilating effects and is thought to protect organs from ischemia. Despite the aforementioned cardiovascular implication, preventive treatment with CGRP antibodies has shown no relevant cardiovascular side effects. Results from long-term trials and from real life are now needed.

Keywords: CGRP, CGRP antibody, Migraine treatment, Cardiovascular

Introduction

Migraine is one of the leading chronic neurological disorders, considered among the top five causes of long-term disability and affecting 15% of the population, mainly women [1, 2]. Treatments for migraine can be divided into abortive and prophylactic therapy. Calcitonin gene-related peptide (CGRP) blockade has emerged as a therapeutic target for migraine. CGRP is a neuropeptide released from perivascular nerve fibers after trigeminal nerve activation performing a pivotal role in the pathophysiology of migraine [3, 4]. In recent years, monoclonal antibodies against CGRP and its receptors have been developed and tested in clinical trials involving migraine patients. The site of action of these antibodies is still debated. Because they are large molecules, they have limited potential to pass the blood-brain barrier (BBB) and may act at the peripheral level. However, some studies have shown that brain structures involved in the pathophysiology of migraine (e.g. trigeminal ganglion and the paraventricular structures within the brain stem) are not fully protected by the BBB [5–7], hence effective migraine treatment drugs need not to pass through the BBB. Furthermore, the antimigraine action site may reside in areas not protected by the BBB such as the intra- and extracranial vessels, dural mast cells, and the trigeminal system [3]. Interestingly, CGRP receptors are located not only in the central and peripheral nervous system but also in the cardiovascular system including blood vessels and the heart [8]. CGRP acts as a very potent vasodilator and plays an important role in regulating vascular resistance and regional organ blood flow in physiological and also during pathological conditions like cerebral or cardiac ischemia [7, 9–11]. We reviewed the current evidence on the role of CGRP in the cardiovascular system to understand the possible short- and long-term effect of CGRP blockade with monoclonal antibodies in migraineurs.

Methods of review

Two independent reviewers conducted an independent search on PubMed on July 20th, 2018 using the search terms "cgrp" AND "cardiovascular system" OR "cardiovascular" AND "system". This search generated 1585 abstracts, which were reviewed independently, and articles were selected on the basis of relevance to the present topic. Discrepancies between investigators were rechecked and, if necessary, discussed with a third investigator until consensus was achieved. Every author added additional papers when needed in their respective section. The final reference list was generated on the basis of originality and relevance to the topic of this Review.

Calcitonin gene-related peptide and CGRP receptors

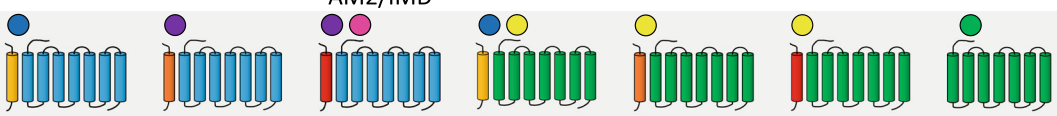
CGRP, a peptide with 37 amino acid residues, exists in humans in two isoforms, α and βCGRP, otherwise known as CGRP I and II. Alternative splicing of the *CACL1* gene (calcitonin gene) produces, most prominently in the central and peripheral nervous system, αCGRP [12, 13]. Transcription of the *CACLII* gene leads to βCGRP, most abundantly found in the enteric sensory system [12, 13]. αCGRP and βCGRP share > 90% homology in humans (with only three amino acids being different) [14]. Therefore, it is logical that their biological activity is similar. CGRP is expressed in the peripheral nervous system in thin unmyelinated C fibers, and at numerous sites in the central nervous system [4, 15–17].The synthesis and release of CGRP can be triggered by activation of the transient receptor potential vanilloid subfamily member 1 (TRPV1). One of the ligands of TRPV1, capsaicin, was first used to demonstrate the release of CGRP from sensory neurons [10]. However, the synthesis and release of CGRP is mediated by many factors, which are still being investigated.

CGRP acts by activating multiple receptors [18–20]. The functional CGRP receptor consists of three components: calcitonin-like receptor (CLR), receptor component protein (RCP) which defines the G-protein to which the receptor binds, and receptor activity-modifying protein 1 (RAMP1) [19–21]. RCP links the receptor to an intracellular C protein-mediated signaling pathway, which increases cyclic adenosine monophosphate (cAMP) levels [22]. For updated classification and nomenclature of calcitonin/CGRP family of peptides and receptors see Table 1. CGRP receptors are also present on the smooth muscle cells of human cranial and coronary arteries [9, 23]. It remains unclear if there is a difference in the expression of CGRP receptors between cranial and coronary arteries, but functional studies suggest a higher expression of CGRP receptors in cranial arteries. Receptor components of CGRP have also been identified in the trigeminal ganglion, cerebral cortex, hippocampus, thalamus, hypothalamus, brainstem, spinal cord and cerebellum [24–26]. As such, CGRP probably has both neural and vascular actions.

Endothelial dysfunction and CGRP in migraineurs

Various vascular mechanisms have been described in order to explain the role of CGRP in vasodilation of peripheral vascular beds. The presence of an NO- and endothelium-independent pathway, which leads to vascular relaxation, has been observed in smooth muscle cells of most tissues [27, 28]. However, CGRP also has the capability to stimulate the production of NO by acting via a receptor located on the endothelium. This endothelium-dependent relaxation pathway results in an accumulation of cAMP and production of NO through endothelial protein kinase A/endothelial NO Synthase (PKA/eNOS) signaling. Eventually, NO diffuses into adjacent smooth muscle cells and activates guanylate cyclase. This finally leads to the production of cGMP and relaxation of vessels [11, 28, 29]. The role of endothelium in migraine pathophysiology is still debated. Some studies indicate that migraineurs have an impaired

Table 1 Current classification of human calcitonin-family receptors, subunit composition and respective ligands

Receptor name	CGRP	AM_1	AM_2	AMY_1	AMY_2	AMY_3	CTR
Receptor composition	CLR + RAMP1	CLR + RAMP2	CLR + RAMP3	CTR + RAMP1	CTR + RAMP2	CTR + RAMP3	CTR
Ligand	CGRP	AM	AM - AM2/IMD	CGRP - AMY	AMY	AMY	CT
Structure							

CGRP Calcitonin Gene-Related Peptide, *AM* Adrenomedullin, *AMY* Amylin, *CTR* Calcitonin Receptor, *CLR* Calcitonin receptor-like receptor, *RAMP* receptor activity-modifying proteins, *AM2/IMD* Adrenomedullin 2/Intermedin

arterial and endothelial function as compared to non-migraineurs [30]. On the contrary, a recent study suggested that the contribution of endothelium to CGRP-induced vasodilation may not be significant [31]. In fact, cutaneous microvascular sensitivity to endothelial and non-endothelial donors including CGRP showed no difference between a group of patients with migraine compared to controls [32]. It has been speculated that alterations at the endothelial level may contribute to the increased risk (approximately 50%) of several cardiovascular diseases such as ischemic and hemorrhagic stroke, angina and myocardial infarction, which has been observed in several studies that compared migraineurs (with aura) to non-migraineurs [33–38].

Physiological and pathological influence of CGRP on the cardiovascular system

CGRP release induces relaxation of smooth muscle cells due to an increase in cAMP and leads to activation of protein kinase A, which phosphorylates and opens potassium channels [39, 40]. In blood vessels, CGRP acts as an extremely potent vasodilator when compared to several known vasodilators such as histamine, prostaglandin E2 and substance P [41]. Even so, CGRP seems to have no pivotal role in the physiological regulation of systemic blood pressure. For instance, blocking CGRP does not affect systemic blood pressure in healthy volunteers [42]. In the heart, CGRP is localized in sensory nerve fibers and around peripheral arteries [9]. There are specific binding sites for CGRP linked to stimulation of adenylate cyclase activity more concentrated in the atrium [43]. In both rats and humans, in addition to its vasodilator effect, intravenous CGRP administration has been shown to cause positive inotropic and chronotropic effects on the heart [44–47]. In physiological conditions, CGRP might act on a more local level, regulating vascular responsiveness and protecting organs from injury. Thus, CGRP may have a cardiovascular protective role. In pathophysiological situations, like hypertension, conflicting observations have been made. Both decreased, increased and unchanged plasma levels of CGRP have been observed in patients with essential hypertension [48, 49]. While CGRP does not seem to be involved in the physiological regulation of blood pressure, it has a protective role against the development of hypertension. It exerts its action mainly directly on smooth muscle cells in the vessel wall, most prominently in the microvasculature, which is responsible for the majority of the peripheral vascular resistance and thus, the blood pressure [9, 50].

Moreover, CGRP given intravenously to patients with congestive heart failure improved myocardial contractility without any consistent change in arterial pressure or heart rate [51]. CGRP causes beneficial effects on physiological cardiac hypertrophy helping the heart to distinguish physiological, exercise-induced from pathological stresses [52].

In addition, CGRP may play an important role in mediation of ischemic preconditioning, the phenomenon in which a tissue is rendered resistant to the deleterious effects of prolonged ischemia. Capsaicin, which evokes CGRP release from sensory nerves, is reported to protect against myocardial injury by ischemia-reperfusion in the isolated perfused rat heart [53]. Moreover, pretreatment with CGRP for 5 min produces a significant protective effect on the ischemic myocardium, as shown by the enhanced post-ischemic myocardial function, the reduced incidence of ventricular arrhythmia, and the attenuated release of creatine phosphate kinase [54]. Some studies have also suggested that the protective role of CGRP against ischemia may be due to induced vasodilation [55]. In the setting of brain ischemia, it might reduce the extent of the infarct zone [56], while in the case of subarachnoid hemorrhage, there is evidence that CGRP is protective against cerebral vasospasm [57–59]. CGRP might be protective also in the setting of chronic cerebrovascular disease (as induced by bilateral carotid stenosis) and the subsequent neuronal injury and cognitive impairment [56].

Sex differences and CGRP pathophysiology

CGRP plasma levels are higher in women than in men [60]. Cardiovascular benefits of CGRP, such as vasodilatory and hypotensive effects on the arteries [61] and the positive inotropic effects on the myocardium are strongly influenced by fluctuations in female sex hormone levels [62]. Furthermore, sex hormone receptors are found in the trigeminovascular and cardiovascular system and, therefore, it is likely that there is an interaction between female sex hormones and CGRP, but the exact mechanism is still not fully understood [63, 64]. In animal models, females had higher CGRP levels in the medulla and lower expression of CLR, RAMP1 and RCP-encoding mRNA in tissues, compared to males, suggesting that CGRP receptor synthesis, expression or release in the trigeminovascular system may be regulated by fluctuating female sex hormones. Numerous animal and human studies have shown that cyclic fluctuations of ovarian hormones (mainly estrogen) modulate CGRP both in peripheral and central nervous system [65–67]. It is, therefore, reasonable to think that females, in particular, are sensitive to therapeutic effects of CGRP blockade, but also to adverse events. In clinical practice, it would be useful to know whether female migraineurs have an additional higher cardiovascular risk if they are prescribed CGRP monoclonal antibodies for the treatment of migraine. Future studies should assess possible sex differences in the benefits and harms of drugs acting on the CGRP and its receptor.

Blocking CGRP

The blockade of the CGRP system has been obtained by different molecules: non-peptide CGRP antagonists also known as "gepants" (olcegepant, telcagepant, ubrogepant, atogepant), monoclonal antibodies against CGRP (eptinezumab, fremanezumab, galcanezumab) and monoclonal antibodies against CGRP receptor (erenumab).

Gepants have demonstrated efficacy in relieving migraine in clinical trials and do not cause direct vasoconstriction. However, olcegepant had to be administered intravenously due to its low oral bioavailability [68, 69]. Encouraged by the efficacy of blocking CGRP for the treatment of migraine, monoclonal antibodies able to block either CGRP or its receptor were developed. CGRP antibodies have a slower onset of action compared with the CGRP receptor antagonists, which is consistent with the idea of a slower penetration into the interstitial space of the vascular smooth muscle tissue. The inhibition is evident one week after dosing [70]. Moreover, CGRP antibodies might scavenge CGRP for up to 1.5 months [7].

Short-term effects of blocking CGRP

The cardiovascular safety of short-term CGRP blockade has been widely explored for both CGRP antagonists and for monoclonal antibodies. In animal models, several studies conducted on non-peptidic CGRP-R antagonists (olcegepant) evidenced that short-term blockade of CGRP have no effects on hemodynamic parameters such as heart rate, blood pressure, cardiac output, coronary flow or severity of ischemia were observed in different animal species [71–73]. CGRP antagonism is safe in healthy volunteers; a study demonstrated that the administration of telcagepant at supra-therapeutic dosage did not induce vasoconstriction both in peripheral and central vascular beds in healthy men [74]. Moreover, this drug did not influence treadmill-exercise-time in patients with stable angina [75].

Clinical trials of single-doses of oral telcagepant administered for acute treatment of migraine showed a total absence of cardiovascular side effects in migraine patients [76, 77]. Only minor adverse events were registered (dry mouth, somnolence, dizziness, nausea, fatigue) [78].

Since the half-life of monoclonal antibodies is longer (21–50 days) [79] than that of non-peptidic CGRP antagonists, the blockade of CGRP has a longer duration. In rats CGRP blocking antibodies inhibit the neurogenic vasodilation, confirming the role of these molecules in treating migraine, but no effect on heart rate and arterial blood pressure was observed [70]. Similar results were obtained using fremanezumab in monkeys, where the effect of single or multiple (once weekly for 14 weeks) injections on cardiovascular parameters were evaluated. No meaningful modifications of ECG parameters, heart rate, and systolic blood pressure were observed in both situations [80]. In another trial, healthy women over 40 years old (mean age 56 years) were monitored for 24 weeks after administration of a single dose of fremanezumab at different dosages. No changes in ECG parameters, nor heart rate or blood pressure were registered [81].

Safety and tolerability data from clinical trials are encouraging for the anti-CGRP monoclonal antibodies for the treatment of both episodic and chronic migraine. All phase II and phase III clinical trials completed so far for the four developed monoclonal antibodies did not show any safety problem concerning the cardiovascular system [82, 83]. It must be noted that the patients recruited for clinical trials were young (age range 18–65, with a mean of about 40 years) usually without any significant cardiovascular disease. Therefore, the safety profile of this class of drugs in high-risk patients has to be specifically addressed. A randomized, double-blind placebo-controlled study was performed for studying the cardiovascular effect of erenumab in patients with stable angina. In particular, the investigators evaluated the impact of a dose of the drug (iv infusion of 140 mg) on exercise time during a treadmill test. There was no decrease in treadmill test, so they concluded that the inhibition of CGRP receptor does not worsen myocardial ischemia [84]. One major criticism about this study regards the population selected, which was composed of non-migraineurs; data indicate that migraineurs are at risk for cardiovascular events [34, 36]. Thus, safety of anti-CGRP monoclonal antibodies in migraineurs may be different from that of the general population. Additionally, in that study most patients (80%) were males, while migraine is more prevalent in women. As previously discussed, sex hormones influence the activity of CGRP on the vascular tone and female migraineurs are at increased risk of myocardial infarction [85], possibly exposing them to a specific sensitivity to CGRP blockade [77].

Long-term effects of blocking CGRP

Pre-registration trials are mostly limited to a maximum of 6 months. Considering the role of CGRP in cardiovascular physiology and in the pathophysiology, this time frame could not be enough to exclude effects of blockade in the long run. There is just one published article about a trial longer than 6 months using anti-CGRP drugs [86]. The interim analysis after one year of open label extension of an erenumab trial (EudraCT 2012–005331-90, NCT01952574) among 383 subjects exposed for a median of 575 days reported one case of death in a 52-year-old man with pre-existing cardiovascular risk factors (hypertension, hypercholesterolemia, obesity, familial history) and post-mortem evidence of severe coronary atherosclerosis and use of sympathomimetics. A case of transient exercise-induced myocardial ischemia during a treadmill

test was confounded by sumatriptan intake 4 h prior to the event [86]. Considering the presence of confounding factors, these adverse events may be not related to the treatment. However, a limitation of the study is the lack of a placebo group, which makes it difficult to differentiate spontaneously occurring adverse events from adverse events due to erenumab.

In all short- and long-term studies published, investigators have not observed any hypertensive effect of anti-CGRP drugs, nor were any negative effects observed regarding the development or aggravation of cardiac failure, although this last issue was not specifically addressed, there was no specific monitoring, and it is not clear if any patient with heart failure was treated. Moreover, the time frame might be not enough to observe a clinical effect of organ remodeling.

Regarding the cerebrovascular risk of anti-CGRP drugs, no safety issues have emerged from all the trials completed so far.

Conclusions

In conclusion, CGRP plays an important role in migraine but also in physiological and pathological cardiovascular conditions. We can speculate that CGRP may act as a link between the brain and the heart. Data emerging from trials with CGRP antibodies suggest that this specific blockade of the CGRP pathway is a safe treatment. To our knowledge, no serious adverse events have been reported since approval of anti-CGRP monoclonal antibodies for migraine treatment in May 2018. However, results from long-term trials and real life are particularly awaited in order confirm these encouraging data on the long-term safety of the new migraine preventive drugs.

Competing interests

The authors declare that they have no competing interests related to the content of the manuscript.

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Patterns of medicinal cannabis use, strain analysis, and substitution effect among patients with migraine, headache, arthritis, and chronic pain in a medicinal cannabis cohort

Abstract

Background: Medicinal cannabis registries typically report pain as the most common reason for use. It would be clinically useful to identify patterns of cannabis treatment in migraine and headache, as compared to arthritis and chronic pain, and to analyze preferred cannabis strains, biochemical profiles, and prescription medication substitutions with cannabis.

Methods: Via electronic survey in medicinal cannabis patients with headache, arthritis, and chronic pain, demographics and patterns of cannabis use including methods, frequency, quantity, preferred strains, cannabinoid and terpene profiles, and prescription substitutions were recorded. Cannabis use for migraine among headache patients was assessed via the ID Migraine™ questionnaire, a validated screen used to predict the probability of migraine.

Results: Of 2032 patients, 21 illnesses were treated with cannabis. Pain syndromes accounted for 42.4% ($n = 861$) overall; chronic pain 29.4% ($n = 598$;), arthritis 9.3% ($n = 188$), and headache 3.7% ($n = 75$;). Across all 21 illnesses, headache was a symptom treated with cannabis in 24.9% ($n = 505$). These patients were given the ID Migraine™ questionnaire, with 68% ($n = 343$) giving 3 "Yes" responses, 20% ($n = 102$) giving 2 "Yes" responses (97% and 93% probability of migraine, respectively). Therefore, 88% ($n = 445$) of headache patients were treating probable migraine with cannabis. Hybrid strains were most preferred across all pain subtypes, with "OG Shark" the most preferred strain in the ID Migraine™ and headache groups. Many pain patients substituted prescription medications with cannabis (41.2–59.5%), most commonly opiates/opioids (40.5–72.8%). Prescription substitution in headache patients included opiates/opioids (43.4%), anti-depressant/anti-anxiety (39%), NSAIDs (21%), triptans (8.1%), anti-convulsants (7.7%), muscle relaxers (7%), ergots (0.4%).

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Conclusions: Chronic pain was the most common reason for cannabis use, consistent with most registries. The majority of headache patients treating with cannabis were positive for migraine. Hybrid strains were preferred in ID Migraine™, headache, and most pain groups, with "OG Shark", a high THC (Δ9-tetrahydrocannabinol)/THCA (tetrahydrocannabinolic acid), low CBD (cannabidiol)/CBDA (cannabidiolic acid), strain with predominant terpenes β-caryophyllene and β-myrcene, most preferred in the headache and ID Migraine™ groups. This could reflect the potent analgesic, anti-inflammatory, and anti-emetic properties of THC, with anti-inflammatory and analgesic properties of β-caryophyllene and β-myrcene. Opiates/opioids were most commonly substituted with cannabis. Prospective studies are needed, but results may provide early insight into optimizing crossbred cannabis strains, synergistic biochemical profiles, dosing, and patterns of use in the treatment of headache, migraine, and chronic pain syndromes.

Keywords: Cannabis, Cannabinoids, Marijuana, CBD, Cannabidiol, THC, Δ^9-tetrahydrocannabinol, Migraine, Headache, Terpenes, Arthritis, Pain

Background

The legal use of medicinal cannabis continues to increase globally, including the United States. At the time of this writing, there are currently 29 states which have legalized medicinal cannabis, 9 states and Washington DC which have legalized both medicinal and recreational cannabis use, and 18 states which have legalized cannabidiol (CBD)-only bills.

The use of medicinal cannabis for a multitude of health maladies, particularly chronic pain, has been well described through ancient, historical, and current times, and well supported through the medical literature [1–28]. In 2017, The National Academies of Sciences, Engineering, and Medicine published a statement that the use of cannabis for the treatment of pain is supported by well-controlled clinical trials and that there is substantial evidence that cannabis is an effective treatment for chronic pain in adults [24]. In 2014, the Canadian Pain Society revised their consensus statement to recommend cannabinoids as a third-level therapy for chronic neuropathic pain given the evidence of cannabinoid efficacy in the treatment of pain with a combined number needed to treat (NNT) of 3.4 [25]. Most medicinal cannabis registries report that chronic pain is the most common indication for use [29–39]. However, most of these registries do not further differentiate chronic pain into different pain subsets.

Supporting evidence also exists for cannabis/cannabinoids in the treatment of migraine and/or chronic migraine [1, 40–56], cluster headache [56–59], chronic headaches [13, 44, 60, 61], medication overuse headache [62], idiopathic intracranial hypertension [63], and multiple sclerosis associated trigeminal neuralgia [64]. Publications detailing this headache, migraine, and facial pain literature, as well as described mechanisms of pain relief with cannabis and cannabinoids are available and should be reviewed, but are beyond the scope of this paper [1, 2, 28, 51, 65]. At the time of this writing, the limited supporting headache literature consists of one retrospective analysis, numerous case series, case studies, and case reports, clinical/anecdotal reports, and surveys. There are no placebo-controlled studies of cannabis for headache disorders, although a multicenter, double-blind, placebo-controlled study evaluating efficacy and safety of a synthetic Δ^9-tetrahydrocannabinol (THC), Dronabinol, in a metered dose inhaler for the treatment of migraine with and without aura has been completed, but results not available [66]. There are only two prospective trials containing a control group evaluating the use of cannabinoids in the treatment of headache disorders, specifically chronic migraine, cluster headache, and medication overuse headache [56, 62].

The first of these two prospective trials was a randomized, double-blind, active-controlled crossover trial with treatment refractory medication overuse headache (MOH) with daily analgesic intake for at least 5 years and several failed detoxification attempts. Patients completed a course of either Ibuprofen 400 mg or Nabilone 0.5 mg daily for 8 weeks, had a 1 week washout, then a second 8 weeks of the other medication. Results showed that Nabilone 0.5 mg daily, a synthetic cannabinoid, was superior in reducing daily analgesic intake, pain intensity, level of medication dependence, and improved quality of life in these patients [62].

The second prospective trial evaluated the use of cannabinoids as both a prophylaxis and acute treatment for both chronic migraine and chronic cluster headache [56]. Patients were given one of two compounds containing 19% THC or a combination of 0.4% THC + 9% CBD. In phase 1, dose finding observations to determine effective dosing was performed with a group of 48 chronic migraineurs. It was found that doses less than 100 mg produced no benefit, while an oral dose of 200 mg administered during a migraine attack decreased acute pain intensity by 55%, which was the dose used in phase 2. In phase 2, chronic migraine patients were assigned to 3 months prophylaxis treatment with either

25 mg per day of Amitriptyline or THC + CBD 200 mg per day. Chronic cluster headache patients were assigned to 1 month prophylaxis treatment with either Verapamil 480 mg per day or THC + CBD 200 mg per day. For acute pain attacks, additional dosing of THC + CBD 200 mg was allowed in both groups. In the migraine patients, the THC + CBD 200 mg prophylaxis provided a 40.4% improvement versus 40.1% with Amitriptyline. In the cluster headache patients, the THC + CBD 200 mg prophylaxis gave minimal to no benefit. Additional acute THC + CBD 200 mg dosing decreased pain intensity in migraine patients by 43.5%. This same result was seen in cluster headache patients, but only if they had a history of migraine in childhood. In cluster headache patients without a previous history of childhood migraine, the additional THC-CBD 200 mg abortive treatment provided no benefit as an acute treatment.

It is unclear whether certain types of pain may respond better to certain cannabis strains with specific combinations of cannabinoids, terpenes, or other biochemical properties. There have been a multitude of studies showing benefit in many forms of chronic pain, but there have been no studies attempting to differentiate which types and strains of cannabis along with associated compositions of cannabinoids and terpenes may be more effective for certain subsets of pain. This information would be of great clinical use in providing direction for treatment recommendations by healthcare providers.

