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New Targets in Migraine Therapy

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Every head has its own headache
Arab Proverb

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Chapter 1

General introduction on migraine and existing and emerging therapies for the treatment of Migraine

Introduction to migraine

Migraine is a recurrent, incapacitating neurovascular disorder with a high prevalence affecting up to 10% of the general population (1). It presents a major public health concern and remains the most costly neurological disorder in the European Community, costing more than € 27 billion each year (2). Since the hereditary nature of the disorder would imply that the prevalence of migraine will remain constant, new therapeutic approaches for migraine are greatly needed (3).

Migraine is as old as human history itself and descriptions suggestive of migraine can be found in Babylonian manuscripts dating back to 3000 BC and Egyptian papyri from 1550 BC (4). In the 19th century, as scientific medicine developed, several physicians were preoccupied with the pathophysiology of migraine. At the time two rather straightforward theories dominated the thinking about migraine: the neural theory and the vascular theory (5). The neural theory sought to explain migraine as a central nervous system disorder, while the vascular theory assumed migraine resulted from either vasoconstriction or vasodilation (6, 7). Current research still attempts to elucidate the aberrant processes that characterize migraine and whether to place them within the brain or the vasculature. It is anticipated that a better understanding of the disorder's origin will lead to better management of patients with migraine and help develop new therapeutic agents.

Clinical features and diagnostic criteria

Migraine headache is a clinical diagnosis, based on careful history taking and the exclusion of other disorders that may cause headache (8). One of most important advances in the twentieth century concerning the management of headache, was the acceptance of the International Headache Society IHS classification (9). According to the IHS, migraine is a collection of symptoms and is best characterized as a headache with features of sensory sensitivity, sensitivity to light, sounds, smells and head movement. Migraine typically presents as a throbbing headache that is most likely the result of movement of cranial blood vessels (10). The IHS classification did not only improve the diagnosis of headache disorders, it also provided a uniform tool for conducting clinical studies. Current diagnostic criteria for migraine are shown in Table 1.

Table 1 Classification and diagnostic criteria for migraine according to the International Classification of Headache Disorders, 2nd edition (9)

Migraine without aura:

A. At least 5 attacks fulfilling criteria B-D

B. Headache attacks lasting 4-72 hours (untreated or unsuccessfully treated)

C. Headache has at least two of the following characteristics:

1. Unilateral location
2. Pulsating quality
3. Moderate or severe pain intensity
4. Aggravation by or causing avoidance of routine physical activity

D. During headache at least one of the following:

1. Nausea and/or vomiting
2. Photophobia and phonophobia

E. Not attributed to another disorder

Typical Aura with migraine headache:

A. At least 2 attacks fulfilling criteria B-D

B. Aura consisting of at least one of the following, but no motor weakness:

1. fully reversible visual symptoms including positive features and / or negative features
2. fully reversible sensory symptoms including positive features and / or negative features
3. fully reversible dysphasic speech disturbance

C. At least two of the following:

1. homonymous visual symptoms an
2. at least one aura symptom develops gradually over ≥5 minutes and / or different aura symptoms occur in succession over ≥5 minutes each symptom lasts ≥5 and ≤60 minutes

D. Headache fulfilling criteria B-D for *Migraine without aura* begins during the aura or follows aura within 6O minutes

E. Not attributed to another disorder

Migraine headaches are divided by the IHS into two varieties: “typical aura with migraine headache” and “migraine without aura”. To establish a diagnosis of migraine without aura, the patient must have at least five attacks, each lasting 4 – 72 hours and having at least two of the following characteristics: unilateral location, pulsating quality, moderate to severe pain intensity and aggravation by or causing avoidance of routine physical activity. In addition, the attacks must be accompanied by at least one of the following symptoms: nausea and / or vomiting and photophobia and phonophobia. To diagnose typical aura with migraine headache, a minimum of two attacks is required in which a typical migraine headache begins during or follows a typical aura, consisting of visual and / or sensory and / or speech symptoms. The aura develops gradually, lasts no longer than 60 minutes, is typically a mix of positive (e.g. fortification spectra) and negative (e.g. scotoma) features and is completely reversible. Several rare types of migraine with aura have been defined: typical aura with non-migraine headache, typical aura without headache, familial and sporadic hemiplegic migraine (characterized by an aura that includes motor weakness) and basilar-type migraine (characterized by an aura that includes symptoms originating from the brain stem and / or affecting both hemispheres of the brain at the same time but with no motor weakness, e.g. decreased level of consciousness, temporal blindness, diplopia).