Methods

Appropriate Investigational Review Boards approved the survey. A French and English electronic survey was sent to 16,675 Tilray medicinal cannabis patients. Tilray is a federally authorized medical cannabis production, distribution, and research company in Nanaimo, British Columbia. Data gathering was performed with REDCap (Research Electronic Data Capture), a HIPAA and PIPEDA compliant secure web application for building and managing online surveys and databases. A $10 account credit was offered to each patient completing the online survey, funded by Tilray. There was a response of 3405 (3390 English and 15 French), 2032 of which provided a verifiable Tilray patient number and were therefore included in the final analysis. The responses represent 12% of those reached. Recruitment was deliberately halted at 2000 (overlap with additional 32 subjects represents participants who were in the middle of completing the survey when it was halted). The survey launched at 9 AM PST on Monday January 9th 2017 and closed on Wednesday January 11th 2017 at 5 PM PST. The limit to responses was due to financial constraints, and patients were informed that the survey would be available for a two-week period or until limit was reached, whichever came first.

An estimation of migraine prevalence among those surveyed was obtained by incorporating the ID Migraine™ questionnaire [67] into the survey, which is used to predict the probability of migraine. In the ID Migraine™ questionnaire, the patient is given 3 questions. If the patient answers "Yes" to 3 of these questions, there is a 97% chance they have migraine. If they answer "Yes" to 2 of these questions, there is a 93% chance they have migraine. The questions are: 1) Have your headaches interfered with your ability to work, study, or do what you needed to do? 2) Have you felt nauseated or sick to your stomach when you have a headache? 3) Does light bother you when you have a headache (a lot more than when you don't have a headache)?

Patients were asked a multitude of additional questions involving demographics, primary illnesses and symptoms treated with cannabis, frequency and quantity of use, favorite cannabis types and strains, methods of use, and prescription drugs substituted with cannabis.

Patients who reported headache as the primary illness were compared with each patient group reporting a diagnosis other than headache as the primary illness. Separately, patients who reported headache as the primary symptom (regardless of diagnosis) were compared with each patient group who both reported a diagnosis other than headache as the primary illness and also did not report headache as the primary symptom. Statistical methods were the same for each set of comparisons. Pearson chi-squared tests, or Fisher's exact tests where appropriate, were used to compare headache patients with each non-headache patient group, with regards to five cannabis strains: Hybrid, Indica, Sativa, 3:1 CBD:THC, and 1:1 CBD:THC. Significance for omnibus chi-squared tests was designated by $p < .05$. When omnibus chi-squared tests were found to be significant, pairwise comparisons were carried out using a Bonferroni correction. Given ten pairwise comparisons per omnibus test, significance for each pairwise comparison was indicated by $p < .005$. Methods chosen to control for multiple comparisons allow a moderately conservative level of control, and reflect the exploratory nature of the study. Analyses were two-tailed and performed using SAS Studio v 3.5.

Results

Of the 2032 patients included in the survey, 1271 (62.6%) were male, 758 (37.3%) were female, and 3 (0.15%) did not specify gender. Ages ranged from 9 to 85 years old, with an average age of 40. Reported ethnicities in the overall cohort revealed 1839 (90.5%) Caucasian, 62 (3.1%) Metis, 60 (3%) Aboriginal/First Nation, 39 (1.9%) Other, 37 (1.8%) South Asian (East Indian, Pakistani,

Sri Lankan, etc.), 35 (1.7%) Asian (Chinese, Japanese, Korean, Vietnamese, etc.), 25 (1.2%) Black (African, Caribbean, etc.), and 24 (1.2%) Hispanic (Mexican, Central American, South America, etc.), with some patients reporting more than one ethnicity. Relationship status showed 833 (41%) were married, 507 (25%) were single and never married, 274 (13.5%) were in a domestic partnership or civil union, 203 (10%) were single but cohabiting with a significant other, 132 (6.5%) were divorced, 64 (3.2%) were separated, and 19 (0.94%) were widowed. Habitation showed 883 (43.5%) to be living in an urban area, 795 (39.1%) in a suburban area, and 354 (17.4%) in a rural or remote area.

There were 21 primary illnesses that were reported as being treated with medicinal cannabis, as seen in Table 1. The subsets analyzed further were headache, chronic pain, and arthritis. Chronic pain was the most frequently reported primary illness for which medicinal cannabis was being used at 29.4% ($n = 598$), arthritis was 9.3% ($n = 188$), and headache was 3.7% ($n = 75$). Notably, when combined these three categories of pain syndromes accounted for 42.4% ($n = 861$) of the entire medicinal cannabis users.

Headache was then evaluated as a primary symptom being treated by medicinal cannabis across all primary illnesses (headache was the major symptom being treated with medicinal cannabis, among the primary illness categories), as seen in Table 2. There were 505 patients within the entire group surveyed (24.9%) who reported headache as a primary symptom for which they were using medicinal cannabis across all primary illness categories. Of these patients, 262 (51.9%) were male, 241 (47.7%) were female, and 2 (0.40%) did not specify gender. Ages ranged from 10 to 86 years old with an average age of 38. Reported ethnicities revealed 453 (89.7%) Caucasian, 23 (4.6%) Metis, 21 (4.2%) Aboriginal/First Nation, 12 (2.4%) Other, 11 (2.2%) Hispanic (Mexican, Central American, South America, etc.), 10 (2%) Asian (Chinese, Japanese, Korean, Vietnamese, etc.), 8 (1.6%) South Asian (East Indian, Pakistani, Sri Lankan, etc.), and 4 (0.8%) Black (African, Caribbean, etc.), with many patients reporting more than one ethnicity. Relationship status showed 181 (36%) were married, 125 (24.8%) were single and never married, 88 (17.4%) were in a domestic partnership or civil union, 62 (12.3%) were single but cohabiting with a significant other, 28 (5.5%) were divorced, 18 (3.6%) were separated, and 3 (0.6%) were

Table 1 Primary illness treated with medicinal cannabis

Primary Illness	Total	Male	Female	Unspecified
n	2032	1271 (62.6%)	758 (37.3%)	3 (0.15%)
Chronic Pain	598 (29.4%)	371 (62%)	227 (38%)	
Mental Health Condition	548 (27%)	319 (58.2%)	228 (41.6%)	1 (0.2%)
Insomnia/Sleep Disorder	198 (9.7%)	145 (73.2%)	53 (26.8%)	
Arthritis/Musculoskeletal	188 (9.3%)	112 (59.6%)	76 (40.4%)	
PTSD	93 (4.6%)	59 (63.4%)	33 (35.5%)	1 (1.1%)
Headache	75 (3.7%)	44 (58.7%)	31 (41.3)	
Gastrointestinal Disorder	62 (3.1%)	34 (54.8%)	28 (45.2%)	
Multiple sclerosis	45 (2.2%)	26 (57.8%)	19 (42.2%)	
Other	38 (1.9%)	23 (60.5%)	15 (39.5%)	
Cancer/Leukemia	35 (1.7%)	24 (68.6%)	11 (31.4%)	
Crohn's Disease	35 (1.7%)	27 (77.1%)	8 (22.9%)	
Brain Injury	24 (1.3%)	16 (66.7%)	8 (33.3%)	
Epilepsy/Seizure Disorder	21 (1.0%)	18 (85.7%)	3 (14.3%)	
Eating Disorder	20 (1.0%)	10 (50%)	10 (50%)	
Diabetes	16 (0.79%)	13 (81.3%)	3 (18.7%)	
Movement Disorder	10 (0.49%)	8 (80%)	1 (10%)	1 (10%)
AIDS/HIV	8 (0.39%)	7 (87.5%)	1 (12.5%)	
Hepatitis	6 (0.30%)	6 (100%)	0 (0%)	
Glaucoma	5 (0.25%)	5 (100%)	0 (0%)	
Osteoporosis	4 (0.20%)	3 (75%)	1 (25%)	
Skin Condition	3 (0.15%)	1 (33.3%)	2 (66.7%)	

Table 2 Headache as primary symptom treated with medicinal cannabis among various primary illnesses reported

Primary Illness	Total	Male	Female	Unspecified
n	505	262 (51.9%)	241 (47.7%)	2 (0.40%)
Chronic pain	148 (29.3%)	70 (47.3%)	78 (52.7%)	
Mental Health Condition	131 (25.9%)	65 (49.6%)	66 (50.4%)	
Headache	75 (14.9%)	44 (58.7%)	31 (41.3%)	
Insomnia	32 (6.3%)	25 (78.1%)	7 (21.9%)	
Arthritis/Musculoskeletal	29 (5.7%)	12 (41.4%)	17 (58.6%)	
PTSD	24 (4.8%)	9 (37.5%)	14 (58.3%)	1 (4.2%)
MS	13 (2.6%)	3 (23.1%)	10 (76.9%)	
Brain Injury	12 (2.4%)	8 (66.7%)	4 (33.3%)	
Gastrointestinal Disorder	11 (2.2%)	5 (45.5%)	6 (54.5%)	
Cancer/Leukemia	6 (1.2%)	3 (50%)	3 (50%)	
Movement Disorder	5 (1.0%)	4 (80%)	0 (0%)	1 (20%)
Other	4 (0.79%)	2 (50%)	2 (50%)	
Epilepsy/Seizure Disorder	3 (0.59%)	2 (66.7%)	1 (33.3%)	
Crohn's Disease	3 (0.59%)	3 (100%)	0 (0%)	
Diabetes	2 (0.40%)	1 (50%)	1 (50%)	
Glaucoma	2 (0.40%)	2 (100%)	0 (0%)	
Hepatitis	2 (0.40%)	2 (100%)	0 (0%)	
Eating Disorder	1 (0.20%)	1 (100%)	0 (0%)	
AIDS/HIV	1 (0.20%)	1 (100%)	0 (0%)	
Osteoporosis	1 (0.20%)	0 (0%)	1 (100%)	

widowed. Habitation showed 218 (43.2%) to be living in an urban area, 205 (40.6%) in a suburban area, and 82 (16.2%) in a rural or remote area. Chronic pain was the most common primary illness in which headache was reported to be a primary symptom being treated with medicinal cannabis (29.3%), followed by mental health condition (25.9%) and headache (14.9%).

The 505 patients who reported headache as a primary symptom being treated by medicinal cannabis were then analyzed to estimate how many of those patients had probable migraine, and thus, how many were using medicinal cannabis for probable migraine management. This data was obtained via responses to the ID Migraine™ questionnaire. There were 343 (68%) who gave 3 "Yes" responses, and 102 (20%) who gave 2 "Yes" responses. Based on these responses, 445 of these 505 patients (88%) had a very high probability between 93 and 97% that the headaches they were treating with medicinal cannabis represented migraine.

Data was collected among patients to determine the most commonly used and preferred types of cannabis, as well as preferred specific strains. The preferred types of cannabis included Indica, Sativa, Hybrid, 3:1 CBD:THC, or 1:1 CBD:THC. Indicas, Sativas and Hybrids were all high THC/low CBD strains or extracts, while 1:1 and 3:1 strains and extracts represent the CBD:THC ratio, and were considered high CBD strains. The Indica, Sativa, and Hybrid types were further divided into specific strains within each of these cannabis types.

There were 42 different preferred treatment strains reported by patients and these included: Afghani, Afghani CBD, Alien OG, Barbara Bud, Black Tuna, Blueberry, Bubba Kush, Cannatonic, CBD House Blend, Cheese, Churchill, Dig Weed, Elwyn, Green Cush, Girl Scout Cookies (GSC), Harmony, Headband, Hybrid House Blend, Indica House Blend, Island Sweet Skunk, Jack Herer, Jean Guy, Lemon Sour Diesel, Limonene House Blend, Mango, Master Kush, Myrcene Blend, OG Kush, OG Shark, Pinene House Blend, Pink Kush, Purple Kush, Rockstar, Sativa House Blend, Sirius, Strawberry Cough (SBC), Skywalker OG, Sour Diesel, Sweet Skunk CBD, Warlock CBD, Watermelon, and White Widow.

Preferred cannabis types and strains were first analyzed between the headache as primary symptom, headache as primary illness, chronic pain as primary illness, and arthritis as primary illness groups. Hybrid strains were the most commonly preferred cannabis types across all pain groups. However, when patients with headache as a primary symptom were excluded from the groups, the arthritis group preferred Indica strains, while the others still preferred Hybrid strains. The top 15 preferred cannabis strains within each of these pain groups are seen in Tables 3 and 5. Preferred cannabis types and

Table 3 Preferred medicinal cannabis types and strains among headache patients and probable migraineurs based on "Yes" responses on ID Migraine™ questionnaire

	Preferred Cannabis Type				
	Headache as primary illness (75)	3 Yes[a] (343)	2 Yes[b] (102)	3 + 2 Yes (445)	Headache as primary symptom (505)
Hybrid	26 (34.7%)	118 (34.4%)	35 (34.3%)	153 (34.4%)	165 (32.7%)
Indica	19 (25.3%)	106 (30.9%)	20 (19.6%)	126 (28.3%)	144 (28.5%)
Sativa	20 (26.7%)	76 (22.2%)	36 (35.3%)	112 (25.2%)	136 (26.9%)
3:1 CBD:THC	5 (6.7%)	22 (6.4%)	7 (6.9%)	29 (6.5%)	34 (6.7%)
1:1 CBD:THC	5 (6.7%)	20 (5.8%)	4 (3.9%)	24 (5.4%)	25 (5%)
No response	0 (0%)	1 (0.3%)	0 (0%)	1 (0.2%)	1 (0.2%)
	Preferred Cannabis Strains (Top 15)				
	Headache as primary illness	3 Yes	2 Yes	3 + 2 Yes	Headache as primary symptom
	Skywalker OG (7; 10.6%)	OG Shark (20; 8.4%)	OG Shark (9; 11%)	OG Shark (29; 8.9%)	OG Shark (34; 9.6%)
	Headband (5; 7.6%)	Afghani (19; 8.0%)	Skywalker OG (8; 9.8%)	Afghani (25; 7.7%)	Jean Guy (29; 8.2%)
	Cannatonic (5; 7.6%)	Jack Herer (19; 8.0%)	White Widow (8; 9.8%)	Skywalker OG (25; 7.7%)	Skywalker OG (28; 7.9%)
	Jack Herer (5; 7.6%)	Jean Guy (19; 8.0%)	Lemon Sour Diesel (7; 8.5%)	Lemon Sour Diesel (25; 7.7%)	Lemon Sour Diesel (28; 7.9%)
	Afghani (4; 6.1%)	Lemon Sour Diesel (18; 7.6%)	Afghani (6; 7.3%)	Jack Herer (24; 7.3%)	Afghani (26; 7.4%)
	Indica House Blend (4; 6.1%)	Skywalker OG (17; 7.1%)	Pink Kush (6; 7.3%)	Jean Guy (24; 7.3%)	White Widow (26; 7.4%)
	Rock Star (4; 6.1%)	Master Kush (16; 6.7%)	Island Sweet Skunk (6; 7.3%)	White Widow (24; 7.3%)	Jack Herer (26; 7.4%)
	Warlock CBD (3; 4.6%)	White Widow (16; 6.7%)	Jack Herer (5; 6.1%)	Pink Kush (21; 6.4%)	Pink Kush (22; 6.2%)
	Sweet Skunk CBD (3; 4.6%)	Sweet Skunk CBD (15; 6.3%)	Jean Guy (5; 6.1%)	Master Kush (20; 6.1%)	Sweet Skunk CBD (21; 5.9%)
	Jean Guy (3; 4.6%)	Pink Kush (15; 6.3%)	Headband (4; 4.9%)	Sweet Skunk CBD (18; 5.5%)	Island Sweet Skunk (21; 5.9%)
	Girl Scout Cookies (GSC) (3; 4.6%)	Headband (13; 5.5%)	Master Kush (4; 4.9%)	Headband (17; 5.2%)	Master Kush (21; 5.9%)
	OG Shark (2; 3%)	Cannatonic (13; 5.5%)	Sour Diesel (4; 4.9%)	Island Sweet Skunk (17; 5.2%)	Black Tuna (20; 5.7%)
	Black Tuna (2; 3%)	Warlock CBD (13; 5.5%)	Black Tuna (4; 4.9%)	Black Tuna (16; 4.9%)	Headband (19; 5.4%)
	Bubba Kush (2; 3%)	Blueberry (13; 5.5%)	Hybrid House Blend (3; 3.7%)	Warlock CBD (14; 4.3%)	Cannatonic (18; 5.1%)
	CBD House Blend (2; 3%), Elwyn (2; 3%), Island Sweet Skunk (2; 3%), Mango (2; 3%), Master Kush (2; 3%), Blueberry (2; 3%), Pink Kush (2; 3%)	Black Tuna (12; 5.0%)	Sweet Skunk CBD (3; 3.7%)	Cannatonic (14; 4.3%), Blueberry (14; 4.3%)	Hybrid House Blend (15; 4.2%)

[a]3 "Yes" responses = 97% probability of migraine
[b]2 "Yes" responses = 93% probability of migraine

strains were then analyzed in the positive ID Migraine™ patients who answered 3 "Yes" responses (343), 2 "Yes" responses (102), or combined 3 + 2 "Yes" responses (445) to the ID Migraine™ questionnaire. Thus, they were the most probable group of headache patients who were treating migraine with medicinal cannabis. Hybrid strains were the most commonly preferred cannabis types across the positive ID Migraine™ groups with the exception that the 2 "Yes" group had a slight preference for Sativa, followed by Hybrid strains. The top 15 preferred cannabis strains within each positive ID Migraine™ group are seen in Table 3. "OG Shark" was the most commonly preferred strain across all of the positive ID Migraine™ and headache as primary symptom groups. Quantification and comparison of the cannabinoids and terpenes present in these top 15 preferred strains is seen in Table 4. The cannabinoids analyzed were Δ^9-tetrahydrocannabinol (THC), tetrahydrocannabinolic acid (THCA), cannabidiol (CBD), and cannabidiolic acid (CBDA). The terpenes analyzed were α-pinene, β-myrcene, D-limonene, linalool, β-caryophyllene, humulene, trans-nerolidol, and bisabolol. Notably, "OG Shark", a high THC/THCA, low CBD/CBDA strain with β-caryophyllene followed by β-myrcene as the predominant terpenes, was the most preferred strain in both the positive ID Migraine™ and headache as primary symptom groups.

For further comparison purposes, preferred cannabis types and strains were also analyzed for the three most common non-pain subsets of patients, which included mental health condition/PTSD, insomnia/sleep disorder, gastrointestinal disorder/Crohn's Disease, and the overall patient cohort, as seen in Table 5. Indica strains were preferred in the insomnia/sleep disorders group, Sativa strains in the mental health condition/PTSD group, and Hybrid strains in the gastrointestinal disorder/Crohn's Disease group, regardless of whether patients with headache as a primary symptom were included or not. Table 6 shows these same groups, as well as the arthritis and chronic pain groups, with all groups excluding patients with headache as a primary symptom.

Statistical analysis was performed to determine if there were significant differences in preferred cannabis types reported by headache patients. The data were insufficient for statistical analysis of specific strain preferences. There were no statistically significant differences found between patients with headache as primary illness and those with chronic pain, arthritis, or mental health condition/PTSD. When compared to insomnia/sleep disorder patients, headache as primary illness patients were 7.7 times as likely to prefer 3:1 CBD:THC over Indica (OR 7.7, 95% CI 1.7-35.11, p = .003).

Patients with headache as primary symptom were 2.7 times as likely to prefer Sativa over 1:1 CBD:THC (OR 2.66, 95% CI 1.52-4.66, p < .001) when compared to chronic pain patients. When compared to arthritis patients, headache as primary symptom patients were 3.4 times as likely to prefer Sativa over 1:1: CBD:THC (OR 3.35, 95% CI 1.57-7.12, p = .001). When compared to insomnia patients, headache as primary symptom

Table 4 Terpenes and cannabinoids present in top 15 preferred medicinal cannabis strains in headache patients who replied with 3 or 2 "Yes" responses on ID Migraine™ questionnaire

Strain	Terpenes (%)								Cannabinoids (%)			
	α-Pinene	β-Myrcene	D-Limonene	Linalool	β-Caryophyllene	Humulene	Trans-nerolidol	Bisabolol	THCA	THC	CBDA	CBD
OG Shark	0.022	0.194	0.191	0.136	0.263	0.078	0.023	0.107	22.8	21.4	0.1	0
Afghani	0.024	0.101	0.036	0.033	0.132	0.055	0.032	0.066	16.9	15.6	0.1	0
Skywalker OG	0.037	0.217	0.208	0.159	0.319	0.149	0.024	0.110	24.2	22.9	0.2	0
Lemon Sour Diesel	0.127	0.235	0.037	0.026	0.169	0.067	0.022	0.026	19.9	18.3	0.1	0
Jack Herer	0.369	0.612	0.023	0.021	0.132	0.039	0.046	0.013	18.8	17.9	0.2	0
Jean Guy	0.031	0.066	0.069	0.063	0.156	0.047	0.050	0.052	18.1	17.3	0.1	0
White Widow	0.032	0.093	0.195	0.006	0.106	0.032	0.034	0.051	20.1	18.7	0.1	0
Pink Kush	0.019	0.187	0.178	0.148	0.317	0.093	0.058	0.124	27.7	25.8	0.1	0
Master Kush	0.045	0.168	0.192	0.203	0.353	0.169	0.039	0.130	28	25.6	0.1	0
Sweet Skunk CBD	0.054	0.162	0.042	0.014	0.051	0.019	0.015	0.028		9.1		11.2
Headband	0.028	0.238	0.230	0.138	0.318	0.094	0.065	0.124	25.1	23.4	0.1	0
Black Tuna	0.026	0.139	0.149	0.077	0.267	0.088	0.033	0.054	21.8	0.2	0.1	0
Warlock CBD	0.050	0.298	0.199	0.051	0.173	0.102	0.023	0.032	11.4	11	12.6	11.4
Cannatonic	0.059	0.152	0.038	0.022	0.099	0.032	0.015	0.035	10.9	9.4	7.6	7.5
Blueberry	0.000	0.333	0.000	0.052	0.324	0.089	0.021	0.023		21.7		0.1

Table 5 Preferred medicinal cannabis types and strains in all non-headache groups, including patients with headache as primary symptom

	Preferred Cannabis Type					
	Chronic pain as primary illness (598)	Arthritis as primary illness (188)	Mental Health Condition (548) /PTSD (93) = (641)	Insomnia/Sleep Disorder (198)	Gastrointestinal Disorder (62) /Crohn's Disease (35) = (97)	Overall Medicinal Cannabis Cohort (2032)
Hybrid	221 (37%)	57 (30.3%)	177 (27.6%)	61 (30.8%)	37 (38.1%)	651 (32%)
Indica	152 (25.4%)	56 (29.8%)	173 (27%)	88 (44.4%)	16 (16.5%)	569 (28%)
Sativa	121 (20.2%)	34 (18.1%)	207 (32.3%)	39 (19.7%)	23 (23.7%)	502 (24.7%)
3:1 CBD:THC	49 (8.2%)	22 (11.7%)	46 (7.2%)	3 (1.5%)	11 (11.3%)	154 (7.6%)
1:1 CBD:THC	52 (8.7%)	16 (8.5%)	35 (5.5%)	7 (3.5%)	10 (10.3%)	146 (7.2%)
No response	3 (0.5%)	3 (1.6%)	3 (0.5%)	0 (0%)	0 (0%)	10 (0.49%)
	Preferred Cannabis Strains (top 15)					
	Chronic pain as primary illness	Arthritis as primary illness	Mental Health Condition/PTSD	Insomnia/Sleep Disorder	Gastrointestinal Disorder/ Crohn's Disease	Overall Medicinal Cannabis Cohort
	OG Shark (43; 10.5%)	Sweet Skunk CBD (13; 8.8%)	Jack Herer (52; 10.8%)	Lemon Sour Diesel (20; 13.8%)	Island Sweet Skunk (8; 9.8%)	OG Shark (120; 8.6%)
	CBD House Blend (34; 8.3%)	OG Shark (12; 8.1%)	Island Sweet Skunk (50; 10.4%)	OG shark (15; 10.4%)	Jack Herer (8; 9.8%)	Jack Herer (119; 8.5%)
	Pink Kush (34; 8.3%)	Cannatonic (11; 7.4%)	White Widow (46; 9.6%)	Skywalker OG (13; 9%)	Black Tuna (7; 8.5%)	White Widow (109; 7.8%)
	Skywalker OG (29; 7.1%)	CBD House Blend (10; 6.8%)	Jean Guy (41; 8.5%)	Pink Kush (12; 8.3%)	Afghani (6; 7.3%)	Lemon Sour Diesel (109; 7.8%)
	Master Kush (28; 6.8%)	Indica House Blend (9; 6.1%)	Lemon Sour Diesel (37; 7.7%)	Jack Herer (10; 6.9%)	Warlock CBD (6; 7.3%)	Pink Kush (109; 7.8%)
	Warlock CBD (28; 6.8%)	Jack Herer (9; 6.1%)	Pink Kush (35; 7.3%)	White Widow (9; 6.2%)	White Widow (6; 7.3%)	Island Sweet Skunk (107; 7.6%)
	Black Tuna (27; 6.6%)	Warlock CBD (8; 5.4%)	OG Shark (34; 7.1%)	Afghani (8; 5.5%)	CBD House Blend (5; 6.1%)	Jean Guy (95; 6.8%)
	Jean Guy (26; 6.3%)	Lemon Sour Diesel (8; 5.4%)	Sweet Skunk CBD (30; 6.2%)	Indica House Blend (7; 4.8%)	Sweet Skunk CBD (5; 6.1%)	Skywalker OG (90; 6.4%)
	Lemon Sour Diesel (26; 6.3%)	White Widow (8; 5.4%)	Afghani (28; 5.8%)	Sweet Skunk CBD (7; 4.8%)	Hybrid House Blend (5; 6.1%)	Afghani (87; 6.2%)
	Jack Herer (25; 6.1%)	Island Sweet Skunk (8; 5.4%)	Skywalker OG (24; 5%)	Island Sweet Skunk (7; 4.8%)	Pink Kush (5; 6.1%)	Sweet Skunk CBD (81; 5.8%)
	Cannatonic (24; 5.8%)	Hybrid House Blend (7; 4.7%)	Master Kush (24; 5%)	Black Tuna (7; 4.8%)	Cannatonic (4; 4.9%)	Cannatonic (77; 5.5%)
	White Widow (24; 5.8%)	Master Kush (7; 4.7%)	Hybrid House Blend (23; 4.8%)	Jean Guy (6; 4.1%)	Lemon Sour Diesel (4; 4.9%)	Warlock CBD (77; 5.5%)

Table 5 Preferred medicinal cannabis types and strains in all non-headache groups, including patients with headache as primary symptom *(Continued)*

	Island Sweet Skunk (22; 5.4%)	Pink Kush (7; 4.7%)	Warlock CBD (21; 4.4%)	Rock Star (6; 4.1%)	Headband (4; 4.9%)	CBD House Blend (76; 5.4%)
	Sweet Skunk CBD (21; 5.1%)	Skywalker OG (7; 4.7%)	Cannatonic (20; 4.2%)	Sour Diesel (6; 4.1%)	OG Shark (3; 3.7%)	Master Kush (75; 5.4%)
	Headband (20; 4.9%)	Afghani (6; 4.1%), Blueberry (6; 4.1%), Girl Scout Cookies (GSC) (6; 4.1%), Jean Guy (6; 4.1%)	Black Tuna (16; 3.3%)	Master Kush (6; 4.1%), Mango (6; 4.1%)	Jean Guy (3; 3.7%), Blueberry (3; 3.7%), Purple Kush (3; 3.7%)	Black Tuna (70; 5%)

Table 6 Preferred medicinal cannabis types and strains in all non-headache groups, excluding patients with headache as primary symptom

	Preferred Cannabis Type					
	Chronic pain as primary illness (450)	Arthritis as primary illness (159)	Mental Health Condition (417)/ PTSD (69) = (486)	Insomnia/Sleep Disorder (166)	Gastrointestinal Disorder (51)/Crohn's Disease (32) = (83)	Overall Medicinal Cannabis Cohort (1527)
Hybrid	162 (36%)	46 (28.9%)	138 (28.4%)	52 (31.3%)	33 (39.8%)	486 (31.8%)
Indica	114 (25.3%)	51 (32.1%)	125 (25.7%)	74 (44.6%)	10 (12.1%)	426 (27.9%)
Sativa	88 (19.6%)	26 (16.4%)	154 (31.7%)	32 (19.3%)	20 (24.1%)	366 (24%)
3:1 CBD:THC	40 (8.9%)	17 (10.7%)	37 (7.6%)	2 (1.2%)	10 (12.1%)	120 (7.9%)
1:1 CBD:THC	43 (9.6%)	16 (10.1%)	30 (6.2%)	6 (3.6%)	10 (12.1%)	121 (7.9%)
No response	3 (0.7%)	3 (1.9%)	2 (0.4%)	0 (0%)	0 (0%)	8 (0.5%)
	Preferred Cannabis Strains (top 15)					
	Chronic pain as primary illness	Arthritis as primary illness	Mental Health Condition + PTSD	Insomnia/Sleep Disorder	Gastrointestinal Disorder + Crohn's Disease	Overall Medicinal Cannabis Cohort
	OG Shark (33; 10.5%)	OG Shark (11; 9.3%)	Jack Herer (42; 11.6%)	Lemon Sour Diesel (17; 14.4%)	Island Sweet Skunk (7; 10.6%)	Jack Herer (93; 8.9%)
	Pink Kush (30; 9.6%)	Cannatonic (10; 8.5%)	Island Sweet Skunk (39; 10.7%)	OG shark (10; 8.5%)	Jack Herer (6; 9%)	Pink Kush (87; 8.3%)
	CBD House Blend (29; 9.3%)	Sweet Skunk CBD (9; 7.6%)	White Widow (38; 10.5%)	Skywalker OG (10; 8.5%)	Warlock CBD (6; 9%)	OG Shark (86; 8.2%)
	Skywalker OG (22; 7%)	CBD House Blend (9; 7.6%)	Jean Guy (28; 7.7%)	Pink Kush (10; 8.5%)	Sweet Skunk CBD (5; 7.6%)	Island Sweet Skunk (86; 8.2%)
	Warlock CBD (21; 6.7%)	Jack Herer (9; 7.6%)	Pink Kush (27; 7.4%)	Jack Herer (9; 7.6%)	White Widow (5; 7.6%)	White Widow (83; 7.9%)
	Jack Herer (20; 6.4%)	Indica House Blend (8; 6.8%)	Lemon Sour Diesel (26; 7.2%)	White Widow (9; 7.6%)	Hybrid House Blend (5; 7.6%)	Lemon Sour Diesel (81; 7.7%)
	Master Kush (19; 6.1%)	Warlock CBD (7; 5.9%)	OG Shark (23; 6.3%)	Afghani (7; 5.9%)	Afghani (4; 6%)	Jean Guy (65; 6.2%)
	Black Tuna (19; 6.1%)	Lemon Sour Diesel (7; 5.9%)	Sweet Skunk CBD (21; 5.8%)	Black Tuna (7; 5.9%)	Black Tuna (4; 6%)	Warlock CBD (63; 6%)
	Afghani (18; 5.8%)	White Widow (7; 5.9%)	Afghani (20; 5.5%)	Sweet Skunk CBD (6; 5.1%)	Lemon Sour Diesel (4; 6%)	CBD House Blend (63; 6%)
	Lemon Sour Diesel (18; 5.8%)	Pink Kush (7; 5.9%)	Warlock CBD (20; 5.5%)	Island Sweet Skunk (6; 5.1%)	Headband (4; 6%)	Skywalker OG (62; 5.9%)
	Island Sweet Skunk (18; 5.8%)	Hybrid House Blend (6; 5.1%)	Cannatonic (18; 5%)	Indica House Blend (6; 5.1%)	Cannatonic (4; 6%)	Sweet Skunk CBD (60; 5.7%)
	Sweet Skunk CBD (17; 5.4%)	Master Kush (6; 5.1%)	Master Kush (17; 4.7%)	Master Kush (6; 5.1%)		Afghani (59; 5.6%)

Table 6 Preferred medicinal cannabis types and strains in all non-headache groups, excluding patients with headache as primary symptom *(Continued)*

				CBD House Blend (3; 4.6%)	
Cannatonic (17; 5.4%)	Island Sweet Skunk (6; 5.1%)	Skywalker OG (16; 4.4%)	Jean Guy (5; 4.2%)	Purple Kush (3; 4.6%)	Cannatonic (59; 5.6%)
Jean Guy (17; 5.4%)	Girl Scout Cookies (GSC) (6; 5.1%)	Hybrid House Blend (15; 4.1%)	Blueberry (5; 4.2%)	Jean Guy (3; 4.6%)	Master Kush (54; 5.1%)
Girl Scout Cookies (GSC) (15; 4.8%)	Skywalker OG (5; 4.2%), Jean Guy (5; 4.2%)	Black Tuna (13; 3.6%)	Mango (5; 4.2%)	Pink Kush (3; 4.6%)	Black Tuna (50; 4.8%)

patients were over twice as likely to prefer Sativa over Indica (OR 2.18, 95% CI 1.36-3.52, $p = .001$) and 8.7 times as likely to prefer 3:1 CBD:THC over Indica (OR 8.74, 95% CI 2.04-37.37, $p < .001$). When compared to gastrointestinal disorder/Crohn's disease patients, headache as primary symptom patients were almost three times as likely to prefer Indica over Hybrid (OR 2.88, 95% CI 1.37-6.05, $p = .004$), 4.2 times as likely to prefer Indica over 3:1 CBD:THC (OR 4.24, 95% CI 1.63-10.98, $p = .002$), and 5.8 times as likely to prefer Indica over 1:1 THC:CBD (OR 5.76, 95% CI 2.17-15.26, $p < .001$). There were no statistically significant differences found between headache as primary symptom patients and mental health condition/PTSD patients, nor between all non-headache patients as a group.

A number of variables were assessed across all pain groups. These variables included primary method of cannabis use, prevalence of cannabis extract (drops, capsules) use and preferences, cannabis quantity and frequency of use, highest level of education completed, employment status, and prescription medications replaced with medicinal cannabis. The most common primary methods of use across all pain groups were vaporizing and joint use, although additional methods included waterpipe/bong, oral (edibles such as oil drops/extracts, baked goods, butter, tincture), pipe, juicing, tea, or topical use, as seen in Table 7. In the 505 patients with headache as a primary symptom, the most common primary methods of use were joint in 170 (33.7%), and vaporizing in 162 (32.1%), and this pattern was similar in the positive ID Migraine™ groups. In general, primary methods of use were similar to the top non-pain related primary illnesses, and the overall patient cohort.

The majority of patients using cannabis extracts (drops, capsules) across pain groups preferred the 3:1 CBD:THC extract with the exception that the chronic pain group preferred 1:1 CBD:THC extract, the 3 "Yes" positive ID Migraine™ group preferred Indica extract, and the combined 3 + 2 "Yes" positive ID Migraine™ group equally preferred 3:1 CBD:THC and Indica extracts, as seen in Table 8. Overall, in the headache as primary symptom group, 195 (38.6%) were using cannabis extracts, and the 3:1 CBD:THC extract was most commonly used in 53 (27.2%) followed by the Indica extract in 51 (26.2%).

Quantity of cannabis used was estimated as one joint = 0.3-0.5 g, one eighth = 3.5 g, one quarter = 7 g, and one ounce = 28 g. The quantity and frequency of medicinal cannabis use across the groups ranged from 9.6-11.4 g/week, 1.4-1.7 g/day, 0.58-0.76 g/treatment, 5.9-6.5 days/week and 3.2-3.9 times/day. The quantity of medicinal cannabis use in the headache group averaged 11.4 g/week, 1.7 g/day, and 0.66 g/treatment, with a frequency of 6.4 days/week, and 3.9 times/day. The positive ID Migraine™ patients averaged similar patterns of use, although at the upper ranges of use. These results can all be seen in Table 9.

Table 7 Primary method of medicinal cannabis use among various pain syndromes, "Yes" responses on ID Migraine™ questionnaire, top non-pain related primary illnesses, and overall cohort

Primary method of use								
	Vaporizer	Pipe	Joint	Oral/ Edible	Waterpipe/ Bong	Juicing	Tea	Topical
Headache as primary symptom (505)	162 (32.1%)	50 (9.9%)	170 (33.7%)	58 (11.5%)	63 (12.5%)	1 (0.2%)	1 (0.2%)	
Headache as primary illness (75)	26 (34.7%)	8 (10.7%)	22 (29.3%)	9 (12%)	8 (10.7%)	1 (1.3%)	1 (1.3%)	
Chronic pain as primary illness (598)	179 (29.9%)	56 (9.4%)	183 (30.6%)	120 (20.1%)	56 (9.4%)	1 (0.17%)		3 (0.5%)
Arthritis as primary illness (188)	70 (37.2%)	16 (8.5%)	60 (31.9%)	36 (19.2%)	4 (2.1%)			2 (1.1%)
3 Yes (343)[a]	109 (31.8%)	37 (10.8%)	120 (35%)	37 (10.8%)	39 (11.4%)		1 (0.29%)	
2 Yes (102)[b]	34 (33.3%)	9 (8.8%)	29 (28.4%)	11 (10.8%)	19 (18.6%)			
3 + 2 Yes (445)	143 (32.1%)	46 (10.3%)	149 (33.5%)	48 (10.8%)	58 (13%)			
Mental Health Condition (548) + PTSD (93)	184 (28.7%)	89 (13.9%)	195 (30.4%)	74 (11.5%)	97 (15.1%)	1 (0.16%)	1 (0.16%)	
Insomnia/Sleep Disorder (198)	63 (31.8%)	19 (9.6%)	65 (32.8%)	30 (15.2%)	19 (9.6%)	1 (0.51%)		1 (0.51%)
Gastrointestinal Disorder (62) + Crohn's Disease (35)	34 (35.1%)	12 (12.4%)	26 (26.8%)	11 (11.3%)	14 (14.4%)			
Overall Medicinal Cannabis Cohort (2032)	632 (31.1%)	229 (11.3%)	617 (30.4%)	330 (16.2%)	212 (10.4%)	4 (0.20%)	2 (0.10%)	6 (0.30%)

[a]3 "Yes" responses = 97% probability of migraine
[b]2 "Yes" responses = 93% probability of migraine

Table 8 Medicinal cannabis extract use preferences among various pain syndromes and "Yes" responses on ID Migraine™ questionnaire

Cannabis extracts (drops, capsules)						
	Total	Hybrid	Indica	Sativa	3:1 CBD:THC	1:1 CBD:THC
Headache as primary symptom (505)	195 (38.6%)	36 (18.5%)	51 (26.2%)	15 (7.7%)	53 (27.2%)	40 (20.5%)
Headache as primary illness (75)	26 (34.7%)	7 (26.9%)	5 (19.2%)	1 (3.9%)	9 (34.6%)	4 (15.4%)
Chronic pain as primary illness (598)	248 (41.5%)	44 (17.7%)	56 (22.6%)	18 (7.3%)	60 (24.2%)	66 (26.6%)
Arthritis as primary illness (188)	80 (42.6%)	14 (17.5%)	11 (13.8%)	5 (6.3%)	26 (32.5%)	24 (30%)
3 Yes (343)[a]	143 (41.7%)	25 (17.5%)	41 (28.7%)	6 (4.2%)	39 (27.3%)	32 (22.4%)
2 Yes (102)[b]	33 (32.4%)	6 (18.2%)	7 (21.2%)	5 (15.2%)	9 (27.3%)	6 (18.2%)
3 + 2 Yes (445)	176 (39.6%)	31 (17.6%)	48 (27.3%)	11 (6.3%)	48 (27.3%)	38 (21.6%)

[a]3 "Yes" responses = 97% probability of migraine
[b]2 "Yes" responses = 93% probability of migraine

The highest level of education completed across medicinal cannabis user groups can be seen in Table 10. Options included graduate degree, university degree (Bachelors' degree or equivalent), some college/university but no degree/certificate, technical/non-university degree, high school degree or equivalent (GED), and less than high school degree. The most common education level completed across all pain groups was technical/non-university degree, including the headache group, $n = 158$ (31.3%). The exception was in the 2 "Yes" positive ID Migraine™ group, which most commonly reported some college/university but no degree/certificate.

Employment status among medicinal cannabis users was assessed, and can be seen in Table 10. The options were employed working full-time, employed working part-time, retired, not employed looking for work, not employed not looking for work, and disabled not able to work. The vast majority of patients across all pain groups were employed working full time, including the headache group, $n = 268$ (53.1%).

Prescription medications that were replaced with medicinal cannabis were also recorded, as seen in Table 11, and included opiates/opioids, NSAIDs/analgesics, triptans, ergots, anti-depressant/anti-anxiety, anti-convulsant, and muscle relaxers. Many patients across all groups had replaced prescription medications with medicinal cannabis, including headache as primary symptom $n = 272$ (53.9%). Ranges of prescription medication replacement across pain groups varied between 41.2%-59.5% of patients. The most common prescription medications replaced by medicinal cannabis were opiates/opioids in every pain group, including headache as primary symptom $n = 118$ (43.4%). Ranges of opiate/opioid replacement across pain groups varied between 40.5%-72.8% of patients. Notably, additional prescription medications replaced by medicinal cannabis in headache patients included 106 (39%) anti-depressant/anti-anxiety, 57 (21%) NSAIDs, 22 (8.1%) triptans, 21 (7.7%) anticonvulsants, 19 (7%) muscle relaxers, and 1 (0.4%) ergots.

Discussion

The neurobiological pathways of cannabinoids and pain, including migraine and headache, have been detailed, summarized and should be reviewed [1, 2, 51, 65, 68–70]. Briefly, the endocannabinoid system is distributed throughout the central and peripheral nervous system, is involved

Table 9 Quantity and frequency of medicinal cannabis use among various pain syndromes and "Yes" responses on ID Migraine™ questionnaire

Cannabis quantity and frequency used					
	Grams per week (Average)	Grams per day (Average)	Grams per treatment (Average)	Days used per week (Average)	Times used per day (Average)
Headache as primary symptom (505)	1 to > 28 (11.4)	≤0.25 to ≥4 (1.7)	≤0.25 to ≥4 (0.66)	1-7 (6.4)	1 to > 10 (3.9)
Headache as primary illness (75)	1 to > 28 (9.6)	≤0.25 to ≥4 (1.4)	≤0.25 to ≥4 (0.67)	1-7 (5.9)	1 to > 10 (3.3)
Chronic pain as primary illness (598)	1 to > 28 (10.8)	≤0.25 to ≥4 (1.6)	≤0.25 to ≥4 (0.68)	1-7 (6.2)	1 to > 10 (3.7)
Arthritis as primary illness (188)	1 to > 28 (9.8)	≤0.25 to ≥4 (1.4)	≤0.25 to ≥4 (0.58)	1-7 (6.1)	1 to > 10 (3.2)
3 Yes (343)[a]	1 to > 28 (11.2)	≤0.25 to ≥4 (1.7)	≤0.25 to ≥4 (0.63)	1-7 (6.4)	1 to > 10 (3.9)
2 Yes (102)[b]	1 to > 28 (11.3)	≤0.25 to ≥4 (1.7)	≤0.25 to ≥4 (0.76)	1-7 (6.5)	1 to > 10 (3.8)
3 + 2 Yes (445)	1 to > 28 (11.3)	≤0.25 to ≥4 (1.7)	≤0.25 to ≥4 (0.70)	1-7 (6.5)	1 to > 10 (3.9)

[a]3 "Yes" responses = 97% probability of migraine
[b]2 "Yes" responses = 93% probability of migraine

Table 10 Highest education level completed and employment status in medicinal cannabis users among various pain syndromes and "Yes" responses on ID Migraine™ questionnaire

	Highest level of education completed					
	Graduate degree	University degree (Bachelors' degree or equivalent)	Some college/ university, but no degree/certificate	Technical and non-university degree	High school degree or equivalent (GED)	Less than high school degree
All patients (2032)	122 (6%)	322 (15.9%)	432 (21.3%)	642 (31.6%)	375 (18.5%)	139 (6.8%)
Headache as primary symptom (505)	17 (3.4%)	81 (16%)	124 (24.6%)	158 (31.3%)	91 (18%)	34 (6.7%)
Headache as primary illness (75)	5 (6.7%)	18 (24%)	16 (21.3%)	22 (29.3%)	9 (12%)	5 (6.7%)
Chronic pain as primary illness (598)	39 (6.5%)	74 (12.4%)	131 (21.9%)	196 (32.8%)	107 (17.9%)	51 (8.5%)
Arthritis as primary illness (188)	10 (5.3%)	31 (16.5%)	36 (19.2%)	65 (34.6%)	38 (20.2%)	8 (4.3%)
3 Yes (343)[a]	10 (2.9%)	54 (15.7%)	87 (25.4%)	114 (33.2%)	53 (15.5%)	25 (7.3%)
2 Yes (102)[b]	4 (3.9%)	13 (12.8%)	30 (29.4%)	28 (27.5%)	21 (20.6%)	6 (5.9%)
3 + 2 Yes (445)	14 (3.2%)	67 (15.1%)	117 (26.3%)	142 (31.9%)	74 (16.6%)	31 (7.0%)
	Employment status					
	Employed, working full-time	Employed, working part-time	Retired	Not employed, looking for work	Not employed, not looking for work	Disabled, not able to work
All patients (2032)	1045 (51.4%)	231 (11.4%)	120 (5.9%)	164 (8.1%)	88 (4.3%)	384 (18.9%)
Headache as primary symptom (505)	268 (53.1%)	50 (9.9%)	10 (2%)	36 (7.1%)	30 (5.9%)	111 (22%)
Headache as primary illness (75)	56 (74.7%)	4 (5.3%)	1 (1.3%)	1 (1.3%)	5 (6.7%)	8 (10.7%)
Chronic pain as primary illness (598)	278 (46.5%)	64 (10.7%)	33 (5.5%)	30 (5%)	24 (4%)	169 (28.3%)
Arthritis as primary illness (188)	94 (50%)	18 (9.6%)	38 (20.2%)	13 (6.9%)	4 (2.1%)	21 (11.2%)
3 Yes (343)[a]	172 (50.2%)	31 (9%)	6 (1.8%)	24 (7%)	21 (6.1%)	89 (26%)
2 Yes (102)[b]	59 (57.8%)	12 (11.8%)	2 (2%)	9 (8.8%)	3 (2.9%)	17 (16.7%)
3 + 2 Yes (445)	231 (51.9%)	43 (9.7%)	8 (1.8%)	33 (7.4%)	24 (5.4%)	106 (23.8%)

[a]3 "Yes" responses = 97% probability of migraine
[b]2 "Yes" responses = 93% probability of migraine

in inflammatory and pain processing, and plays regulatory physiological roles across virtually every organ system [19, 46, 71–74]. The endocannabinoid system interacts within its own pathways, as well as within major endogenous pain pathways, including inflammatory, endorphin/enkephalin, vanilloid/transient receptor potential cation channel subfamily V (TRPV), subfamily M (TRPM), subfamily A (TRPA), and nuclear receptors/transcription factors called the peroxisome proliferator-activated receptors (PPAR) [75].

The activities of the endocannabinoid system are based on the pre-synaptic G protein-coupled cannabinoid 1 (CB1) and 2 (CB2) receptors [76]. There is also a presumed third cannabinoid receptor, G protein-coupled receptor 55 (GPR55), termed CB3 [77]. The primary endogenous cannabinoid receptor ligands (endogenous cannabinoids, or endocannabinoids) are arachidonic acid derivatives, and they work via retrograde signaling receptor activation. The primary mediator of endocannabinoid signaling is N-arachidonoylethanolamine (anandamide, or AEA), and 2-arachidonoylglycerol (2-AG) is another primary endocannabinoid [71, 78–80]. Cannabis-based phytocannabinoids, as well as inherent endocannabinoids interact at the CB1 and CB2 receptors with variable affinities and actions [81–83].

The CB1 receptor is the most abundant G protein-coupled receptor in the brain and one of the most abundant in both the peripheral and central nervous system [81]. CB1 receptors are expressed primarily on presynaptic peripheral and central nerve terminals, and are found extensively through the anatomical pain pathways as well as many other neurological central and peripheral

Table 11 Medicinal cannabis reported as a substitute for prescription drugs among various pain syndromes and "Yes" responses on ID Migraine™ questionnaire

Prescription drugs replaced							
	Yes	Opiates, opioids	NSAIDs, Analgesics	Triptans/Ergots	Anti-depressant, Anti-anxiety	Anti-convulsant	Muscle Relaxers
Headache as primary symptom (505)	272 (53.9%)	118 (43.4%)	57 (21%)	22 (8.1%)/1 (0.4%)	106 (39%)	21 (7.7%)	19 (7%)
Headache as primary illness (75)	36 (48%)	19 (52.8%)	11 (30.6%)	14 (38.9%)	5 (13.9%)	1 (2.8%)	4 (11.1%)
Chronic pain as primary illness (598)	316 (52.8%)	230 (72.8%)	64 (20.3%)	3 (1%)	74 (23.4%)	41 (13%)	30 (9.5%)
Arthritis as primary illness (188)	90 (47.9%)	48 (53.3%)	37 (41.1%)	2 (2.2%)	15 (16.7%)	5 (5.6%)	7 (7.8%)
3 Yes (343)[a]	204 (59.5%)	92 (45.1%)	45 (22.1%)	20 (9.8%)/1 (0.5%)	84 (41%)	13 (6%)	15 (7.4%)
2 Yes (102)[b]	42 (41.2%)	17 (40.5%)	6 (14.3%)	2 (4.8%)	15 (35.7%)	6 (14.3%)	4 (9.5%)
3 + 2 Yes (445)	246 (55.3%)	109 (44.3%)	51 (20.7%)	22 (8.9%)/1 (0.4%)	99 (40.2%)	19 (7.7%)	19 (7.7%)

[a]3 "Yes" responses = 97% probability of migraine
[b]2 "Yes" responses = 93% probability of migraine

locations [19, 84–87]. CB1 receptors are associated with the "high" felt with some cannabis strains, activated by THC. Activation leads to hyperpolarization of the pre-synaptic terminal, closing of calcium channels with subsequent inhibition of released stored inhibitory and excitatory neurotransmitters, including glutamate, 5-hydroxytryptamine (5-HT; serotonin), gamma-aminobutyric acid (GABA), noradrenaline, dopamine, acetylcholine, D-aspartate, and cholecystokinin at inhibitory and excitatory synapses [19, 71, 73, 80, 86, 88–90], and can modulate pain pathways involving opioid, serotonin, and N-methyl-d-aspartate (NMDA) receptors through other indirect mechanisms [91].

The CB2 receptors are located primarily in the peripheral tissues and immune cells where they influence the release of cytokines, chemokines, and cell migration including neutrophils and macrophages, but do have some presence in the central nervous system [18, 86, 92–95], and may also contribute to pain relief by dopamine release modulation [96, 97].

Over 540 phytochemicals have been described in cannabis [98], 18 different chemical classes, and more than 100 different phytocannabinoids, although some are breakdown products [99, 100]. THC and CBD have been the most researched and are considered the major cannabinoids. There are many additional cannabinoids referred to as minor cannabinoids. The quantities of major and minor cannabinoids are widely variable between different types of cannabis strains. There is evidence for analgesic and anti-inflammatory effects in many of the cannabinoids, and this publication will focus primarily on these properties for the cannabinoids assessed in this study. However, a more extensive discussion and a comprehensive review of other medicinal properties of these, as well as many other cannabinoids, has been summarized and is available [28]. The cannabinoids analyzed in this study were limited to THC, THCA, CBD, and CBDA.

THC is one of the most researched cannabinoids, and the cause of the psychoactive side effects of cannabis, suspected from modulation of glutamate and GABA systems [18, 83, 101–103]. It is a partial agonist at CB1 greater than CB2 receptors, which are its primary mechanisms of action. However, other mechanisms of action reflect its activity as an agonist at the PPAR-γ and TRPA1 receptors [83], a 5HT3A antagonist, a glycine receptor activation enhancer via allosteric modification, reduces elevated intracellular calcium levels from TRPM8 activity (cold and menthol receptor 1 (CMR1)), elevates calcium levels by TRPA1 or TRPV2, and stimulates G Protein Receptor 18 and other nuclear receptors [104–113]. It reduces NMDA responses by 30-40% [114–116], blocks capsaicin-induced hyperalgesia [117], inhibits CGRP activity [118], increases cerebral 5HT production, decreases 5HT reuptake, and inhibits 5HT release from platelets, all of which may influence trigeminovascular migraine circuitry [1, 68, 69, 119]. THC enhances analgesia from kappa opioid receptor agonist medications [120–123], stimulates production of beta-endorphin and increases proenkephalin mRNA levels in brainstem regions involved in pain processing [124–126], and intraventricular and intrathecal administration of THC produces analgesia similar to opioids [127].

THC is 20 times more anti-inflammatory than aspirin, twice as anti-inflammatory as hydrocortisone [128], and has well documented analgesic and anti-inflammatory benefits including arthritic and inflammatory conditions [83, 114, 127, 129–156]. There have been many positive studies across various chronic pain syndromes, showing benefit of THC in trials with smoked or vaporized cannabis comparing between different doses of THC, with

benefit often noted at higher percentages [28, 47, 157–169]. However, compositions of other cannabinoids including CBD, minor cannabinoids, and other important compounds such as terpenes were not assessed in most of these trials. Given the entourage effects of cannabis [100, 170], where cannabinoids and terpenes influence activity of one another, resulting in strain-specific characteristics, effects and responses, it is often unclear if these studies showing positive (or negative) effects of cannabis are due to the THC alone, or due to synergy between undefined compositions of other cannabinoids and terpenes.

There have been a multitude of studies confirming benefit in various chronic pain syndromes with an oral-mucosal spray called Nabiximols (Sativex) [171–196], approved in 30 countries for various neurological symptoms. This is a tincture of cannabis made from cannabis plants [197]. Each spray delivers a standardized dose of 2.7 mg THC and 2.5 mg CBD, along with additional cannabinoids, flavonoids, and terpenes in unmeasured small amounts. Despite the standardized THC:CBD ratio, the actual concentrations of terpenes and other compounds are unknown. This again creates uncertainty as to what components are providing most of the benefit, although entourage effects are again suspected. There was also a study comparing between three varieties of this spray; 1:1 THC:CBD vs. THC alone vs. CBD alone and the sprays that contained THC showed the most pain benefit, over CBD alone [179]. Other cannabis extract studies of only THC and CBD in varying doses also showed pain benefit, although these did not evaluate each cannabinoid individually [187, 198].

The strong anti-emetic benefits of THC have also been well documented in adults [26, 83, 129, 130, 199–238] and children [235, 239–241], and migraine associated nausea and vomiting would certainly be another benefit of THC. In fact, the FDA has approved two synthetic forms of THC in the treatment of chemotherapy related nausea and vomiting; Dronabinol [242] and Nabilone [243]. Notably, these synthetic THC medications have also shown analgesic effects [55, 57, 62, 188, 244–256].

Besides THC, CBD is the other major cannabinoid. It has gained a lot of attention over the past several years due to its lack of any psychoactivity, as opposed to THC. In November 2017, The World Health Organization announced that in humans, CBD exhibits no evidence for abuse or dependence potential, and there is no evidence of public health related problems associated with the use of pure CBD [257]. In January 2018, the World Anti-Doping Agency (WADA) removed CBD from their prohibited list, no longer banning use by athletes [258]. CBD has powerful analgesic and anti-inflammatory effects [23, 83, 114, 129–131, 137–140, 149, 259–281] mediated by both cyclooxygenase and lipoxygenase inhibition. Its anti-inflammatory effect is several hundred times more potent than aspirin [128, 282], although to date, there have been no clinical studies evaluating pure CBD in headache or chronic pain disorders. CBD has much lower affinity for CB1 or CB2 receptors, and acts as an antagonist of CB1 and CB2 agonists such as THC [276]. At low concentrations, its antagonism of CB1 underlies its neutralizing effects on the CB1 agonist THC side effects such as anxiety, tachycardia, and sedation [283–288]. CBD appears to attenuate some of these negative side effects of THC when the CBD:THC ratio is at least 8:1 (± 11.1), but may potentiate some of the THC side effects when the CBD:THC ratio is around 2:1 (± 1.4) [286, 288]. It is also an inverse agonist at the CB2 receptor, which may contribute to its anti-inflammatory effects [276].

CBD also interacts with a multitude of ion channels, enzymes, and other receptors [18, 83, 129, 130, 225, 259]. It acts as a TRPV1 agonist, similar to capsaicin, although without the noxious sides effects, and also inhibits AEA uptake and metabolism [108–110, 289, 290]. It acts as a positive allosteric modulator at α1 and α1β glycine receptors [291], suggested to play a role in chronic pain after inflammation or nerve injury since glycine acts as an inhibitory postsynaptic neurotransmitter in the dorsal horn of the spinal cord. CBD acts as a μ opioid receptor ligand and a positive allosteric modulator at μ and δ opioid receptors suggesting that it may enhance opiate effects [83]. Additional mechanisms of action suggested to reflect its anti-inflammatory and analgesic effects, as well as other medicinal benefits, include TRPA1 agonist, TRPV1 agonist, TRPM8 antagonist [108–110], TRPV2 agonist in which it may mediate CGRP release from dorsal root ganglion neurons [292], T-type calcium^{2+} channel inhibitor [293], suppression of tryptophan degradation (precursor to 5HT) [294], phospholipase A2 modulator [295], 5-HT1A agonist [83, 296], regulator of intracellular calcium^{2+} [297, 298], fatty acid amide hydrolase (FAAH; breaks down AEA) inhibition [290], GPR55 antagonist [77], adenosine uptake competitive inhibitor [299], PPARγ agonist [300], 5-lipoxygenase and 15-lipoxygenase inhibitors [301], and antagonism of the abnormal-CBD receptor [83, 302].

Cannabinoid acids are the precursors to the cannabinoids in raw and live cannabis, and have no psychotropic qualities. They are decarboxylated by heat, UV exposure, and prolonged storage to form the active cannabinoids, although heat such as from smoking or vaporizing is the primary conversion factor. The two cannabinoid acids assessed in this study were THCA, which converts to THC, and CBDA, which converts to CBD.

THCA is a TRPA1 partial agonist [108], and TRPM8 antagonist [108] which may underlie a potential role in analgesia, and has been shown to have anti-inflammatory [140] and anti-nausea properties [303]. CBDA is often

obtained through consumption of raw cannabis juice. It is a TRPA1 agonist [108], TRPV1 agonist [290], and TRPM8 antagonist [108] which may also reflect its potential as an analgesic. It is also anti-inflammatory [130, 140, 304] via selective COX2 inhibition, and has anti-nausea properties [237, 305].

The terpenes, or terpenoids, form the largest group of phytochemicals [99], and account for some pharmacological properties of cannabis, as well as many medicinal herbs, plants and essential oils. They are the source of flavors, aromas, and other characteristics that help differentiate cannabis strains. The terms terpenes and terpenoids are often used interchangeably in the literature, although technically, terpenes are basic hydrocarbons, while terpenoids contain extra functional groups of a wide range of chemical elements. Cannabis contains up to 200 different terpenes [100], and they are generally classified as primary and secondary terpenes, based on how frequent they occur in cannabis. They are lipophilic with wide ranging mechanisms of action sites including neurotransmitter receptors, G-protein receptors, muscle and neuronal ion channels, enzymes, cell membranes, and second messenger systems [100, 306, 307]. The terpenes work synergistically with the cannabinoids for a variety of therapeutic effects, and this phenomenon is known as the cannabis entourage effects [100, 170]. They have shown many medicinal benefits, including anti-inflammatory and analgesic properties [308]. This publication will focus primarily on the anti-inflammatory and analgesic evidence for the terpenes analyzed in this study, although a more extensive discussion and a comprehensive review of other medicinal properties of these, as well as many other terpenes has been summarized and is available [28]. The majority of this data comes from preclinical studies involving animal models or in vitro studies, and some of the reported benefits attributed to individual terpenes come from studies evaluating whole essential oils or plants in which the specified terpene may be a predominant constituent. However, therapeutic contribution from some of the other terpenes in some of these studies cannot be excluded. The terpenes analyzed in this study were limited to α-pinene, β-myrcene, D-limonene, linalool, β-caryophyllene, humulene, trans-nerolidol, and bisabolol.

Alpha-pinene (α-pinene) is the most commonly occurring terpene in nature [309], and accounts for the aroma of fresh sage, pine needles, and conifers, but is produced by many herbs such as basil, parsley, and dill as well. It has anti-inflammatory effects in human chondrocytes, suggesting anti-osteoarthritic activity [310, 311], anti-inflammatory effects by PGE-1 [312], and anti-nociception properties [313].

Beta-myrcene (β-myrcene), or myrcene, is common in lemongrass, basil, bay leaves, wild thyme, parsley, hops, and tropical fruits such as mango. It has potent anti-inflammatory, analgesic, and anxiolytic properties [314–316], and has benefit in muscle relaxation [317], and prominent sedation/hypnotic, helpful in sleep [317, 318]. Its analgesic effects were antagonized by naloxone suggesting an opioid-mediated mechanism [315, 316]. Its significant anti-inflammatory effects [319] occur via prostaglandin E2 [315] and it has anti-catabolic effects in human chondrocytes suggesting anti-osteoarthritic activity and the ability to halt or slow down cartilage destruction and osteoarthritis progression [320].

D-limonene (limonene) is prominent in the rinds of citrus fruits, and the second most commonly occurring terpene in nature [309]. It has analgesic [321], anti-inflammatory [320, 322–325], and antidepressant effects [321, 326]. It contributes to muscle relaxation and sleep [317], and is a powerful anxiolytic [327–330], which extended anxiolytic benefit to patients with chronic myeloid leukemia (CML) [331]. It increases the metabolic turnover of dopamine in the hippocampus and serotonin in the prefrontal cortex and striatum, suggesting that anxiolytic and antidepressant-like effects may occur by the suppression of dopamine activity related to enhanced serotonergic neurons, especially via 5-HT1A [332].

Linalool is found in flowers and spices including citrus, lavender, rosewood, birch trees, and coriander. It exhibits anti-inflammatory and analgesic activity [333–335] as well as anti-nociception via activation of opioidergic and cholinergic systems [333], anticonvulsant via anti-glutamatergic and GABA neurotransmitter systems [336–340], anti-anxiety/stress [341–344], sedation [343, 345–347], and anti-insomnia properties [100]. Its local anesthetic effects [348] were equivalent to procaine and menthol [349], and analgesic effects have been attributed to adenosine A_{2A} activity [350] and ionotropic glutamate receptors including AMPA, NMDA and kainate [351]. Morphine opioid usage in gastric banding surgical patients was significantly decreased following lavender inhalation vs. placebo, and this was attributed to the linalool concentration [352].

Beta-caryophyllene (β-caryophyllene) is found in spices and plants including cloves, cinnamon, black pepper, hops, rosemary, oregano, and basil. It has analgesic effects in inflammatory and neuropathic pain [353], and has potent anti-inflammatory effects [354–357], with local anesthetic properties [358]. Anti-inflammatory effects appear to occur via PGE-1 [359], with similar efficacy as indomethacin and etodolac [360, 361], and comparable to phenylbutazone [359, 360]. β-caryophyllene is a selective cannabinoid receptor 2 (CB2) agonist [362–364]. CB2 receptors have been implicated in anxiety and depression, and β-caryophyllene has shown anxiolytic and antidepressant effects [365].

Humulene (α-caryophyllene) is an isomer of β-caryophyllene and plays a role in many of the distinguishing characteristics between different cannabis strains. It is found in herbs and spices such as clove, basil, hops, sage, spearmint and ginseng, in addition to some vegetables and fruits. It has strong anti-inflammatory properties comparable to dexamethasone systemically, topically, and in allergic airway inflammation [354–356, 366, 367], as well as anti-nociceptive and analgesic properties [367].

Nerolidol (trans-nerolidol) is found in many herbs and spices including lavender, lemon grass, ginger, jasmine, tea tree, oranges, and present in orange and other citrus peels. It has anti-insomnia and sedative properties [368].

Alpha-bisabolol (α-bisabolol, bisabolol, levomenol) is produced by some flowers used in making tea, such as the chamomile flower. It has anti-inflammatory effects in the skin [369], as well as anti-nociceptive properties [370].

Cannabis sativa strains are generally described by patients as uplifting, energetic, creative, euphoria, spacey, cerebrally-focused effects, and better for day use, while *Cannabis indica* strains are typically described as calming, relaxing, sedative, full body effects such as "body buzz", and better for night use. Research suggests these effects are not likely due purely to CBD:THC ratios, as there are no significant differences in CBD:THC ratios between Sativa and Indica strains. Rather these different subjective effects are likely due to varying ratios of major cannabinoids as well as minor cannabinoids, terpenes and probably additional phytochemicals [100, 371–374]. High CBD strains are Sativa or Indica strains that have been crossed with high CBD hemp strains (1:1 CBD:THC up to approximately 5:1 CBD:THC), while pure CBD strains (ratios of > 10:1 CBD:THC, which can be up to approximately 50:1 CBD:THC) are considered hemp strains. Most strains utilized today are Hybrids designed with standardized ratios of CBD, THC, other cannabinoids, and other compounds such as terpenes and flavonoids, targeting specific symptoms, responses, and end user effects.

Although not of statistical significance, there were some pattern use trends noted. The majority of patients across all pain groups including the positive ID Migraine™, headache as primary symptom, chronic pain, and arthritis groups all preferred Hybrid cannabis strains followed by Indica, Sativa, and higher CBD strains (1:1 CBD:THC, 3:1 CBD:THC) when patients with headache as primary symptom were included. However, when these patients were excluded, the arthritis group preferred Indica strains. When comparing headache and migraine to non-headache groups, Indica strains were preferred in the insomnia/sleep disorders group, Sativa strains in the mental health condition/PTSD group, and Hybrid strains were still preferred in the gastrointestinal disorder/Crohn's Disease group. Perhaps the headache, chronic pain, and gastrointestinal disorder/Crohn's groups preferred similar Hybrid strains due to underlying inflammatory pathophysiology. The positive ID Migraine™ and headache as primary symptom patients most commonly preferred the "OG Shark" Hybrid strain specifically, although this pattern was also noted in the chronic pain and arthritis groups, so was not unique to headache and migraine. This is a high THC/THCA, low CBD/CBDA strain with β-caryophyllene followed by β-myrcene as the predominant terpenes. This could reflect the potent analgesic, anti-inflammatory, and anti-emetic properties of THC, along with documented anti-inflammatory and analgesic properties of β-caryophyllene and β-myrcene. Given the prominent features of pain with nausea and vomiting in migraine headache, the fact that headache and migraine patients preferred a strain such as this, with its associated cannabinoid and terpene profile, would make sense given the known therapeutic effects of this cannabinoid and these terpenes. Furthermore, there were additional terpenes present in this strain of lower percentages, some of which also have analgesic and anti-inflammatory properties.

Substituting cannabis for alcohol, illicit drugs and/or prescription medications has been commonly observed in cross sectional surveys, suggesting a harm reduction role in the use of these substances, as well as implications for abstinence-based substance use treatment strategies [375–377]. The "opioid-sparing effect" of cannabinoids has been well described with extensive supporting evidence showing that combining cannabis with opiates decreases opiate dose requirements [166, 378]. CB1 receptors are 10 times more concentrated then mu-opioid receptors in the brain, and cannabinoid receptors co-localize with opioid receptors in many regions involved in pain pathways. This is suspected to contribute to synergistic augmentation of the analgesic opioid effects and decreased opioid dose requirements [8, 122–125, 166, 379–384], and studies have shown cannabis use did not affect blood levels of oxycodone or morphine [8, 166]. Cannabinoid receptor agonists increase endogenous opioid peptide release, and chronic THC use increases endogenous opioid precursor gene expression in supraspinal and spinal structures involved in pain perception [119, 126, 166, 379].

The synergistic effect of concomitant cannabis/cannabinoids and opioids in lowering both pain and opioid dose requirements without affecting serum opioid levels has been demonstrated prospectively [166]. A large meta-analysis showed that 17 of 19 pre-clinical studies provided good evidence of these synergistic effects from opioid and cannabinoid co-administration and that the median effective dose (ED50) of morphine administered with THC is 3.6 times lower than the ED50 of morphine

alone, while the ED50 for codeine administered with THC was 9.5 times lower than the ED50 of codeine alone [378]. The combination of cannabis/cannabinoids and opioids appears to allow for opioid treatment at lower doses with fewer side effects, allowing easier detoxification and weaning due to lessening of tolerance and withdrawal from opiates, and rekindling of opiate analgesia after prior dosages have worn off [124]. Some pain specialists have suggested the use of medicinal cannabis treatment in addition to or in replacement of opiate treatments to help reduce overdose mortality and morbidity associated with opiate use [385]. Prospective studies have shown that chronic pain patients who use cannabis have improved pain and functional outcomes, and a significant reduction in opioid use [386], and medical cannabis use was associated with decreased opiate use, improvement in quality of life, and better side effect profile in a retrospective cross-sectional survey of chronic pain patients [387].

Notably, the most common prescription medications replaced by medicinal cannabis in this study were opiates/opioids in a large percentage within every pain group, up to 72.8% of patients in the chronic pain as primary illness group. Given the opioid epidemic, particularly in the United States, cannabis has been discussed as an option that may help in the opioid/opiate detoxification and weaning process and perhaps assist in combating the epidemic of opioid related death [377, 385, 388–390]. States with medicinal cannabis laws have been shown to have a 24.8% decreased annual opioid overdose mortality rate compared with states without medicinal cannabis laws. The association between medicinal cannabis law implementation and decrease in annual opioid overdose mortality strengthened over time to a decrease of 33.7% by year 5 [391].

The synergistic interactions between the phytocannabinoids, terpenes and other cannabis compounds resulting in various therapeutic benefits and responses have been termed the cannabis entourage effects [100, 170]. This synergy between the cannabinoids, terpenes, and other compounds leads to variable benefits, user effects, and strain characteristics. In addition, synergistic interactions between cannabis and opioid pathways may be a promising new weapon in the battle of the opioid epidemic. Further study is needed to determine optimal combinations for specific synergies and composition ratios of the cannabis constituents to best target different symptoms and diseases. Medicinal cannabis production has become a very sterile, scientific, standardized production process, and an emerging new industry. Similar to the broad category of anticonvulsants with many varieties targeting variable neurochemical pathways and channels with different responses and side effects, cannabis should also be thought of a broad category of medicine, of which further therapeutic delineations and disease targeting differentiations between strains is necessary.

There are multiple limitations to this study beginning with its survey design and inherent limitations. Many of the patients who reported headache as a primary symptom for which they were treating with medicinal cannabis, had also reported other diseases or symptoms that they were using medicinal cannabis for. So, some of the answers provided may not have been specific for only headache treatment, but potentially other symptoms or a combination of symptoms including headache. This could also influence reported preferred strains being used since some strains are used more commonly for some symptoms, while other strains may be used for other symptoms. There may be some inaccuracy of patient numbers within the different pain groups of chronic pain, arthritis, and headache. For example, some patients who reported chronic pain as the primary illness for which they were using medicinal cannabis did not specify their type of chronic pain further. It is unknown if some of these patients may have been treating chronic pain of arthritis or headache types, but reporting it as chronic pain, and therefore some of these patients may have been more accurately listed in a different more specific category. Variability in patients' cannabis knowledge could potentially influence self-reporting accuracy. When documenting the preferred cannabis types and strains within each of the pain and non-pain groups, many patients did not provide an answer for their preferred type or strain. If a preferred cannabis type was not provided, but a preferred strain was provided, then their preferred type was presumed to correlate to their reported preferred strain, and counted as such. In addition, reported preferred cannabis types and strains sometimes did not correlate (reported strain did not fall under the correct reported type). Therefore, the preferred cannabis types and strains listed within each category, and their inferred potential benefits, may be inaccurate based on this inconsistent reporting by some patients, and the validity of the preferred cannabis type and strain data requires prospective validation.

Conclusions

Chronic pain was the most common reason for use of medicinal cannabis, consistent with the statistics of most registries. Identifying differences in use patterns between migraine, headache, arthritis, and chronic pain syndromes may be helpful in optimizing crossbred cannabis strains, synergistic biochemical profiles, or dosing differences between these pain subsets. The majority of patients treating headache with medicinal cannabis were positive for migraine (88%) according to the ID

Migraine™ questionnaire. This suggests that most headaches being treated with medicinal cannabis were likely of migrainous pathophysiology.

Hybrid cannabis strains were preferred across most pain groups. "OG Shark", a high THC/THCA, low CBD/CBDA strain with β-caryophyllene followed by β-myrcene as the predominant terpenes, was the most preferred strain in the positive ID Migraine™ and headache as primary symptom groups. This could reflect the potent analgesic, anti-inflammatory, and anti-emetic properties of THC, along with documented anti-inflammatory and analgesic properties of β-caryophyllene and β-myrcene. Since migraines also involve nausea and vomiting, the potent antiemetic properties of THC may be a reason for this preference. Vaporizing or joint use were the primary methods of use across all groups, including migraine and headache, likely reflecting the need for a quick acting inhaled or non-orally ingested therapy in migraine attacks before severe pain and nausea/vomiting become prominent.

Most patients in the pain groups reported replacing prescription medications with medicinal cannabis, the most common of which were opiates/opioids across all pain groups. This is notable given the well-described "opioid-sparing effect" of cannabinoids and growing abundance of literature suggesting that cannabis may help in weaning from these medications and perhaps providing a means of combating the opioid epidemic. There are several limitations to the data in this study, and these results require further confirmation with more sophisticated prospective study methods. However, these results may provide early insight and a framework for direction into optimizing crossbred cannabis strains, synergistic biochemical profiles, dosing, and patterns of use that may be of clinical benefit in the treatment of headache and migraine, as well as other chronic pain syndromes.

Abbreviations

2-AG: 2-arachidonoylglycerol; 5-HT: 5-hydroxytryptamine (serotonin); AEA: N-arachidonoylethanolamine (anandamide); AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CB1: Cannabinoid 1 receptor; CB2: Cannabinoid 2 receptor; CB3: Cannabinoid 3 receptor; CBD: Cannabidiol; CBDA: Cannabidiolic acid; CGRP: Calcitonin gene related peptide; CML: Chronic myeloid leukemia; CMR1: Cold and menthol receptor 1; COX2: Cyclooxygenase-2; ED50: Median effective dose; FAAH: Fatty acid amide hydrolase; FDA: Federal drug administration; GABA: Gamma-aminobutyric acid; GPR55: G protein-coupled receptor 55; NMDA: N-methyl-d-aspartate; NNT: Number needed to treat; NSAID: Non-steroidal anti-inflammatory drug; PGE-1: Prostaglandin E1; PPAR: Peroxisome proliferator-activated receptors; PTSD: Post-Traumatic Stress Disorder; THC: Δ9-Tetrahydrocannabinol; THCA: Tetrahydrocannabinolic acid; TRPA: Transient receptor potential cation channel, subfamily A; TRPM: Transient receptor potential cation channel, subfamily M; TRPV: Transient receptor potential cation channel subfamily V; WADA: World Anti-Doping Agency

Competing interests

PL: Vice-President of Patient Research and Access for Tilray, ownership interest (stocks, stock options, or other ownership interest excluding diversified mutual funds), salary.
JE: Vice-President and Chief Science Officer for Tilray, ownership interest (stocks, stock options, or other ownership interest excluding diversified mutual funds), salary.

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Efficacy and safety of exogenous ketone bodies for preventive treatment of migraine: A study protocol for a single-centred, randomised, placebo-controlled, double-blind crossover trial

Abstract

Background: Currently available prophylactic migraine treatment options are limited and are associated with many, often intolerable, side-effects. Various lines of research suggest that abnormalities in energy metabolism are likely to be part of migraine pathophysiology. Previously, a ketogenic diet (KD) has been reported to lead to a drastic reduction in migraine frequency. An alternative method to a strict KD is inducing a mild nutritional ketosis (0.4–2 mmol/l) with exogenous ketogenic substances. The aim of this randomised, placebo-controlled, double-blind, crossover, single-centre trial is to demonstrate safety and superiority of beta-hydroxybutyrate (βHB) in mineral salt form over placebo in migraine prevention.

Methods/design: Forty-five episodic migraineurs (5–14 migraine days/months), with or without aura, aged between 18 and 65 years, will be recruited at headache clinics in Switzerland, Germany and Austria and via Internet announcements. After a 4-week baseline period, patients will be randomly allocated to one of the two trial arms and receive either the βHB mineral salt or placebo for 12 weeks. This will be followed by a 4-week wash-out period, a subsequent second baseline period and, finally, another 12-week intervention with the alternative treatment. Co-medication with triptans (10 days per months) or analgesics (14 days per months) is permitted. The primary outcome is the mean change from baseline in the number of migraine days (meeting International Classification of Headache Disorders version 3 criteria) during the last 4 weeks of intervention compared to placebo. Secondary endpoints include mean changes in headache days of any severity, acute migraine medication use, migraine intensity and migraine and headache-related disability. Exploratory outcomes are (in addition to routine laboratory analysis) genetic profiling and expression analysis, oxidative and nitrosative stress, as well as serum cytokine analysis, and blood βHB and glucose analysis (pharmacokinetics).

Discussion: A crossover design was chosen as it greatly improves statistical power and participation rates, without increasing costs. To our knowledge this is the first RCT using βHB salts worldwide. If proven effective and safe, βHB might not only offer a new prophylactic treatment option for migraine patients, but might additionally pave the way for clinical trials assessing its use in related diseases.

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Trial registration: ClinicalTrials.gov, NCT03132233. Registered on 27 April 2017.

Keywords: Migraine, Migraine prevention, Exogenous ketone bodies, Beta-hydroxybutyrate, 3-Hydroxybutyrate, Ketosis, Randomised controlled trial, Placebo-controlled, Crossover, Clinical trial

Background

Migraine is a complex, common and debilitating neurological disorder [1] that affects approximately 17% of women and 8% of men in Europe [2]. With a peak incidence during the most productive years of life, migraine not only causes a huge amount of suffering, but also inflicts a substantial amount of costs on society: approximately €18.5 billion per year in Europe alone [3].

Various lines of research suggest that brain energy metabolism abnormalities are likely to be part of migraine pathophysiology [4–9]. Specifically, there is some evidence for reversible abnormalities in mitochondrial functioning in migraine [7, 8, 10]. For example, treatment with riboflavin and coenzyme Q10 has been shown to have migraine-protective effects [4, 7, 9–12], probably via a positive effect on energy metabolism [7, 10]. Lactic and pyruvic acid, markers of mitochondrial disease, have been found to be increased in migraineurs [13]; ^{31}P-MRS patterns seen in migraine are consistent with what is seen in mitochondrial disorders [5, 14–16]; and COX-negative fibres typical of mitochondrial diseases have also been seen in some patients with migraine [6]. A breakdown of the resting membrane potential due to lack of ATP could explain cortical abnormalities in excitability, which have been reported in migraine [17–21] and would offer a mechanism by which the trigeminal pain pathway, whose afferents densely innervate the meninges and its associated blood vessels, could be activated or sensitised in migraine. The activation and sensitisation of the trigeminal pain pathway is considered the current understanding for the origin of the migraine headache [22–24].

Despite causing a huge amount of suffering and a substantial amount of costs for society [3, 25], current migraine treatment options are limited and their mechanisms of action are also not completely understood [26]. Most of the prophylactic agents licensed to date are not migraine specific and are additionally associated with significant, sometimes intolerable, side-effects. Furthermore, their migraine-preventive properties tend to be moderate at most. Hence, there is a need for developing alternative anti-migraine therapies.

The ketogenic diet (KD) was developed about 100 years ago after the observation that prolonged fasting has anticonvulsive properties [27]. With its high fat, low carbohydrate and medium protein content, the KD simulates the metabolic effects of starvation. With the advent of antiepileptic medication the rather complicated KD fell out of favour. However, within recent years it has received new interest, in particular since ketone bodies (KBs) might be beneficial for a variety of neurological and even psychiatric disorders due to various different mechanisms [28–30], including improved energy metabolism.

Recently, some case studies [31–34] and a first short proof-of-concept study [35] have demonstrated a reduction in migraine attack frequency, severity and use of acute anti-migraine medication during ketosis—with effect sizes ranging from total absence of attacks [31] to a reduction to 1/5th of the run-in period [35]. In addition, preliminary evidence suggests that the migraine-protective effect may outlast the duration of ketosis [31, 32, 35]. This might be a result of longer-lasting gene expression changes [28, 36]. Elevated KB levels in humans have been shown to be well tolerated for extended periods of time (up to several years) [34, 37–48]. However, a strict KD might not provide a feasible long-term solution for all episodic migraine patients, because patient adherence may be limited and it is not easily implemented in an ambulatory setting.

An alternative means to induce a state of mild to medium nutritional ketosis (0.4–2 mmol/l), irrespective of blood glucose levels, is dietary supplementation with ketogenic substances, such as beta-hydroxybutyrate (βHB) salts [45, 49–52]. This approach could be easily implemented with intake of a ketogenic powder dissolved in water (consisting of a calcium–magnesium–βHB salt three times a day). This intervention seems much more feasible than a strict KD in larger patient populations and avoids the complications of a very restricted high-fat diet. These considerations led us to examine the efficacy and safety of KB mineral salts in migraine prevention within the scopes of a double-blind, randomised, placebo-controlled, efficacy and safety trial with a crossover design.

Material and methods

Study design and setting

The study is an investigator-initiated, double-blind, randomised, placebo-controlled, efficacy and safety trial with a crossover design (see Fig. 1) and a treatment period of 36 weeks. It is a single-centre study;

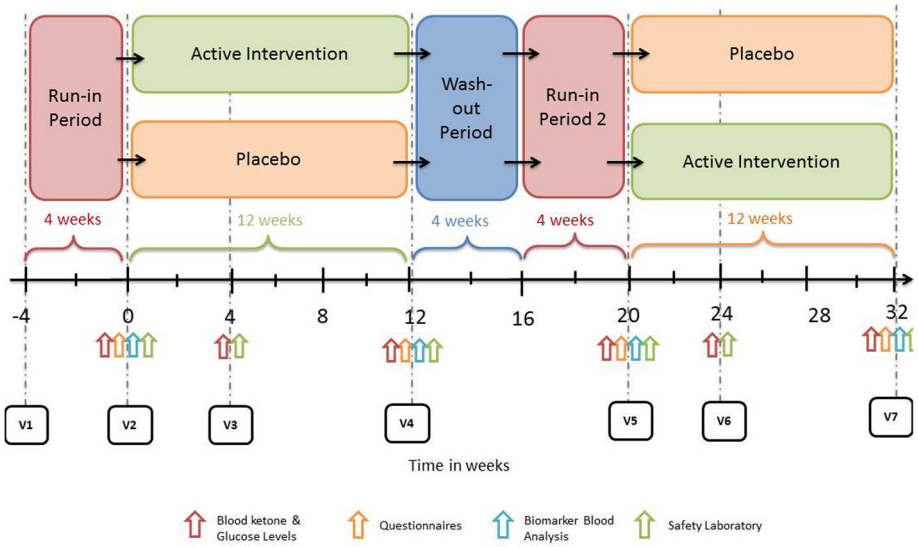

Fig. 1 Flowchart of study design, including timing of measurements and procedures. V = visit

all investigations will take place at the clinical trial unit (CTU) of the University Hospital Basel (USB), Switzerland.

We plan to enrol 45 medium to high-frequency episodic migraineurs (5–14 migraine days/months), with or without aura, aged between 18 and 65 years.

The study period will begin with a 4-week run-in period, during which there is no investigational treatment. The purpose of the run-in period will be observation for baseline comparison. The run-in period will be followed by a 12-week intervention period, when the subjects will receive the investigational medicinal product (IMP) or placebo (orally, three times a day). The intervention period will be followed by a 4-week wash-out and a 4-week second run-in period, during which the subjects will receive no further intervention.

As the study medicament has a half-life of less than 4 h and the outcome measures are based on the last 4 weeks of the 12 weeks intervention only, a 4-week wash-out period was judged to be sufficient. This will be followed by a second 12-week intervention period of the alternative treatment (patients who first received placebo will now receive IMP and vice versa).

Ethics approval has been obtained from the local Ethics Committee (EKNZ 2015-304) and the corresponding competent authority (CA): the National Swiss Drug Agency (2016DR2109). The trial was registered at ClinicalTrials.gov (NCT03132233) prior to starting recruitment. Funding for the study has been received from the Swiss National Science Foundation (SNSF).

Eligibility criteria

Inclusion criteria

Visit 1 (prior to the 4-week run in period) The patient:

1. is between the ages of 18 and 65 years;
2. has been previously diagnosed with migraine (with or without aura) in accordance with the International Classification of Headache Disorders version 3 (ICHD-3) Beta Classification criteria;
3. experiences between 5 and 14 migraine days per month (over the last 4 months) with at least two of the migraines lasting more than 4 h;
4. has an age of onset of migraine younger than 50 years;
5. agrees to refrain from initiating or changing the type, dosage or frequency of any prophylactic medications (exclusive of medications taken for acute relief of migraine symptoms) as well as dietary supplements (such as Q10, riboflavin, etc.) against migraine and for indications other than migraine that in the opinion of the clinician may interfere with the study objectives (e.g. antidepressant, anticonvulsants, beta blockers, etc.) for the duration of the study;
6. has not changed type, dosage or frequency of any prophylactic medications (exclusive of medications taken for acute relief of migraine symptoms) as well as dietary supplements (such as Q10, riboflavin, etc.) against migraine and for indications other than migraine that in the opinion of the clinician may interfere with the study objectives (e.g. antidepressant,

anticonvulsants, beta blockers, etc.) for at least 3 months prior to study onset;
7. refrains from making any drastic changes to their diet for the duration of the study, including periods of fasting;
8. agrees to use the study medication as intended, follow all of the requirements of the study including follow-up visit requirements, record required study data in the subject dairy and other self-assessment questionnaires, and is okay with drawing blood samples; and
9. is able to provide written informed consent.

Visit 2 (baseline visit, just prior to 12-week intervention) Before starting the intervention, the study patient must meet all of the following inclusion criteria.

The patient:

1. continues to meet all baseline (Visit 1) eligibility criteria;
2. has experienced between 5 and 14 migraine days; and
3. has demonstrated compliance with the headache diary during the run-in period.

Exclusion criteria

Visit 1 (prior to the 4-week run-in period) Subjects meeting any of the following criteria cannot be included in this research study.

The patient:

1. has a concomitant medical condition that will require oral or injectable steroids during the study;
2. has a history of any significant neurological, psychiatric or other medical condition that in the opinion of the investigator may confound the study assessments (no liver or kidney diseases in particular);
3. has a cardiovascular disease (hypertension in particular) or a history thereof;
4. has a known history of suspected secondary headache;
5. currently takes simple analgesics or non-steroidal anti-inflammatory drugs (NSAIDs) for more than 14 days per 4 weeks or triptans for more than 10 days per 4 weeks for headaches or other body pain;
6. currently takes prescription opioids;
7. has previous diagnosis of medication overuse headache (MoH), which has reverted to episodic migraine within the last 6 months;
8. meets the ICHD-3 Beta Classification criteria for chronic migraine (> 15 headache days per month);
9. has failed an adequate trial (2 months or longer) of at least three classes of a drug therapy for the prophylaxis of migraine;
10. has had surgery for migraine prevention;
11. has received Botox injections within the last 6 months;
12. is pregnant or thinking of becoming pregnant during the study period, or is of childbearing years and unwilling to use an accepted form of birth control;
13. is participating in any other therapeutic clinical investigation or has participated in a clinical trial in the preceding 30 days;
14. belongs to a vulnerable population or has any condition such that his or her ability to provide informed consent, comply with the follow-up requirements or provide self-assessments is compromised (e.g. homeless, developmentally disabled or prisoner); and
15. is thinking to start, change or stop a hormone-based contraception.

Visit 2 (baseline visit, just prior to 12-week intervention) Before starting the intervention, the study patient must meet none of the following exclusion criteria.

The patient:

1. has initiated or changed the type, dose or frequency of any prophylactic medication for indications other than migraine that in the opinion of the clinician may interfere with the study objectives during the 4-week run-in period

Interventions

Experimental intervention

The investigational medicinal product (IMP) used in this clinical trial is D-L-beta-hydroxybutyrate (βHB) in powdered calcium (Ca^{2+})–magnesium (Mg^{2+})–salt form (Ca-Mg-βHB). D-L-Beta-hydroxybutyrate calcium salt (Ca-βHB) dissolves in water (i.e. in the body) into Ca^{2+} and D-L-beta-hydroxybutyrate (βHB), the compound of interest. D-L-Beta-hydroxybutyrate magnesium salt trihydrate (Mg-βHB) dissolves in water (i.e. in the body) into Mg^{2+} and βHB. Also known as beta-hydroxybutyric acid, 3-hydroxybutyric acid or 3-hydroxybutyrate, βHB is an endogenous metabolite with the formula $CH_3CH(OH)CH_2CO_2H$. It is a beta-hydroxy acid and a keto acid. The IMP was purchased from Ergomax (https://www.ergomaxsupplements.com) in bulk powder of GMP quality and packaged at Hänseler AG (Herisau, Switzerland). It does not contain anything else other than the βHB mineral salts. The flavour is masked using a sucralose-based sugar-free syrup. The daily dose of 9 g Ca-βHB contains 7.54 g of βHB and 1.47 g Ca^{2+}, and will be divided into three servings

supplied in individual sachets containing 2.51 g βHB and 0.49 g Ca^{2+}, respectively. The daily dose of 9 g Mg-βHB contains 6.6 g βHB and 0.77 g Mg^{2+}, and will also be divided into three servings supplied in individual sachets containing 2.2 g βHB and 0.26 g Mg^{2+}. Both IMPs are provided as a water-soluble powder. During the 12-week intervention period participants will consume the IMPs in three oral doses, to be taken with or after breakfast, lunch and dinner, respectively. This adds up to less than 100 kcal per day. Each serving will raise KB levels for approximately 3 h. To minimise possible gastrointestinal symptoms such as bloating or diarrhoea, patients are instructed to increase the dosage over time, starting with half the dose during the first week before reaching the maximum dosage by day 7.

Elevated ketone body (KB) levels have been shown to be well tolerated for extended periods of time (up to several years) [31–35, 37–49]. During fasting, the healthy adult is capable of producing up to 185 g of KBs [53]. Previously, orally administered sodium βHB salts with higher doses ranging between 0.5 and 1 g per kg have been shown to be tolerated in both the short term [39, 50, 54, 55] and the long term [45, 49, 52, 56, 57] with no significant side-effects. The rather conservative dose of 18 g βHB mineral salt per day (as compared to endogenous production during starvation) was determined largely by the mineral load of Mg^{2+} and Ca^{2+}, which we wanted to keep within acceptable ranges. Not going over the suggested maximum supplemental guidelines meant 9 g of Mg-βHB and 9 g of Ca-βHB, respectively. A similar dose of 5 g βHB/day was shown to lead to a modest elevation in blood KB (up to 0.4 mmol/l) [52], supporting the safety of our chosen dose. A Ca-βHB and Mg-βHB salt was chosen to avoid the potentially negative long-term consequences of high sodium intake.

To the best of our knowledge, no human controlled trials using βHB mineral salts have been done, either for migraine or for any other indication, and there seems to not yet be other human published data on specifically Ca-βHB and Mg-βHB. However, recently, βHB supplements, mostly in mineral salt form similar to our IMP, are being produced and sold in the USA, marketed as a sport/life-style supplement. A couple of million servings with a similar dosing to our IMP have been consumed without any incidents reported.

Control intervention

The placebo powder consists of mannitol, a sugar alcohol, which has the same texture, colour and packaging. Taste and smell are masked in the applied form (both are diluted in sugar-free syrup) and therefore similar. It is used by the USB Pharmacy as the standard placebo substance. In higher doses it can also lead to gastrointestinal symptoms [58], which means it has similar potential side-effects to the IMP.

Packaging, labelling and supply

The IMP and placebo are provided in sachets containing either one dose of Ca-βHB (3 g) or one dose of Mg-βHB (3 g), respectively, in powder form (see earlier). The whole supply for the study (ca. 3 kg per patient, > approximately 70 kg IMP and 70 kg placebo in total) will be delivered to and stored at the pharmacy of the University Hospital Basel. Patients will be provided with sufficient quantity to last from each visit to the next. The IMP will be labelled in accordance with regulatory requirements.

Storage conditions

The IMP is stored at room temperature. After delivery it will be stored at the USB Pharmacy until the end of the study.

Concomitant interventions (treatments)

The use of analgesics and triptans is allowed for less than either 14 days (analgesics) or 10 days (triptans), respectively, per month. They are not predicated to have an effect on the study outcomes. Steroids (oral or injectable) as well as prescription opioids are not permitted for the duration of the study period, including run-in and follow-up. Migraine-related surgery and Botox injections within the last 6 months are also not permitted. Prophylactic medications (exclusive of medications taken for acute relief of migraine symptoms) as well as dietary supplements (such as CoenzymeQ10, riboflavin, etc.) against migraine and for indications other than migraine that in the opinion of the clinician may interfere with the study objectives (e.g. antidepressant, anticonvulsants, beta blockers, etc.) are permitted as long as the type, dosage or frequency is not changed for the duration of the study and has not been changed at least 3 months prior to study onset. Hormone-based contraception is permitted as long as the patient does not intend to start, stop or change it for the duration of the study and at least 3 months prior to the intervention. Other hormonal treatment is not permitted.

Outcome measures

Primary outcome measure

Mean change from baseline in number of migraine days (meeting ICHD-3 criteria) during the last 4 weeks of intervention compared to placebo In order to assess the therapeutic efficacy of externally induced mild ketosis over placebo in migraine prevention, a detailed headache diary in pen and paper form is used to record the change in monthly migraine frequency. The headache diary includes: month, days 1–31, distinction migraine/headache, pain intensity (Likert scale 0–10), medication, dosage, treatment effectiveness of acute

medication used (Likert scale 0–10), migraine-associated symptoms, days with menstruation and potential trigger factors (if known).

A day with head pain will only be classified as a migraine day if it meets ICHD-3 classification criteria. According to the International Headache Association and the European Medical Association Guidelines, the recommended measure to assess migraine frequency reduction is the change in migraine days per 4 weeks compared to baseline. This approach has one major advantage over the other frequently used method of recording the number migraine attacks: attack duration is also taken into consideration.

Secondary outcome measures

Mean change from baseline in number of headache days of any severity (meeting ICHD-3 criteria) during the last 4 weeks of intervention compared to placebo The same headache diary in pen and paper form is used to record the change in 4-week headache frequency. A day with headache will only be classified as a headache day if it does not meet ICHD-3 migraine classification criteria. According to the International Headache Association and the European Medical Association Guidelines, the change in migraine days versus headache days per 4 weeks compared to baseline should be recorded separately in migraine patients who experience both headache types.

Mean change from baseline in consumption of acute migraine medication (analgesics or triptans) measured in days with acute headache medication use during the last 4 weeks of the intervention The same headache diary in pen and paper form is used to record the change in days with acute headache medication use (analgesics or triptans). With this approach the number of tablets is not of primary interest, but rather the number of days on which one or more analgesics or triptans were consumed. A clinically meaningful migraine preventative is predicted to lower the days during which migraine acute medication is necessary.

Mean change from baseline in migraine intensity (measured with a numerical rating scale from 1 to 10) during the last of 4 weeks of the intervention period The same headache diary in pen and paper form is used to record a potential change in migraine intensity, as measured with a numerical rating scale from 1 to 10. Each migraine or headache day, respectively, is given an intensity score, with 0 being not painful at all and 10 being an operation without anaesthesia.

Change in disability from baseline during any treatment period, as assessed with the Migraine Disability Assessment and the Headache Impact Test (comparison baseline and post-intervention score) In order to assess a change in migraine and headache-related disability, two commonly used validated and reliable questionnaires are used: the Migraine Disability Assessment (MIDAS) and the Headache Impact Test (HIT-6) [59–63]. The German translations were also shown to have adequate reliability and validity [60, 63]. Both questionnaires will be provided as pen and paper versions and will be filled out at the baseline visits (V2 and V5) and the end of innervation visits (V4 and V7), respectively.

Exploratory outcome measures

The demographic characteristics and neurological examination will be assessed at one time point (Visit 1). To determine the potential mechanisms of action of successful migraine treatment, we are going to examine single nucleotide polymorphism (SNP) markers in order to assess the genetic background of migraine patients involved in this study. In addition to this, we also plan to conduct gene expression analysis. SNP and gene expression analysis will be conducted using microarrays. In our analysis strategy we especially focus on, but not limit to, genes coding for mitochondrial-related enzymes (citrate synthase, cytochrome C oxidase subunit 1, succinate dehydrogenase subunit A).

We will also examine the serum concentration of oxidative and nitrosative stress markers (malondialdehyde (MDA), carbonylated proteins, nitrate, nitrite, nitrotyrosine) using enzyme-linked immunosorbent assay (ELISA) and mass spectroscopy. In addition to Hba1c, insulin, cortisol, lactate and markers of functioning, cytokines will be analysed using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel—Premixed 41 Plex—Immunology Multiplex Assay.

Optionally, patients will also receive an Abbott FreeStyle Libre Blood Glucose Monitoring System for 2 weeks at visits V2, V3, V5 and V6, respectively, which will allow permanent tissue glucose monitoring without finger pricking. This allows us to examine a potential association between blood glucose levels (hyperglycaemia or hypoglycaemia) and migraine, and the potential effect of the study medication on glucose levels.

Safety outcomes measures

Safety and tolerability will be determined by:

1. comparison of treatment-emergent adverse events (any event regardless of potential causality with the drug) and treatment-related adverse events (such as gastrointestinal upset) as imputed by the principal

investigator between active treatment and placebo; and

2. examination for potential effects of the intervention on routine laboratory parameters (renal and liver function tests, electrolytes, full blood count, lipids, glucose, CRP, Hba1c, insulin, cortisol, lactate, TSH, FT4 and FT3) in the treatment group compared to the control group.

Study procedure

At screening (Visit 1 (V1), week − 4), patients are informed about preclinical data, alternative treatments, potential risks and benefits of the study (see Fig. 2). Further, written informed consent, including consent for the collection of blood for genetic analysis, from the patients is obtained by the trial physician. After signing the informed consent form, the inclusion and exclusion criteria are verified. If the criteria are fulfilled, the patient will be enrolled in the study under reserve. During V1 the following additional procedures are performed: a detailed first clinical interview/examination, vital signs, migraine diary explanation, where necessary a pregnancy test and a neurological examination. After screening, visits will be scheduled for baseline (V2, week 0). V1 will last approximately 30 min.

At the start of the intervention (baseline/V2, week 0) the following procedures are performed: check inclusion/exclusion criteria and, if met, confirmation of enrolment, migraine diary check, diet check, consumption of first dose of IMP/placebo, adverse events, vital signs, physical examination if necessary, blood draw (for safety, biomarkers and genetic analysis), standardised migraine questionnaires, KB and glucose concentration measurements using a portable point-of-care blood ketone meter (precision xtra from Abbot) and/or the Abbott FreeStyle Libre Blood Glucose Monitoring System. Patients will be randomly assigned to the treatment or control group and receive the according study medication, which will be consumed three times daily for the following 12 weeks. V2 takes approximately 60 min.

After 4 weeks of intervention, there will be another visit (V3, week 4), during which KB and glucose levels will be measured, adverse events will be recorded, vital signs, diet and migraine diary will be checked, a dose of IMP/placebo will be consumed, blood for safety will be drawn, physical examination will be performed if necessary, participants will be provided with the rest of the study medication for the first intervention period and sachets of used study medication will be collected for compliance control. V3 takes about 30 min.

During the visit after the first intervention period (V4, week 12), the following procedures are performed: migraine questionnaires, migraine diary and diet check, consumption of IMP/placebo, KB and glucose measurements, vital signs, blood draw for biomarker and safety analysis, physical examination if necessary and sachets of used study medication will be collected for compliance control. V4 takes about 60 min.

After 8 weeks without intervention, V5 (week 20) takes place, which is identical to the baseline visit (V2). V5 includes the following procedures: standardised migraine questionnaires, migraine diary and diet check, consumption of IMP/placebo, blood draw for biomarker and safety analysis, physical examination if necessary, KB and glucose concentration and vital signs. At this visit the patients will receive the alternative treatment to the first intervention.

After 4 weeks of the second intervention, V6 (week 24, analogous to V3) takes place. The following procedures are performed: migraine diary and diet check, consumption of IMP/placebo, KB and glucose measurements, adverse effects, vital signs, blood draw for safety and physical examination, if necessary. Participants will be provided with the rest of the study medicament and sachets of used study medication will be collected for compliance control. V5 takes about 30 min.

After completion of the second intervention, the last visit (V7, week 32, analogous to V4) takes place. The following procedures are performed: migraine questionnaires, migraine diary and diet check, consumption of IMP/placebo, KB and glucose measurements, vital signs and blood draw for biomarker and safety analysis, physical examination if necessary and collection of sachets of used study medication for compliance control.

All investigations will take place at the clinical trial unit of the University Hospital Basel, Switzerland. Participants are required to keep a detailed headache diary for the entire duration of the study.

Sample size estimation

Determination of sample size

Sample size is estimated to be able to show the superiority of IMP over placebo. A crossover design with 1:1 IMP/placebo:placebo/IMP randomisation is planned.

Fixed sample size estimation

Assumptions Sample size estimation is based on the following assumptions:

- We expect the baseline number of migraine days per 4 weeks to be 10 days in our patient population.
- Placebo effect: based on recent findings [64], we assume a rather strong placebo effect of 32% reduction in the primary endpoint. This corresponds to an absolute reduction of 3 migraine days per 4 weeks.

STUDY PERIOD	Screening	Baseline	Intervention 1		Wash-out	Baseline 2	Intervention 2	
VISIT	V1	V2	V3	V4		V5	V6	V7
TIMEPOINT (in weeks)	-4 (+/-2)	0 (+/-2)	4 (+/-1)	12 (+/-1)		20 (+/-2)	24 (+/-1)	32 (+/-1)
ENROLLMENT:								
Demographics	X							
Medical History	X							
Pregnancy test	X							
Informed consent	X	X						
Inclusion/Exclusion	X	X						
Randomisation		X						
INTERVENTIONS:								
Observational run-in	◆—	—◆				◆—		
Treatment/ Placebo 1		◆—	—	—◆				
Wash-out					◆—	—◆		
Treatment/ Placebo 2							◆—	—
Dispensing of study medication		X	X	X		X	X	X
Collection of study medication			X	X			X	X
ASSESSMENTS:								
Adverse Events			X	X		X	X	X
Vital Signs[1]	X	X	X	X		X	X	X
Physical examination	X							
Migraine Diary[2]	X	X	X	X		X	X	X
MIDAS & HIT-6 questionnaire[3]		X		X		X		X
Blood ketone & glucose level[4]		X	X	X		X	X	X
Blood draw for safety analyis[5]		X	X	X		X	X	X
Blood draw for genetic analysis[6]		X		X		X		X
Blood draw for markers of oxidative / nitrosative stress and cytokines[7]		X	X	X		X	X	X

Fig. 2 Detailed study schedule. [1]Blood pressure, heart rate, weight and height. [2]Pen and paper headache dairy. [3]Migraine Disability Questionnaire (Migraine Disability Assessment (MIDAS)) and Headache Impact Test (HIT), German versions, standard questionnaires for assessing the extent of migraine-related disability. [4]Blood beta-hydroxybutyrate and glucose levels, measured with a portable ketone meter (precision xtra by Abbot). [5]Routine laboratory (renal and liver function tests, electrolytes, full blood count, C-reactive protein, serum cholesterol, triglycerides, serum proteins, albumin, glucose, Hba1c, insulin, cortisol, lactate, TSH, FT4 and FT4). [6]Blood draw (1 × EDTA, 1 × PAXgene) at each time point for genetic profiling and gene expression analysis using microarrays. [7]Blood draw at each time point for oxidative and nitrosative stress markers (malondialdehyde (MDA), carbonylated proteins, nitrite, nitrotyrosine) and serum cytokine measurements (including, but not limited to, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, MCP-1, TNF-α, TNF-β, TGF-β1). V = visit

- IMP effect: synthesising previous findings [4, 64] and our pilot data [65], we aim to detect a difference of 2 days between placebo and IMP.
- We assumed the absolute reduction in migraine days to be normally distributed with a standard deviation of 3 days.
- We assume a conservative intra-patient correlation between IMP and placebo of 0.4.
- Drop out: a high drop-out rate of 30% is assumed.

Re-sampling The sample size was estimated using a re-sampling method. Each sample size ($n_i = 1,...,49 = 12$,

..., 60) was evaluated by sampling $R = 999$ times the reduction in migraine days from a bivariate normal distribution as already described. For each sample, whether superiority of the IMP over placebo could be shown (i.e. whether a two-sided paired t test resulted in significant $p < 0.05$) was tested.

In order to show the superiority of the IMP over placebo with a statistical power of 90%, 45 patients should be recruited in total to ensure 31 evaluable patients, assuming a drop-out rate of 30%. Figure 3 shows how the sample size depends on the expected reduction in number of migraine days in the IMP arm.

Recruitment

Patients will be informed about the study at the Department of Neurology, University Hospital Basel (USB). Moreover, there will by flyers publicly displayed in the waiting room of the neurology and general medicine department of the University Hospitals in Basel, Bern, Zurich and St Gallen, as well as the University Library. An announcement similar to the flyer will be posted on the webpages of the University of Basel "Marktplatz" dedicated to research studies (https://markt.unibas.ch/nc/inserate/kategorie/job-angebot-studien/) as well as the USB website (https://www.unispital-basel.ch/lehre-forschung/studieninserate/) and the University Children's Hospital Basel (UKBB) website (http://www.ukbb.ch/en/research/research-groups/neuromuscular-research.php), respectively. More flyers will be displayed in local pharmacies and pharmacies in Germany (with a radius of approximately 100 km around Basel), local neurologists, the neurological department of the Bruderholzspital (Kantonsspital Baselland) and the Headache Clinic of RehaClinic, located in Baden as well as Bad Zurzach, and also in the neurological outpatient clinic in Brugg (team of Prof. Sandor). Flyers will also be displayed in local busses and trains. The Swiss Headache Society (SKG) and the German migraine and headache society (DMKG) will advertise the trial on their website. All websites may include the link to a short recruitment video (https://www.youtube.com/watch?v=2YzNjIX-k_eY&t=19s) explaining the clinical trial with similar wording to the flyer. The video and an advertisement with similar wording to the flyer will also be advertised on Facebook (for users in a radius of 200 km of Basel). Patients previously contacted for a migraine–sport intervention study at the USB (EKNZ-Number 194/13) will be contacted again, if they previously agreed and met the inclusion criteria for the current study.

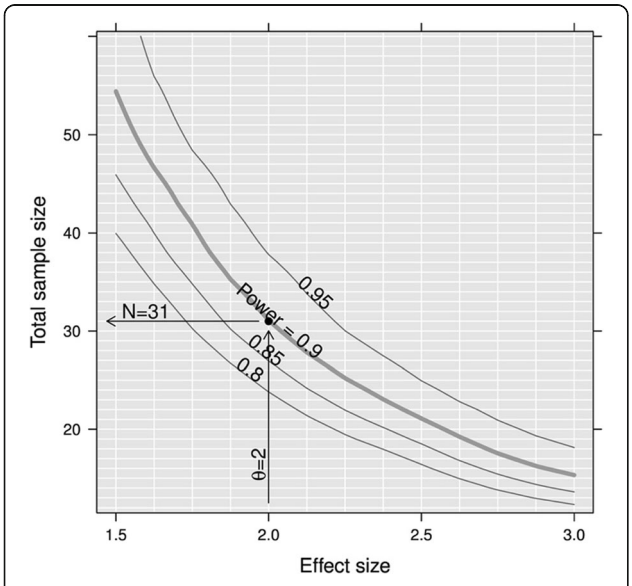

Fig. 3 Sensitivity of sample size with regard to expected difference in reduction in number of migraine days per 4 weeks of IMP compared to placebo. Example given, based on an effect size of 2 and a statistical power of 90%. The curves are smoothed and are for illustrative purposes only

Randomisation and blinding

Methods of minimising bias

Bias will be minimised by randomisation in 1:1 allocation and blinding of patients and investigators to the intervention. Randomisation will be done using an electronic data capture (EDC) system (SecuTrial) through an independent individual. The medication will be numerically labelled at the Pharmacy of the University Hospital of Basel and will then be provided to the ward and applied to the patient. This will allow a double-blinded randomisation (patient and treating physician will be blinded to the treatment).

The placebo powder has the same texture, colour and packaging as the IMP, so they cannot be distinguished in their appearance. The placebo also has a similar side-effect profile to the IMP. Data will be checked for protocol violation by the independent monitoring institution (see Quality assurance and control).

Randomisation

A crossover design with 1:1 AB/BA (IMP/placebo:placebo/IMP) randomisation is planned. The randomisation list will be computer generated and uploaded into the electronic data capture software SecuTrial by the responsible Data Manager at the Clinical Trial Unit (CTU) of the University Hospital Basel. Only unblinded personnel at the Pharmacy of the University Hospital Basel and at the CTU Basel will have access to the randomisation list. Just before the baseline visit, a clinical investigator will use SecuTrial to automatically assign a randomisation number from the randomisation list to the patient.

An additional list with medication numbers complementing the treatment arm of the first intervention

period will be provided by the CTU, in order to allow opposite treatment allocation during the second intervention period without unblinding the trial staff.

Blinding procedures

The study medication (IMP or placebo) will be provided as similar-looking medication in sachets. The medication will be packed by the Pharmacy of the University Hospital of Basel and will be numerically labelled using the randomisation list provided by the responsible Data Manager at CTU Basel. All investigators and patients will remain blinded until the trial is completed and the database has been locked.

Unblinding procedures (code break)

In the case of problems and safety concerns that cannot be solved with ongoing randomisation, the participant's allocated intervention will be revealed. Unblinding can be performed by authorised investigators using the EDC software SecuTrial. Each unblinding is documented in the EDC's integrated audit trail system and automatically reported to the principal investigator.

Data management

The study data recorded in the CRF will be transferred to a corresponding electronic CRF (e-CRF) by the clinical investigators. The principal investigator and co-investigator at the study site will be responsible for assuring that the data entered into the e-CRF is complete and accurate, and that the entry and updates are performed in timely manner. All information recorded in the e-CRFs will be traceable to the source documents in the patient's file and in the date source files.

Data management system

Data management will be conducted fulfilling all ethical and legal requirements according to Good Clinical Practice (GCP) and the Swiss Laws as "Bundesgesetz über die Forschung am Menschen" (Humanforschungsgesetz (HFG)).

The e-CRF will be implemented by the data management group at the Clinical Trial Unit (CTU) of the University Hospital Basel using the electronic data capture (EDC) software SecuTrial. The EDC system runs on a server maintained by the IT department of the University Hospital Basel.

Data entry will be performed by trained clinical investigators at the UKBB.

Data security, access and back-up

The EDC system is accessible via a standard browser on a www-connected device. Password protection and user-right management ensures that only authorised UKBB or CTU staff can enter the system to view, add or edit data according to their permissions. User administration and user training is performed by the CTU Basel according to predefined processes.

Back-up of SecuTrial study data is performed regularly according to the processes of the IT department of the University Hospital Basel. An integrated audit trail system will maintain a record of initial entries and changes made, reasons for change, time and date of entry, and user name of the person authorising entry or change.

Source data will be available at the site to document the existence of the study participants and will include the original documents relating to the study (patient demographics, medical history, medication, neurological examination, informed consent forms).

Analysis and archiving

The EDC system will be locked after e-CRF data entry is completed, all data have been monitored and raised queries have been resolved. The complete study dataset is exported from the database and transferred to the study statistician as well as the principal investigator through a secure channel. The exported data will be archived for 10 years by the principal investigator.

Electronic and central data validation

Data entered into the e-CRF will be validated for completeness and discrepancies automatically. The data will be reviewed by the responsible investigator as well as an independent monitor. The monitor will raise queries using the query management system implemented in SecuTrial. Designated investigators have to respond to the query and confirm or correct the corresponding data. Thereafter, the monitor can close the query.

Data monitoring

To ensure the quality of the study conduct and of the data, monitoring of the study is performed by organisations independent of the study (CTU, USB and Kammermann Monitoring Services GmbH). All inclusion and exclusion criteria are checked, and the monitor controls whether the data have been recorded correctly in the CRF, whether the drug accountability is correct and whether serious adverse events (SAEs) have occurred during the study.

Statistical analyses

Detailed methodology for summaries and statistical analyses of the data collected in this study will be documented in a statistical analysis plan. The statistical analysis plan will be finalised before database closure and will be under version control at the CTU, University Hospital Basel.

The primary endpoint, the number of migraine days in the last 4 weeks of treatment, will be measured twice for

each patient, once after the placebo treatment period and once after the IMP treatment period. The number of migraine days in the 4 weeks before the start of treatment will be assessed for both treatment periods, thus there will be two baseline values that will be used as covariates. This process has the aim of correcting for any potential seasonal variation in baseline migraine frequency or carry-over effects.

Hypothesis

The *null hypothesis* is that there is no difference in the difference in number of migraine days per 4 weeks from baseline to the last 4 weeks of intervention between the IMP and the placebo treatment.

The corresponding *alternative hypothesis* is that the difference in the number of migraine days per 4 weeks from baseline to the last 4 weeks of intervention differs between the IMP and the placebo treatment.

Statistical criteria for termination of trial

No early stopping is planned, either for efficacy or for futility.

Planned analyses

Datasets to be analysed, analysis populations The full analysis set (FAS) consists of all patients who are randomised and for whom the number of migraine days per 4 weeks at baseline is available.

The intention to treat (ITT) will include all randomised patients for whom the number of migraine days of at least the first 4 weeks of the first treatment period is available.

The per protocol (PP) will include all patients from the ITT set for whom the primary endpoint is available for both treatment periods, who are compliant as per the protocol (see later) and who have no protocol violations (to be defined in detail in the statistical analysis plan).

Primary analysis The primary endpoint, the number of migraine days in the last 4 weeks of treatment, will be measured twice for each patient, once after the placebo treatment period and once after the IMP treatment period. The number of migraine days in the 4 weeks before start of treatment will be assessed for both treatment periods, thus there will be two baseline values that will be used as covariates. This process has the aim of correcting for any potential seasonal variation in baseline migraine frequency or carry-over effects.

The primary analysis will be performed using a linear, mixed-effects regression model. The primary model will include the primary endpoint (the number of migraine days in the last 4 weeks of treatment) as the response variable, the respective baseline value as a covariate, treatment (IMP vs placebo) and period (first vs second) as main effects, the two interaction terms "treatment × period" and "treatment × baseline value", and patient as random effects. A significant interaction term between treatment and period would indicate a carry-over effect. Since it is not known how strongly the primary endpoint correlates with the baseline value, it is not known whether including the baselines as covariates in the model is sensible. Therefore, the already described primary model will be compared to models without the interaction term "treatment × baseline value" and without both the interaction term "treatment × baseline value" and the baseline value as a covariate by means of Akaike's Information Criterion (AIC).

The primary analysis will be done on the ITT set.

Subgroup analyses The following a priori defined subgroups will be investigated: sex (male/female), migraine with aura (yes/no) and baseline frequency of migraine days (medium = 5–9 days/4 weeks; high = 10–14 days/4 weeks). For each subgroup, the main effect of the subgroup and the interaction term "subgroup × treatment" will be added to the already described statistical model. In the case of a trend ($p < 0.10$) for an interaction effect—indicating a difference in the treatment effect between the subgroups—separate models will be fit for each subgroup.

Sensitivity analysis The main analysis, without subgroup analyses, will be repeated on the PP set. Potential deviations from the results of the ITT analysis will be described in detail.

Secondary analysis The secondary (exploratory) objectives are to assess the therapeutic efficacy of externally induced mild ketosis by the IMP regarding the following secondary endpoints:

- change in number of headache days of any severity from baseline (meeting ICHD-3 criteria) during the last 4 weeks of intervention;
- change in number of headache days of any severity from baseline (meeting ICHD-3 criteria) during the last 4 weeks of follow-up;
- change in consumption of acute migraine medication from baseline (analgesics or triptans)—measured in days with acute headache medication use—during the last 4 weeks of intervention;
- change in average migraine intensity from baseline—assessed with a VAS from 0 to 10 for each migraine episode—during the last of 4 weeks of the intervention period; and

- change in disability from baseline—assessed with the Migraine Disability Assessment (MIDAS) and the Headache Impact Test (HIT-6)—to the last of 4 weeks of the intervention period.

All of these secondary endpoints will be analysed as described for the primary endpoint with the corresponding baseline measure as covariate, if available.

All secondary analyses are done on the ITT set.

Exploratory analyses The exploratory objectives are to assess the potential mechanisms of action of externally induced mild ketosis by the IMP regarding markers of oxidative stress, markers of inflammation, glucose, fat, protein metabolism and genetic analyses:

- Serum concentration changes from baseline of oxidative and nitrosative stress markers (malondialdehyde (MDA), carbonylated proteins, nitrate, nitrite, nitrotyrosine) using ELISA and mass spectroscopy. This exploratory endpoint will be analysed as described for the primary endpoint with the corresponding baseline measure as a covariate.
- Serum concentration changes from baseline in markers of fat (triglycerides, cholesterol, HDL, LDL) or glucose metabolism (insulin, glucose, cortisol, Hba1c and lactate) during the last 4 weeks of intervention. This exploratory endpoint will be analysed as described for the primary endpoint with the corresponding baseline measure as a covariate.
- Serum concentration changes from baseline in serum inflammatory markers (cytokines including, but not limited to, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, MCP-1, TNF-α, TNF-β, TGF-β1) during the last 4 weeks of intervention, using a multiplex immunoassay analysed with a BioPlex 200. This exploratory endpoint will be analysed as described for the primary endpoint with the corresponding baseline measure as a covariate.

The following exploratory endpoints will be analysed with standard methods for gene and/or gene expression variation analysis:

- genetic profile (single nucleotide polymorphisms (SNPs)) of all patients involved in the study and correlation of the genetic markers with other outcome measures;
- gene expression changes before and after diet using expression microarrays with a special focus on mitochondrial-related genes (citrate synthase, cytochrome C oxidase subunit 1, succinate dehydrogenase subunit A); and
- correlation of gene expression changes with the genetic profile of the patients (eQTL analysis in combination.

All exploratory analysis is done on the ITT set.

Safety analysis Safety and tolerability will be determined by:

- comparison of treatment-emergent adverse events (any event regardless of potential causality with the drug) and treatment-related adverse events as defined by the principal investigator between active treatment and placebo; and
- examination for potential effects of the intervention on routine laboratory parameters (renal and liver function tests, electrolytes, full blood count, CRP, lipids, Hba1c, insulin, cortisol, lactate, TSH, FT4, FT3) in the treatment group compared to the control group.

Deviation(s) from the original statistical plan If substantial deviations of the analysis as outlined in these sections are needed for whatever reason, the protocol will be amended. All deviations of the analysis from the protocol or from the detailed analysis plan will be listed and justified in a separate section of the final statistical report.

Handling of missing data and drop-outs

The frequency of, timing of and reasons for, as well as all side-effects of, drop-outs will be reported for each treatment. Patients who drop out during the first run-in period or during the first 4 weeks of the first treatment period will be excluded. All patients who drop out later will be included in the ITT set.

For patients who drop out after the first 4 weeks and before the end of the first treatment period, the primary endpoint for the first treatment period will be imputed using multiple imputations. If appropriate, imputations will be accounted for baseline value and number of migraine days during the first treatment period, as far as available. The primary endpoint for the second treatment period will not be imputed for these patients.

For patients who drop out after the end of the first treatment period and before the first 4 weeks of the second treatment period are finished, the primary endpoint for the second treatment period will not be imputed. The primary endpoint for the first treatment period will be available.

For patients who drop out after the first 4 weeks and before the end of the second treatment period, the primary endpoint for the second treatment period will be imputed as already described.

Thus, for each patient included in the ITT set, the primary endpoint will be available (whether measured or imputed) for at least the first treatment period and will be taken into account with the proposed mixed effects models.

In case there are indications for missing data not at random, the inverse probability of censoring weights (IPCW) will be considered.

Statistical criteria for termination of trial No early stopping is planned, either for efficacy or for futility.

Quality assurance and control

The principle investigator (PI) is responsible for implementing and maintaining quality assurance and quality control systems with written SOPs and Working Instructions. The PI is responsible for proper training of all involved study personnel. To assess high-quality conduct of the trial in accordance with the protocol, all medical staff involved in this study are certified in good clinical practice (GCP).

Data handling and record-keeping/archiving

Paper documents including the results of the blood analysis, the headache diaries, questionnaires and all study-related documents will be filed in the study files and stored in the hardcopy archive of UKBB on a dedicated shelf.

Case report forms

For each subject included in this study, a case report form (CRF) will be completed, dated and signed by a study investigator. Data will be recorded in the CRF from the source documents, which may include medical notes and results obtained from laboratory reporting systems.

All participants receive a unique identification number (patient ID) and no identifying data such as name, initials or birth date will be collected in the CRF.

Specification of source documents

Source data will be available at the site to document the existence of the study participants. Source data will include the original documents relating to the study (patient demographics, medical history, medication, neurological examination, informed consent forms) as well as the MIDAS and the HIT-6 questionnaire.

Record-keeping/archiving

All study data, including CRFs and informed consent forms, will be archived for a minimum of 10 years after termination (or premature termination) of the clinical research project. Paper documents including the results of the blood analysis and gene expression changes as well as questionnaires will be stored in the hardcopy archive of the UKBB.

Monitoring

To ensure the quality of the study conduct and of the data, monitoring of the study will be performed by a person independent of the study (Kammermann Monitoring Services GmbH, Zug, Switzerland). All inclusion and exclusion criteria will be checked and whether the data have been recorded correctly in the CRF, whether the drug accountability is correct and whether SAEs have occurred during the study.

Audits and inspections

All study documentation and the source data/documents will be accessible to auditors/inspectors (also EKNZ and CA) and questions will be answered during inspections. All involved parties must keep the participant data strictly confidential.

Confidentiality and data protection

Direct access to source documents will be permitted for purposes of audits and inspections (ICHE6, 6.10). The investigators of the study will have access to the protocol, dataset (including questionnaires, demographical/clinical data) and statistical code during and after the study. The patients' identities will never be published in any abstracts or publications. A transfer of data will only take place for study purposes and only in encoded form. Third persons will not gain any insight into original data. For inspection purposes, insight into the original data will be permitted to the members of the appropriate authorities and also for the members of the local ethics committee, EKNZ. During the study, confidentiality will be guaranteed. The principal investigator will guarantee compliance with national and international data security.

Storage of biological material, related health data and returned study medication

Blood samples will be sent immediately to the earlier specified research laboratories. DNA and RNA extraction will be conducted immediately after arrival at the research laboratory. The extracted DNA/RNA will be sent for microarrays analysis on dry ice to Life&Brain, Bonn, Germany.

Biological material and related health data will be stored in an encrypted format for follow-up analyses.

In order to assess compliance of study medication intake, empty and full sachets are returned by the patients at visits 3, 4, 6 and 7. A member of the study team will count and balance the returned containers and can check the correct intake. This will be captured in an appropriate form. A qualified person from the study team will check the number of dispensed/taken

medications and complete a study-specific drug accountability form. After completion of the clinical trial, leftover study medication will be destroyed.

Safety assessments

Adverse events are monitored throughout the study. At every study visit, patients are asked about adverse events and their vital parameters are measured. If an AE is reported, a clinical examination is performed. The following safety parameters amongst other parameters are checked at visits 2, 3, 4, 5, 6 and 7 to determine safety of the treatment: routine laboratory parameters (renal and liver function tests, electrolytes, full blood count, C-reactive protein, serum cholesterol, triglycerides, serum proteins, albumin, glucose, Hba1c, insulin, cortisol, lactate, TSH, FT4 and FT3), blood pressure, heart rate, weight and height, assessed after 5 min of resting in a supine position.

As βHB is an endogenous substance we are not expecting any treatment-related serious adverse events on routine laboratory measures. Nevertheless, the intake of the IMPs will be stopped in the case of clinically significant changes in any of the parameters measured. In the event of any serious adverse events (treatment related or unrelated) occurring during intake of the IMPs, treatment will also be stopped immediately. If pathologic changes should be detected, whether related to or independent of migraine, the affected patients will be informed immediately and the possibilities of further investigation, respectively treatment of these abnormalities according to current medical knowledge, will be discussed.

Reporting of serious adverse events and other safety-related events

Treatment-emergent serious adverse events (any event regardless of potential causality with the drug) and treatment-related adverse events as imputed by the principal investigator (such as gastrointestinal upset) will be recorded. Reporting to the EKNZ will take place according to the clinical trials of medicinal products guidelines for notification and reporting of Swissethics. In brief:

- Serious adverse events (SAEs) with fatal consequences or where a connection is suspected with the intervention will be reported within 7 days.
- Suspected unexpected serious adverse reactions (SUSARs) with fatal consequences will be reported within 7 days, other SUSARs within 15 days.
- SAEs that may be related to the intervention under investigation in other clinical trials will be reported within 15 days.

AEs of this trial are graded in the most recent Common Terminology Criteria for Adverse Events (CTCAE) version 5.0, which was published in November 2017 and became effective in April 2018 [66], published by the National Cancer Institute (NCI) of the National Institutes of Health (NIH).

Follow-up of (serious) adverse events

Patients with adverse reactions which have occurred in the context of the study will be followed up by the investigator up to 30 days after the last visit.

Discussion

We propose a single-centre, randomised, double-blind, placebo-controlled, crossover trial to determine whether treatment with βHB in mineral salt form has a positive effect on migraine frequency and associated symptoms. To our knowledge this is the first RCT using exogenous KB salts worldwide. If proven effective, βHB might offer a new prophylactic treatment option for moderately to strongly affected migraine patients, or at least a subgroup thereof. A demonstration of its safety might additionally pave the way for clinical trials assessing its use in related diseases.

Planning clinical trials in migraine is challenging for the following reasons: migraine is an episodic disease with a fluctuating nature (i.e. in some patients, migraine frequency can vary substantially from one month to the next or one season to the other, which makes it harder to demonstrate a treatment-related effect); the placebo effect is quite large, between 20 and 40% [67], which further adds to this problem; individual migraine attacks are of different length, and in more severely affected patients are sometimes hard to identify [68]; some patients suffer from headache of a different quality in addition to migraine and this distinction must be made by the patient subjectively [68]; and there is no objective biomarker for migraine or disease severity [68].

In order to address these problems, we have: incorporated two baseline periods to account for seasonal changes, and chosen a conservative effect size as well as a study population of moderate to high-frequency episodic migraineurs (5–14 headache days per month), in order to make it easier to demonstrate a sufficiently large effect size within a short timeframe, without introducing any confounds associated with chronic migraine, such as frequent co-morbidities [69]; calculated with a quite large placebo effect of 30%; chosen migraine days versus migraine attack frequency as the primary outcome; included a thorough briefing of each patient on the characteristic features of a migraine versus a headache attack; and included a detailed medical history and

diagnostic consultation by a neurologist, as well as a carefully constructed headache diary.

We have decided in favour of a crossover design in this single-centre RCT for the following reasons. Despite all efforts, recruitment has been slow and screening failures were a little higher than expected; in addition, we found that patients tended to be discouraged when they learned that they had solely a 50% chance of trying the IMP and would only find out which treatment arm they belonged to upon trial completion (in over 2 years time).

A crossover design in migraine is typically not recommended [68] because of the following limitations [67]: the possibility of a carry-over effect; the need for a long total period of treatment (extended by a wash-out period) with concomitant increases in drop-outs over time and in turn loss of statistical power [70]; and the increased likelihood of adverse events, which can unmask the blinding when a subject is exposed to both treatments.

In our case, a crossover design has three key advantages:

(1) A crossover design greatly improves statistical power, as each patient can be his/her own control (within-subject analysis versus between-subject analysis), which can be especially useful in a heterogeneous disease such as migraine, and hence fewer patients would be necessary to demonstrate a given effect. The sample size is effectively halved, even when more conservative a priori assumptions are employed, which is advantageous in single-centre studies. To compensate for some of the aforementioned weaknesses of crossover designs in migraine, we decided to make our a priori assumptions to determine the sample size more conservative than we would have with a parallel group design: a statistical power of 90% and a drop-out rate of 30% were chosen (in addition to a 30% placebo effect).
(2) A crossover design gives each patient the chance to try the IMP, which—from our experience—increases compliance, motivation and participation rates. We asked 25 prospective subjects for their preference and all of them favoured a crossover over a parallel group design. Instead of 6 months including a follow-up period, patients are now participating for a total of 9 months. The longer duration might lead to a slight increase in drop-out rates; however, on the other hand, it also leads to much improved participation rates, while only needing half of the patients. Additionally, it is known that vigilant patient education, monitoring and follow-up may reduce drop-out rates in longer trails [70]. From our experience, the moderate increase in trial duration has nowhere near negatively outweighed the positive impact of being guaranteed exposure to the IMP. A subsequent open-label period at the termination of the parallel group design would have a similar effect, but would also increase the costs substantially, as it does not have any impact on statistical power. This can be problematic, particularly for investigator-initiated trials.
(3) In addition to adding a wash-out period, the crossover design also allowed us to incorporate a second baseline period. This might help control for any potential seasonal effects on migraine frequency.

The possibility of a carry-over effect is always there; however, with a very short half-life of approximately 3–4 h, a 4-week wash-out period was judged to be sufficient.

Finally, we addressed the possibility of unblinding due to exposure to both substances. While there is no way to completely avoid this issue, we chose a placebo with a similar gastrointestinal side-effect profile to the IMP: mannitol, a sugar alcohol, can cause gastrointestinal disturbances, without having any systemic effect as it does not leave the gastrointestinal tract [58].

Various explorative outcomes have been included in order to be able to identify some of the potential protective mechanisms of exogenously induced ketosis in migraine. In addition, we are hoping this might help us distinguish responders and non-responders on both a phenotypical as well as physiological level.

Trial status

The trial started enrolment in May 2017 and is expected to be completed by the end of January 2020.

The newest protocol version is V6 of 5 September 2018. All protocol modifications have been and will be reported to the local ethic committee (Swissethics) and other relevant parties (such as Swissmedics, investigators and trial participants).

Additional file

Additional file 1: SPIRIT 2013 checklist: recommended items to address in a clinical trial protocol and related documents. (DOC 122 kb)

Abbreviations

βHB: Beta-hydroxybutyrate; CA: Competent authority; Ca^{2+}: Calcium ion; CRF: Case report form; CTU: Clinical Trial Unit, University Hospital Basel, University of Basel; EDC: Electronic data capture; EKNZ: Local ethic committee of northern central Switzerland; ELISA: Enzyme-linked immunosorbent assay; FAG: Freie Akademische Gesellschaft; FAS: Full analysis set; GCP: Good clinical practice; HFG: Humanforschungsgesetz; HIT-6: Headache Impact Test; ICHD-3: International Classification of Headache Disorders version 3; IMP: Investigational medicinal product; ITT: Intention to treat; KB: Ketone body; KD: Ketogenic diet; MDA: Malondialdehyde; Mg^{2+}: Magnesium ion; MIDAS: Migraine Disability Assessment; MoH: Medication overuse headache; NSAID: Non-steroidal anti-inflammatory drug; PP: Per protocol; RCT: Randomised controlled trial; SAE: Serious adverse event;

SKG: Swiss Headache Society; SNF: Swiss National Foundation; SNP: Single nucleotide polymorphism; SOP: Standard operating procedure; SUSAR: Suspected unexpected serious adverse reactions; UKBB: University Children's Hospital Basel; USB: University Hospital Basel; V1: Visit 1; VAS: Visual analogue scale

Competing interests

The authors declare that they have no competing interests.

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Saliva molecular inflammatory profiling in female migraine patients responsive to adjunctive cervical non-invasive vagus nerve stimulation: the MOXY Study

Abstract

Background: Rising evidence indicate that oxytocin and IL-1β impact trigemino-nociceptive signaling. Current perspectives on migraine physiopathology emphasize a cytokine bias towards a pro-inflammatory status. The anti-nociceptive impact of oxytocin has been reported in preclinical and human trials. Cervical non-invasive vagus nerve stimulation (nVNS) emerges as an add-on treatment for the preventive and abortive use in migraine. Less is known about its potential to modulate saliva inflammatory signaling in migraine patients. The rationale was to perform inter-ictal saliva measures of oxytocin and IL-1ß along with headache assessment in migraine patients with 10 weeks adjunctive nVNS compared to healthy controls.

Methods: 12 migraineurs and 12 suitably matched healthy control were studied with inter-ictal saliva assay of pro- and anti-neuroinflammatory cytokines using enzyme-linked immuno assay techniques along with assessment of headache severity/frequency and associated functional capacity at baseline and after 10 weeks adjunctive cervical nVNS.

Results: nVNS significantly reduced headache severity (VAS), frequency (headache days and total number of attacks) and significantly improved sleep quality compared to baseline ($p < 0.01$). Inter-ictal saliva oxytocin and IL-1β were significantly elevated pre- as well as post-nVNS compared to healthy controls ($p < 0.01$) and similarly showed changes that may reflect the observed clinical effects.

Conclusions: Our results add to accumulating evidence for a therapeutic efficacy of adjunct cervical non-invasive vagus nerve stimulation in migraine patients. This study failed to provide an evidence-derived conclusion addressed to the predictive value and usefulness of saliva assays due to its uncontrolled study design. However, saliva screening of mediators associated with trigemino-nociceptive traffic represents a novel approach, thus deserve future targeted headache research.

Trial registration This study was indexed at the German Register for Clinical Trials (DRKS No. 00011089) registered on 21.09.2016

Keywords: Migraine, Saliva oxytocin/IL-1β, Cervical non-invasive vagus nerve stimulation, Trigemino-nociceptive signaling, MOXY pilot study

Background

Migraine represents a devastating primary headache disorder affecting approximately 14% of the population with an emerging prevalence and socio-economic burden [1–4]. The distinction and definition of episodic and chronic migraine has been an issue of ongoing debate [5, 6]. For instance, both have been reported to differ in prevalence, symptom profile, socio-demographics, individual/economic burden and co-morbidities [6]. However, preventive and abortive pharmacological/behavioral interventions overlap in both migraine subtypes and failed to achieve favorable response in a considerable proportion of migraineurs [6–8]. Thus, cervical non-invasive vagus stimulation (nVNS) has been approved to represent a reasonable and safe adjunctive treatment option for prevention and abortive migraine therapy [9–16]. In two RCT trials, the EVENT study (chronic migraine prevention with non-invasive vagus nerve stimulation) and PRESTO study (prospective study of nVNS for the acute treatment of migraine) and several prospective observational cohort studies, nVNS demonstrated the capability to effectively act as adjunctive prophylaxis and rescue intervention in episodic and chronic migraine as well as in associated mood and sleep disturbance [10–12, 16–19]. Further long-term follow-up observations by Martelletti and colleagues and an additional post hoc analysis confirmed the initial findings of the PRESTO study [17, 18]. Interestingly, transcutaneous stimulation of the auricular branch of the vagal nerve (t-VNS) at 1 Hz promoted a significantly larger reduction of chronic migraine frequency compared to 25 Hz t-VNS. Of note, t-VNS duration lasted for 4 h per day during the 3 months study period [19]. In order to parallel acute and chronic head pain and to investigate possible VNS-induced changes in the trigemino-nociceptive system, several preclinical studies confirmed the clinical observed VNS responsiveness, although the precise pathways are not fully understood [20–28].

Oxytocin is synthesized in neurons exclusively located within the hypothalamic nuclei (nucleus paraventricularis of the hypothalamus; PVN) and the supraoptic nucleus (SOP). Magnocellular neurons are distributed in the PVN and in the SOP and project to the posterior pituitary lobe (release oxytocin into the blood flow) and secondly, these neurons are connected with brain areas such as the amygdala, hippocampus, and cerebral cortex. A smaller population of parvocellular oxytocinergic neurons associated with the PVN interacts via receptor signaling with the brainstem and the spinal cord (dorsal column layers/dorsal root ganglion), but not via systemic blood circulation. Thus, through both pathways, oxytocin has been suspected to impact central and peripheral nociceptive transmission and neuro-inflammatory pain signaling [29–31]. Observational studies examined migraine relief after oxytocin administration in the past [32, 33]. Tzabazis and colleagues investigated the anti-nociceptive head pain potential of oxytocin in preclinical and human trials [34, 35]. Remarkably, after administration of radiographic labeled oxytocin, high concentrations were tracked in the trigeminal nucleus caudalis (TNC), the trigeminal ganglion (TG) and corresponding three trigeminal branches (V1–V3), which innervate the corresponding mucosa and glandulae [35].

Earlier reports applying VNS for depression and seizure observed peripheral changes of pro-/anti-inflammatory cytokines (IL-1β, TNF-α) in small-scale cohorts [36, 37]. Of interest, ictally elevated IL-1β concentrations were observed in the jugular blood of migraine patients [38]. Perini and et al. demonstrated intra-ictally elevated levels of IL-1β and TNF-α in migraine patients compared to healthy controls, which was re-examined in a most recent cytokine migraine study [39, 40]. In addition, nVNS significantly decreased serum concentrations of IL-1β (pro-inflammatory) and increased anti-inflammatory marker IL-10 compared to sham stimulation in healthy individuals [41]. Although not fully understood, experimental data indicate that IL-1β promotes activation of trigemino-nociception and peripheral/central neuro-inflammatory pathways involved in headache onset [42–46].

This is the first study assessing saliva oxytocin and IL-1β concentrations along with score-based assessment of clinical responsiveness [head pain severity, frequency (headache days-attacks/month), functional state (sleep quality, mood, quality of life)] in migraine patients treated with adjunctive cervical nVNS.

Methods

Study design

The rationale of this prospective observational case control study was to investigate the efficacy of nVNS as an adjunct to medication in patients with treatment-refractory episodic migraine (EM) and chronic migraine (CM). In addition to a variety of functional outcome measures, interictal (defined as 48 h apart from an attack) saliva concentrations of pro-inflammatory cytokine IL-1ß and anti-inflammatory oxytocin were assessed at baseline (pre-nVNS) and re-assessed after 10 weeks of adjunctive nVNS treatment (post-nVNS).

Ethics, consent, permissions

This study was performed according to the guidelines of the latest revision of the declaration of Helsinki. Ethics approval for this study was obtained from the institutional review board (Ethic Commission University Hospital Bonn IRB no.: 296/15). All patients provided

written informed consent. Furthermore, the study was pre-registered at the German Register for Clinical Trials (DRKS No. S00011089).

https://www.drks.de/drks_web/navigate.do?navigationId=trial.HTML&TRIAL_ID=DRKS00011089.

Study population and clinical assessment

The study enrollment was from September to October 2016. The patients were assigned by a headache specialist (anesthesiologist/neurologist) to our university hospital. In addition, the diagnosis of the refractory headache disorder was confirmed from an interdisciplinary internal pain board (including a neurologist, an anesthesiologist, a neurosurgeon, psychiatrist, and pain nurse) in cooperation with a tertiary level headache center according to the criteria listed of the International Classification of Headache Disorders (ICHD; third edition; beta). In particular, the terms refractory and drug-resistant migraine are still highly debated [1, 47, 48]. The patients were refractory to four classes of preventive medication and/or experienced side-effects (β-blockers, anticonvulsants, tricyclic antidepressants, calcium channel blockers) using different dosages of rescue drugs. In addition, 10 (2 CM/8 EM) patients out of 12 patients experienced a less favorable outcome with the usage of botulinum toxin. Of note, botulinum toxin represents off label in EM and probably not effective in EM. Standard medication was stable 4 weeks prior to baseline visit according to the individual's prescriptions and remained unchanged through the entire study period. Depending on their intensity, migraine attacks were categorized as severe (severe = VAS 7–10/10, moderate = VAS 4–6/10 or mild = VAS 1–3/10). Inclusion and exclusion criteria are outlined in Table 1.

Functional outcome measures collected at baseline (pre-nVNS) and after accomplishment of 10 weeks nVNS treatment (post-nVNS) evaluating the mean change from baseline in patient-reported head pain intensity (visual analogue scale, VAS) and frequency (mean change in number of headache days and attacks compared to baseline). Baseline values for number of headache days and migraine attacks per month were assessed on the basis of patient self-report/headache diaries and medical records Participants recorded functional outcome measures during the 10 weeks of nVNS treatment on a daily basis. In addition to patients' self-report (headache diaries), clinical outcome measures were assessed through interviews during the outpatient visits in the 11th week after baseline in an inter-ictal period (defined as 48 h apart from an attack). Data of all reported and treated attacks within the 10 weeks of nVNS therapy were pooled and analyzed. Relevant migraine co-morbidities including impaired sleep quality (Pittsburgh Sleep Quality Index, PSQI), depressive symptoms (Beck Depression Inventory, BDI), health status (EuroQuol EQ-5D-5L), impact of headache on life (Migraine Disability Assessment, MIDAS) and Body Mass Index (BMI) [49, 50]. Pain relief was defined as a $\geq$ 50% reduction in severity and/or frequency of attacks.

Demographic and baseline characteristics

Of the 14 participants initially enrolled in the study, two patients were excluded from analysis due to protocol violation, such that the final analysis included data acquired from 12 patients. One participant changed pain medication and another discontinued nVNS due to temporary skin discomfort. All patients were female with a mean age of 47.6 years (range 34–65 years). The majority presented with EM (n = 10, five with aura) and two patients presented with CM (with aura) (Table 2).

Eleven patients were classified as MIDAS grade III/IV and one patient was classified as grade I. Evaluation of sleep patterns at baseline revealed that 10/12 (83%) patients had a disturbed sleep architecture measured by a PSQI > 5 points. Furthermore, mood disturbances indexed by a BDI score > 12 occurred in 9/12 (75%) patients, with 4/12 (33%) exhibiting at least moderate

Table 1 Inclusion and exclusion criteria according to the study protocol

Inclusion criteria	Exclusion criteria
Chronic refractory headache disorder according to the International Classification of Headache Disorders ICHD (third edition; beta)	No informed consent
Age equal/greater 18	Other concomitant neuropsychiatric comorbidity not adequate classified and/or requiring specific diagnosis or treatment
Informed consent (Study, nVNS)	Pregnancy
Refractory to medical and/or behavioural therapy	Malignancy
Medication overuse headache has been ruled out	Previous performed invasive, noninvasive and ablative procedure
Eligible for vagus nerve stimulation	Not willing to complete pain diary regarding severity and frequency
Willingness to a defined follow-up interval	
Intracranial and cervical pathologies excluded by MR scan	
Standard medication 4 weeks prior to nVNS and within the entire study period according to the individual's prescriptions	

Table 2 Demographic data and baseline characteristics of the study population addressed to severity, frequency and current preventive and abortive medication

Patient No.	Migraine type	Number of attacks per month	Pain intensity (VAS) Score	Number of headache days per month	Prophylactic medication at baseline	Acute medication at baseline
1	CM+	10	7/10	18	None	TRIP + NSAD
2	EM+	12	5/10	12	ß-blocker + TCA	TRIP + NSAD
3	EM−	5	7/10	9	ß-blocker + TCA	TRIP
4	EM−	12	8/10	14	None	TRIP
5	CM+	16	8/10	26	None	TRIP
6	EM−	7	6/10	9	None	TRIP
7	EM+	2	8/10	8	None	TRIP + NSAD
8	EM−	11	7/10	12	None	NSAD
9	EM+	10	8/10	10	Magnesium	TRIP + NSAD
10	EM+	3	8/10	3	SSRI	TRIP + NSAD
11	EM+	8	8/10	14	ß-blocker + TCA	TRIP
12	EM−	10	9/10	14	ß-blocker	TRIP + ASS

f female, *VAS* visual analogue scale, *CM* chronic migraine, *EM* episodic migraine, ± with/without Aura, *TRIP* triptans, *TCA* tricyclic Antidpressants, *SSRI* Selective Serotonin reuptake inhibitor, *NSAD* nonsteroidal anti-inflammatory drugs, *ASS* acetylsalicylic acid, *nVNS* cervical non-invasive vagus nerve stimulation, preVNS medication remained stable 4 weeks prior to study enrollment (see inclusion criteria)

depressive symptoms (BDI score > 19). All patients presented with BMI values < 30 kg/m^2 (Table 3).

Baseline assessment of the healthy control group (HC) demonstrated similar characteristics compared to the migraine group (14 females; mean age, 46.9 years, ranging from 22 to 59 years, BMI 22.1 ± 1.7).

Sample collection and laboratory assessment

Saliva samples from patients were collected at a standardized time (8.00–9.00 a.m.) in the morning (at baseline and again after 10 weeks of nVNS) in fasting condition in an inter-ictal interval (defined as 48 h apart from an ictus). Cytokine levels were assessed using high-sensitivity ELISA kits obtained from BD Biosciences Cell Analysis (IL-1β) (Heidelberg, Germany). Saliva samples were collected using pre-chilled Salivettes (Sarstedt, Nuembrecht, Germany). Salivettes were immediately centrifuged at 4180*g* for 2 min and aliquoted samples were stored at − 80 °C until assayed. Salivary OXY concentrations were determined by using a 96 well commercial

Table 3 Functional state (body weight, sleep, mood, quality of life) and saliva concentrations of oxytocin and IL-1ß at baseline

Patient no	BMI kg/m^2	Migraine Type	MIDAS score/ grade	BDI score	PSQI score	EQ-5D-5L	Oxytocin saliva pg/ml	IL-1ß saliva pg/ml
1	22	CM+	93/IV	25	8	12	61.6	215
2	19	EM+	13/III	2	2	6	27.5	195
3	23	EM−	92/IV	23	10	15	24.1	1000
4	27	EM−	39/IV	20	10	5	29.6	822
5	28	CM+	73/IV	44	13	18	105.3	334
6	20	EM−	40/IV	12	10	8	20.6	194
7	24	EM+	51/IV	7	9	9	16.3	168
8	22	EM−	2/I	9	10	7	41	261
9	28	EM+	47/IV	6	13	7	120.7	1000
10	27	EM+	16/III	12	7	11	16.9	272
11	24	EM+	106/IV	10	19	14	44.2	730
12	19	EM−	17/III	0	4	8	22.7	262

BDI Becks depression inventory, *BMI* body mass index, *CM* chronic migraine, *EM* episodic migraine, *EQ-5D-5L* EuroQol five-dimensional five level scale, *f* female, *MIDAS* Migraine Disability Assessment, *PSQI* Pittsburgh Sleep Quality Index

oxytocin ELISA kit (IBL, Hamburg, Germany). Measurements were performed in duplicate, and samples were treated following kit instructions. According to the manufacturer, the sensitivity limit of the assay is 11.7 pg/ml. The assay's intra-assay and inter-assay coefficients of variability are 9.1–12.4% and 5.2–14.5%, respectively.

Saliva samples for OXY and IL1ß were obtained from a healthy control group (HC) consisting of 14 females (mean age, 46.9 years; range 22–59 years) matching the demographic characteristics of the treatment group. For reliability reasons two saliva samples per person were measured and the mean value was used for further calculations. Lin's concordance correlation coefficient for the two saliva samples showed sufficient reliability ($r = 0.67$, $p < 0.01$). Healthy controls were recruited from the local population by means of online advertisement, public postings and contacts to assisted living facilities. Subjects were free of any current physical or psychiatric illness as assessed by medical history. After completion of the study, participants received monetary compensation.

Cervical nVNS stimulation paradigm

Cervical nVNS (gammaCore) received CE-marked approval for the acute and preventive treatment of primary headache disorders (migraine, cluster headache) and medication-overuse headache and was approved by the US Food and Drug Administration for the acute treatment of episodic cluster headache and acute pain associated with migraine.

Patients self-administered bilateral (first right–second left) nVNS therapy twice daily, i.e. each morning and afternoon. Self-stimulation lasted for 120 s. For attack treatment, patients were instructed to administer one additional bilateral application at the onset of each headache attack in conjunction to medication. An appropriate and standardized patient instruction of the nVNS device was supervised from the same instructor at baseline and throughout the entire study period.

The nVNS device was positioned medially to the sternocleidomastoid muscle and laterally to the larynx, with the following stimulation specifications: 1-ms bursts of 5 kHz sine waves, repeated every 40 ms (25 Hz) with an adjustable stimulation intensity (from 0 to 24 V). A conducting gel was applied in order to ensure transdermal signal conductivity.

Statistical analysis

Normality of the data was assessed by a Shapiro–Wilk test. Normally distributed data are presented as mean ± SEM, while non-normally distributed data are presented as box plots with whiskers showing minimum and maximum values. Pearson's correlation coefficients were used to assess linear associations between different parameters, while Spearman's correlation coefficients were determined to assess non-linear associations. A p value < 0.05 was considered significant. The data was analyzed using GraphPad Prism 5.00 (San Diego California, USA).

Results

nVNS and migraine-associated head pain severity and frequency

The mean VAS score decreased from 7.4 ± 0.3 at baseline to 5 ± 0.2 ($p < 0.01$, 95% CI 4.6–5.4) (Fig. 1a). The mean number of headache days in the nVNS group was 12.5 ± 1.7 at baseline and 8.7 ± 1.3 ($p < 0.01$; 95% CI 5.8–11.5) at the 11th week (end of nVNS treatment), while the number of attacks declined from 9.1 ± 1.5 at baseline to 5.9 ± 0.8 at the end of nVNS therapy ($p < 0.01$; 95% CI 4.1–7.7) (Fig. 1b, c).

The total number of attacks decreased by 1/3 from 109 (24 mild–42 moderate–43 severe) at baseline to 71 (16 mild–38 moderate–17 severe), with a 60% reduction in the percentage of severe attacks (Fig. 2). At baseline, only one patient achieved pain freedom within 2 h after treatment with acute medication. Following 10 weeks of adjunctive nVNS therapy, the proportion of patients achieving pain freedom climbed to 4.

nVNS and migraine-associated depressive symptoms, impairment of functional capacity and sleep architecture

Migraine-related abnormalities in sleep architecture as assessed by the PSQI improved after nVNS therapy (9.6 ± 1.3 to 6.7 ± 1.1; $p < 0.01$; 95% CI 4.3–9.0) (Fig. 1d). Functional capacity measured by the MIDAS score, migraine-associated clinical depressive symptoms quantified by the BDI score and the EQ-5D-5L, however, did not change [BDI (baseline mean 14.2 vs post nVNS 12.6; $p = 0.77$) and EQ-5D-5L (baseline mean 10.0 vs post nVNS 9.3); $p = 0.64$] (Table 4).

nVNS effects on saliva concentrations of oxytocin and IL-1ß

Pre-nVNS oxytocin levels were more than doubled than those measured in healthy controls (HC: 20.4 ± 1.7 pg/ml vs pre-nVNS patients: 44.2 ± 10.1 pg/ml; $p < 0.05$; 95% CI 22.1–66.3) and increased without significant difference after nVNS therapy (pre-nVNS patients: 44.2 ± 10.1 pg/ml vs post-nVNS patients: 46.6 ± 12.6 pg/ml) (Fig. 3a). Cytokine saliva levels of IL-1β increased after nVNS therapy, yielding 2.5 times higher values than those measured in healthy controls ($p < 0.05$) (HC: 199.9 ± 41.4 pg/ml vs pre-VNS patients: 345.3 ± 73.3 pg/ml vs post-nVNS patients: 490.7 ± 113.1 pg/ml; $p < 0.05$, 95% CI 234.6–746.5) (Fig. 3b).

Assessment of cumulative pre- and post-nVNS oxytocin levels showed a trend towards association with the

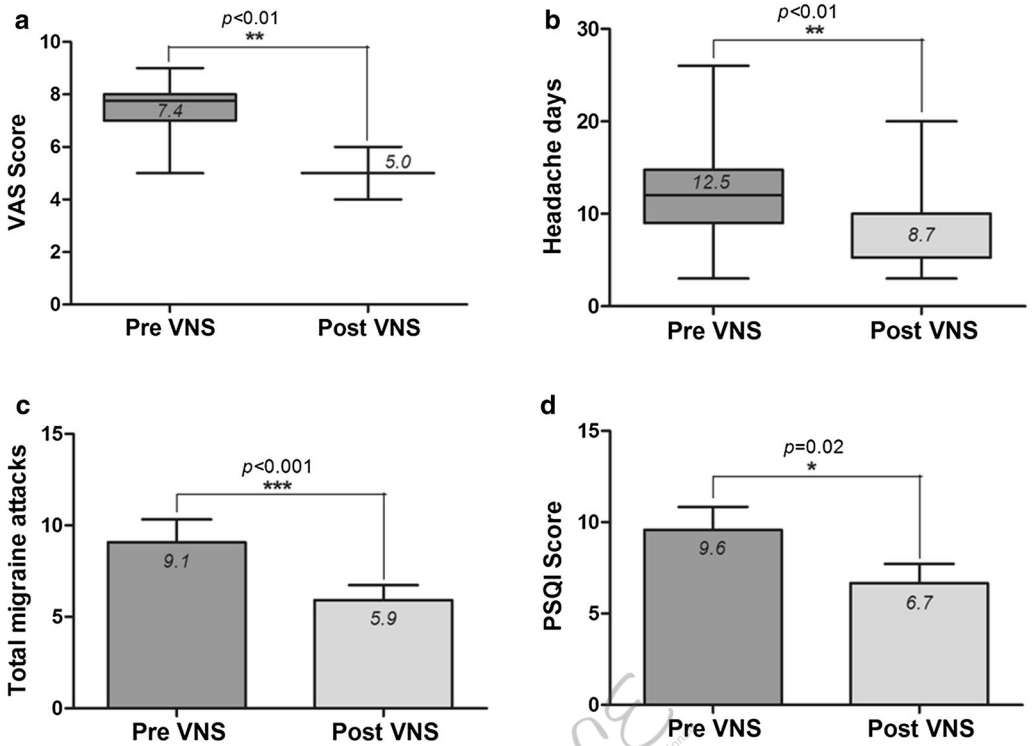

Fig. 1 **a**–**d** Pain intensity (VAS) score, migraine frequency (headache days, total number of attacks) and Pittsburgh Sleep Quality Index (PSQI) score at baseline (preVNS) and follow-up (postVNS) in all patients. Mean values with Standard deviations are presented. "*" Indicate the statistical significance

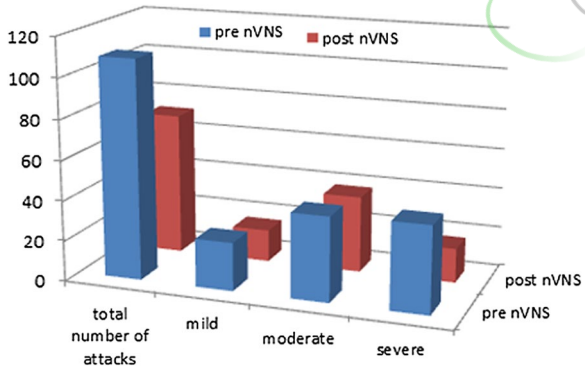

Fig. 2 Total numbers of attacks and distribution of mild/moderate/severe rated attack severity at baseline and post nVNS. Mean values with Standard deviations are presented. "*" Indicate the statistical significance

headache days per month (r = 0.379, p = 0.08) (Fig. 4), but not with number of attacks per month (r = 0.343, p = 0.12) (Fig. 5). By contrast, no significant correlation or trend between global salivary IL-1β and migraine assessment parameters was observed. Prior to nVNS treatment and within the treatment period no clinical systemic disease was observed (CRP below 0.4 mg/dl).

nVNS associated adverse events

Two patients reported mild treatment-related adverse events (AEs), most commonly skin irritation. No severe or serious AEs occurred.

Discussion

Brief summary of study findings and comparison with available literature

Our findings add to pre-existing evidence for the potential therapeutic value and safety of the preventive and acute use of nVNS as an adjunctive to prophylactic and abortive drugs in EM patients. We observed comparable responsiveness for head pain severity and frequency [9–19]. A remarkable reduction in severe attacks was observed in our cohort, similiar responsiveness was observed for pharmacological interventions. Ferrari et al. performed a meta-analysis and reported up to 59% response rates after 2 h (improvement moderate/severe to mild/no pain), 30% pain free state after 2 h and 20% sustained pain free state (no headache recurrence or use of rescue medication 2–24 h after baseline) for acute pharmacological migraine interventions [8]. Most recently, Martelletti et al. assessed additional secondary

Table 4 Mean values of clinical scores at baseline and post-nVNS treatment given for sleep, mood, migraine-associated disability and quality of life

	Pre nVNS	Post nVNS	p-value
PSQI	9.6	6.7	0.02
BDI	14	12.5	0.77
MIDAS	49	38	0.44
EQ-5D-5L	10	9	0.64

BDI Becks depression inventory, *EQ-5D-5L* EuroQol five-dimensional five level scale, *MIDAS* Migraine Disability Assessment, *nVNS* non-invasive vagus nerve stimulation, *PSQI* Pittsburgh Sleep Quality Index

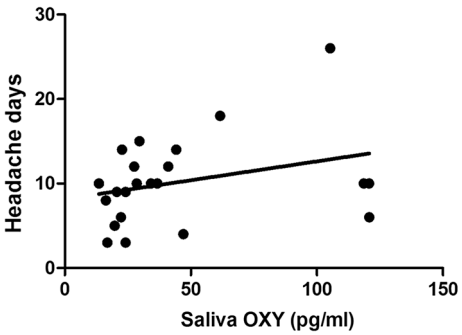

Fig. 4 Cumulative correlation analysis between pre-/postnVNS OXY levels and nVNS outcome in headache frequency (headache days/month). Assessment of cumulative pre- and post-nVNS OXY levels showed a trend towards association with the headache days per month (r = 0.379, p = 0.08). *OXY* Oxytocin, *IL-1ß* Interleukin-1ß, *HC* healthy controls, *ns* not significant

outcome parameter of the PRESTO study and found a higher rate of pain-freedom and pain-relief attacks as well as a higher rate of severity reduction in migraine patients compared to sham stimulation [17]. This meaningful improvement was accompanied by a considerable rescue medication decrease [18]. However, the antinociceptive head pain potential of the vagus nerve was confirmed in a further t-VNS study targeting the auricular branch at low frequencies (1 Hz) with 30% responder rate (responder defined as ≥ 50% reduction in headache days) [19]. Additionally, our feasibility study demonstrated an improved sleep quality with adjunctive nVNS, which is in line with previously published data [10, 11].

No association was found between those with excellent response compared to less favorable outcome and specific saliva OXY and IL-1β measures. Of note, our study enrolled EM and CM participants, as it has been well documented, that both subtypes encompass different characteristics and beyond doubt have been linked to different pathomechanisms [5–7]. Overweight (BMI 25–30 kg/m^2) was present in 4 out of 12 patients, thus it cannot be excluded, that this fact may have a considerable impact on the increased inflammatory levels. In contrast to earlier hypothesis restricting the function of white adipose tissue (WAT) as a metabolic storage organ, current revised concepts consider WAT as an inflammatory endocrine active organ with the capability to promote or suppress peripheral and central inflammation via cross-talks between adipocytes (e.g. synthesis of leptin/adipokines) and the innate and adaptive immune system. Obesity as a low-grade chronic inflammation has been associated with tissue hypoxia/necrosis with consecutively upregulation of the pro-inflammatory response via cellular (M1/2 macrophage—Th1/Th2 cells phenotype transformation) and molecular (IL-1β, IL-6, TNF-α) pathways [51]. Hence, future inflammatory migraine

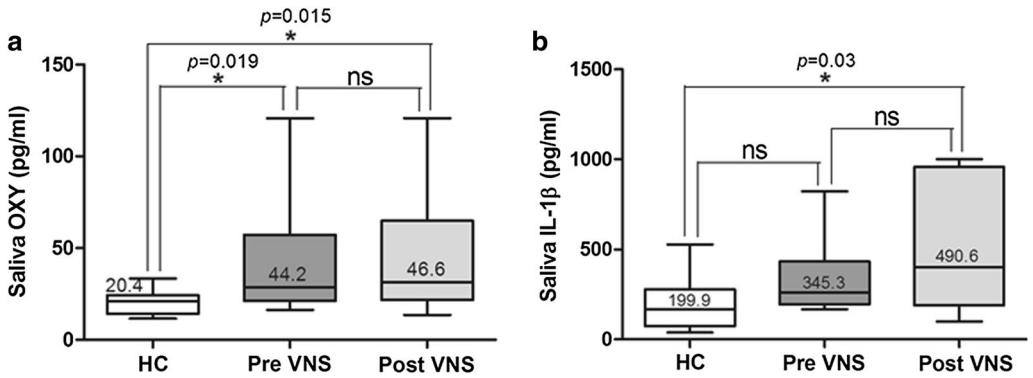

Fig. 3 **a**, **b** Oxytocin and Interleukin-1ß analysis (mean values with standard deviation and p-values). Saliva measurements for Oxytocin and IL-1ß at baseline (preVNS) and follow-up (postVNS) are compared to healthy controls. Mean values with Standard deviations are presented. "*" indicate the statistical significance. Abbreviations: *OXY* Oxytocin, *IL-1ß* Interleukin-1ß, *HC* healthy controls, *ns* not significant

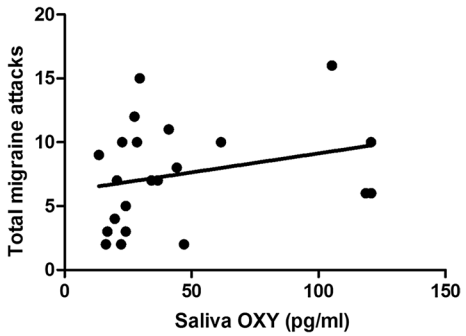

Fig. 5 Cumulative correlation analysis between pre-/postnVNS OXY levels and nVNS outcome in headache frequency (attacks/month). Assessment of cumulative pre- and post-nVNS OXY levels showed no association with number of attacks per month ($r = 0.343$, $p = 0.12$). *OXY* Oxytocin, *IL-1ß* Interleukin-1ß, *HC* healthy controls, *ns* not significant

research should consider targeting peptides of the adipokine superfamily [51].

Inter-ictal oxytocin and saliva levels were significantly higher in migraine patients compared to healthy controls at baseline and subtly increased after nVNS. So far, most of the reported studies determined cytokine, not oxytocin, in serum and compared inter-ictal versus ictal cytokine signaling. Significantly increased ictal IL-1β, IL-6 and TNF-α serum concentrations were measured in migraine patients with/without aura compared to post-ictal (after 1 week treatment) and healthy subjects not clearly indicative for a predictive value. Similar results were published addressed to migraine and peri-ictal cytokine analysis [38–40]. In our trial, oxytocin and IL-1β screening was performed post-ictally and differed in the choice of the investigated biofluid (saliva). Indeed, it would have been of interest to collect ictal values and additional markers relevant for migraine such as CGRP, but was not performed according to our study protocol. As expected we found higher post-ictal concentrations in migraine patients compared to healthy controls. In line with the findings of Perini et al., it is likely, that ictally assessed saliva concentrations would have displayed higher values compared to our post-ictal results [39]. Nevertheless, elevated saliva levels of inflammatory markers may serve as head pain susceptibility screening tool in migraine patients. After nVNS therapy, both mediators further increased along with an improved head pain state by preventive and abortive means. On the one hand, the increased oxytocin levels may be driven by the observed marked pain relief in our cohort. In several experimental studies, oxytocin has been demonstrated to be released in response to activated sensory neurons of the body (pinch, touch). In particular, electrical stimulation of somatic sensory neurons and afferent fibers of the vagal nerves was shown to effectively increase oxytocin plasma levels immediately after stimulation, which may support the observed increased oxytocin levels in our VNS treated study population [52]. In addition, it is important to note, that 50% of our cohort suffered from migraine-associated aura. Cortical spreading depression as the electrophysiological correlate of aura has been suspected to evoke astrocytes (microglia) induced synthetization and immune response via IL-1β among other cytokines [53]. Other human studies found a correlation between oxytocin concentrations and head pain intensity in migraine, contrary, we observed a trend towards association of migraine frequency (headache days/month) and oxytocin saliva levels [53]. On the other hand and to our surprise, concentrations of pro-inflammatory IL-1β was higher after nVNS compared to baseline. Possible explanations may be the fact that although clinically improved none of the nVNS treated subjects could be classified as head pain free suggesting an ongoing inflammatory process. Importantly, preliminary data indicate that intra-nasally administered oxytocin (32 IU) reduces pain in two patients with chronic migraine headache. This effect was reduced in patients who had taken nonsteroidal anti-inflammatory drugs suggesting that the anti-nociceptive effect of oxytocin is cytokine-dependent [33].

Only one human study assessed possible effects of cervical nVNS on the peripheral components of the neuro-immune reflex in healthy humans. Lerman and colleagues measured healthy individuals randomized to verum and sham cervical nVNS treatment. Chemokine levels assessed at baseline, and at 90 min and at 24 h after treatment showed a decrease in pro-inflammatory IL-1β, IL-8, and TNF-α levels and an increase in anti-inflammatory IL-10 levels, indicating that nVNS may inhibit pro-inflammatory cytokine release [41]. Other human studies conceptualized to determine peripheral inflammatory profiles of subjects treated with surgically implanted invasive VNS (iVNS) were limited to depression and focal seizure with limited interpretations due to the uncontrolled study design [36, 37]. However, comparable cytokine/chemokine data under VNS "off"-stimulation remain an open question.

The impact of oxytocin on trigemino-nociceptive signaling

Recently, a preclinical study determined oxytocin receptor expression and co-localization with calcitonin gene-related peptide (CGRP) in the trigeminal ganglion. Application of painful, facial electrocutaneous stimulation and adjunctive capsaicin-driven inflammation increased oxytocin expression in CGRP-containing

trigeminal ganglion neurons, indicating the important role of oxytocin in migraine pathophysiology [34].

The relationship between migraine and oxytocin was under investigation in a sophisticated experimental setting including measurement of electrophysiological (TNC firing response) and gene expression (C-fos) parameters after intranasal oxytocin administration compared to placebo [35]. In the first, increased TNC firing rates after electro-cutaneous stimulation of the face were recorded and of note, attenuated by oxytocin, while in the second an increased C-fos expression in TNC neurons was observed after intra-peritoneal injection of nitroglycerin, which was revised in the oxytocin pre-treated group. In a next translational step, intranasal oxytocin was assessed in sham-controlled trials in EM and CM patients [35]. Although no significant differences were observed after 2 h treatment for both migraine subtypes, the CM subgroup demonstrated a trend in favor of the verum treatment. Additional human trials indicate a stronger effect of oxytocin on frequency, rather than on severity. Interestingly, NSAID use was suspected to interfere with oxytocin effects by inhibiting cytokine synthesis [35]. NSAID as an adjunctive rescue medication was present in 5 out of 12 patients in our study.

Migraine as a multi-network brain disorder displays altered sensory (nociception), cognitive, affective and circadian-dependent autonomic features (sleep, metabolism, thermoregulation), of which each component is able to drive head pain onset/attacks [54–56]. Apart from its ictal functions, inter-ictal endogenous oxytocin has been linked to central sensitization (hyperalgesia, allodynia) and neurogenic inflammation in migraine pathophysiology. Such elevated inter-ictal oxytocin concentrations may reflect modulation of extracephalic pain perception and affective distress symptoms. Indeed, it would have been of great interest to quantify these inter-ical appearing clinical features relevant for migraine [56]. Taken the complex and dynamic nature of migraine into account, the authors speculate, that molecular profiling of oxytocin may have a diagnostic and therapeutic potential for migraine-associated symptoms outside the ictal phase [54, 55].

Pro-inflammatory IL-1 β associated effects in trigemino-nociceptive traffic

Zhang et al. recorded TG neuron response of meningeal nociceptors after local application of IL-1β and IL-6 on dural nociceptors and found that IL-1β, but not IL-6 was able to promote increased activity of TG neurons accompanied with increased mechano-sensitivity of intracranial nociceptors (meningeal afferent signaling) measured von Frey filaments [42].

IL-1β has been suspected to induce upregulation of cyclo-oxygenase 2 mRNA (COX-2) expression in glial cells and neurons of the trigeminal ganglion (TG). These COX-2 dependent pathways lead to prostaglandine release from glial and neuronal TG cells, which in turn stimulates solely neurons of the TG to immediately (1 h after stimulation) produce CGRP, contrary to IL-1β, which demonstrated a delayed CGRP release pattern (24 h after stimulation) suggesting a glia-neuron interaction in the TG [57]. Methylprednisolone reversed the IL-1β effects, but demonstrated no impact on prostaglandine induced CGRP release [58]. Leptin, a metabolic marker produced by WAT cells, has been shown to interact with the COX-2 dependent pathways via crosstalks with IL-1β in glial cells and neurons of the hypothalamic-pituitary axis [59].

The development of acute head pain has been associated with primary afferents activation of the TG driven by dural nociceptors, which are connected with the trigeminal nucleus caudalis (TNC). The TNC itself projects to the trigemino-cervical complex (TCC), and receives reciprocal input from the brainstem, the medulla oblongata, the hypothalamus-pituitary-axis, the thalamic nuclei (intralaminar nucleus of the thalamus) and cortical associated networks.

On the other hand, it has been well described, that the afferent properties of the vagus nerve project via the ncl. tractus solitarii to the locus coeruleus (LC), the dorsal raphe nucleus, the parabrachial plexus, the paraventricular nucleus of the hypothalamus and maybe directly to the TNC and the cervical spinal cord (trigemino-cervical complex, TCC). In view of the anatomic reciprocal connectivity of the vagus nerve, it may be reasonable, that cervical nVNS may impact trigeminovascular nociceptive signaling of pro- and anti-inflammatory markers in different biofluids (plasma, saliva, cerebrospinal fluid) [20, 21, 26–31, 34, 35, 52, 57–59].

Inhaled (olfactory nerve) and/or ingestive (trigeminal nerve) chemical irritants have been suspected to promote pro-inflammatory mediators and to trigger neurogenic inflammation in a broad range of respiratory (asthma) and neurological disorders such as migraine. A dynamic and complex interplay between local (e.g. substance P, bradykinin) and distant efferent effects (adrenergic, cholinergic) characterizes in part the host response, in which mast cell derived immunomodulation plays a pivotal role. Hence, it cannot be excluded, that environmental factors may have an impact on inflammatory phenotyping and quantification of peripheral markers of the neuro-immune axis [60, 61].

In particular, immunomodulatory mast cells (high affinity receptor FceR1) are capable to interact with the innate and adaptive immune response and poses an

important role in the genesis of acute/chronic inflammation associated disorders (e.g. migraine). Immunglobuline E (Ig E), Toll-like receptors, IL-1β and IL-36 are known to activate mast cells and to drive a pro-inflammatory state, while IL-37 and IL-38 (member of the IL-1 cytokine family) act as an inhibitor of inflammation. Gallenga and colleagues reported that IL-38 is able to bind on the IL-36 receptor, which in turn blocks mast cell activation [62]. It is important to note, that mast cell response occur in a time dependent manner with an immediate secretion and a delayed synthesis/release of inflammatory active peptides in order to establish a physiological host response. Among the mentioned IL-1 cytokine family, IL-1β increases IL 33 and TNF-α synthesis derived from mast cells. Furthermore, IL-33 interacts with monocytes and promotes mast cell differentiation, maturation and degranulation with subsequent secretion of pro-inflammatory cytokines/chemokines. Contrary, IL-37, an anti-inflammatory member of the IL-1 cytokine family, has the potential to counterbalance the IL-1β evoked innate and adaptive immune response. Therefore, the therapeutic anti-inflammatory value of IL-37 induced blocking of mast cells deserves further clarification in migraine research [63, 64].

Limitations and future prospects for molecular inflammatory profiling in migraine

This study has several limitations including the uncontrolled design, the small-scale study cohort and a relatively short-term observation period. The main issue is related to the lack of a sham stimulation group (stimulation off) in order to discern what was due to patient's expectation (treatment self-responsibility) in contrast to the real effects of nVNS. With respect to migraine as a complex brain disorder, such expectation associated with a novel device (like nVNS in our study) may represent a confounder. Different dosages of migraine drugs (preventive/abortive) administered in each patient may represent a confounder. Furthermore, cytokine levels may vary and depend on pre-analytic variables including sample processing, environmental factors, intra- and interindividual variability. The cytokine analysis performed in our study did not consider the dynamic nature of neuro-inflammation nor the circadian neurobiology, thus repetitive measurements are recommended in future trials. The role of the brain-immune-communication in primary headache disorders is of major interests. To this end, immune-phenotyping of migraine is in the beginning. Future research in this field should seek to investigate which immune pathways are overactivated in migraine, how immune cell hyperactivity is linked to disease susceptibility, and how environmental and genetic factors influence immune activation and disease manifestation. Immune-phenotyping should consider cell profiling in several flow cytometry panels and whole blood stimulation assays with a range of innate immune stimuli. Can we use molecular profiling to predict and individualize neurostimulation (nVNS) therapy? Can we target cytokines as diagnostic tools? Currently the answer is no as the precise mechanisms of the neuro-immune communication in migraine pathophysiology remains unclarified. Alternatively, advanced statistical methods capable to establish categorical-based dimensions may support the potential and the integration of biobank-based immune-phenotyping, thus help to define migraine specific characteristics and subsets (biotypes) of patients more or less likely to respond to neurostimulation therapy [65, 66]. However, this study firstly approached to screen oxytocin and IL-1β in saliva and attempted to proof the feasibility of saliva analysis and undoubtedly, but was biased by the uncontrolled study design.

Conclusion

Ten weeks of adjunct nVNS therapy significantly decreased migraine severity and frequency in patients with EM (with/without aura). In addition, migraine-associated sleep impairment was improved. Inter-ictal saliva concentrations of oxytocin and IL-1β were significantly higher in migraine patients compared to healthy controls. An evidence-derived conclusion about the predictive value and usefulness of saliva assays is clearly limited by the provided uncontrolled study design. Clearly, our findings along with preclinical and human available literature data point to the necessity to monitor changes in a broad array of anti- and pro-inflammatory markers during nVNS since treatment effects may be reflected in altered ratios of different markers. Thus, the observed salivary levels (and nVNS-induced changes) must be interpreted with caution and currently remain at an experimental stage (feasibility). However, the assessment of cytokine/chemokine plasma and/or saliva levels addressing the pathogenesis and treatment of migraine may represent a novel approach and may be worthy for being re-visited under controlled study condition in order to evaluate its usefulness beyond subjective patient´s self-report.

Abbreviations

EVENT: chronic migraine prevention with non-invasive vagus nerve stimulation; PRESTO: prospective study of nVNS for the acute treatment of migraine; PVN: paraventricular nucleus; SOP: supraoptic nucleus; TNC: trigeminal nucleus caudalis; TCC: trigemino-cervical complex; TG: trigeminal ganglion; LC: locus coeruleus; VNS: vagus nerve stimulation; IL: interleukin; TNF: tumor necrosis factor; CGRP: calcitonin gene-related peptide; COX: cyclooxygenase; WAT : white adipose tissue; EM: episodic migraine; CM: chronic migraine; ICHD: International Classification of Headache Diagnosis; PSQI: Pittsburgh Sleep Quality Index; EQ-5D-5L: Euro quality of life scale; MIDAS: migraine disability score; BMI: body mass index.

Competing interests

Thomas M. Kinfe has received training support and works as a consultant for Abbott (formerly St. Jude Medical, Inc.) and works as a consultant for Medtronic Inc. Joachim K. Krauss works as a consultant for Medtronic Inc. Sajjad Muhammad has received Neuromodulation and Pain Fellowship by Abbott Inc. (formerly St. Jude Medical). Krishnan Chakravarthy works as a consultant to Abbott.

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