Genetics of migraine

It is clear from clinical practice that migraine frequently runs in families, with many patients having first-degree relatives who also suffer from migraine (11, 12). Family and twin studies confirm that both genetic and environmental factors play a role in the aetiology of migraine (13, 14). Investigation of familial hemiplegic migraine (FHM), a rare subtype of migraine with aura with an autosomal dominant mode of inheritance, and of migraine-associated genetic syndromes, would appear to be a promising avenue in unravelling migraine-relevant molecular pathways (15). The direct approach of setting up candidate gene association studies in migraine has proven less successful possibly due to the complex nature and large spectrum of the disease (15).

Three types of FHM have now been indentified based on the affected genes regulating ion transport, suggesting that migraine, or at least the aura, is the result of a “channelopathy” (16, 17). FHM type 1 is the result of mutations in the CACNA1A gene (chromosome 19p13) that encodes the pore-forming α1A subunit of voltage-gated neuronal P/Q type calcium channels (18). FHM type 2 is caused by mutations in the ATP1A2 gene (chromosome 1q23) that encodes the catalytic α2 subunit of sodium-potassium pumps (19). FHM type 3 is caused by a mutation in the SCN1A gene (chromosome 2q24) that encodes a voltage-gated sodium channel (20). Functional studies point towards an increased susceptibility of the brain of affected animals to cortical spreading depression (CSD) caused by either excessive glutamate release (FHM type 1), decreased removal of glutamate from the synaptic cleft (FHM type 2) or persistent sodium influx (FHM type 3) (21). Although it is tempting to extrapolate the pathophysiology of FHM to the more common forms of migraine, this would assume that FHM is an extreme form of migraine, belonging to a continuous migraine spectrum. This assumption is not implausible but needs to be confirmed (22). When studying the genetics of diseases that are co-morbid with migraine, the complexity becomes even more apparent. In the case of migraine and epilepsy, a genetically determined dysfunction of ion transporters seems to point at common

underlying mechanisms for both paroxysmal disorders (23, 24). However, in other monogenetic diseases such as Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), the common etiological background seems to be vascular. For example, one third of the patients affected by CADASIL also has migraine with aura (25). CADASIL patients have mutations in the gene that encodes the Notch3 receptor that plays a key role in functioning of vascular smooth muscle cells in small arteries and arterioles of the brain (26). In addition, some of the polymorphisms associated with migraine clearly affect vascular function (27). This would appear to illustrate that the unravelling of migraine-relevant molecular pathways through genetic findings is hampered by the complex nature of the disease.

Pathophysiology of migraine

In the 1960s and 1970s migraine was considered to be a vascular phenomenon and as such was referred to as a vascular headache (28-30). This term came from the historic notion that migraine aura was due to vasoconstriction while the subsequent headache was due to a reactive vasodilation (7, 31, 32). However, human experimental studies, observation of vascular changes preceding and during a migraine headache and the better understanding of the site of action of existing anti-migraine medication put migraine firmly back into the brain. Firstly, some cranial vasodilators (e.g. nitrates, histamine, calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase activating peptide (PACAP-38)) are capable of triggering delayed migraine in migraine sufferers, while other vasodilators are not (e.g. papaverine, vasoactive intestinal polypeptide (VIP)) (33-39). This indicates that vasodilation is an epiphenomenon, not necessary or sufficient to the development of migraine headache. Secondly, in migraine patients without aura, no focal change in cerebral blood flow is observed and there is no consistent relationship between vascular change and the phase of a migraine attack. Indeed, during the pain phase blood flow can be reduced or normal (40-43). Thirdly, better imaging techniques demonstrate changes in brain function in the brainstem and sub-cortical structures that could readily account for more of the symptoms of migraine than simple vasodilation (44-47). Finally, whereas triptans, which are extremely effective in treating migraine, were developed initially as cranial vasoconstrictors, they are now recognised as affecting neuronal transmission within the brain (48-50).

While the evidence base for the primary vascular theory is shrinking, the notion that pain sensitivity within the skull is mainly restricted to the meninges and meningeal blood vessels does imply an involvement of the cranial vasculature in the pathogenesis of migraine (51, 52). Therefore, rather than a dichotomous view in which migraine is either a disease of the brain or the cranial vasculature, a more pragmatic approach is necessary to define migraine as a paroxysmal neurovascular disorder of which the trigeminovascular system is the anatomical substrate (53).

The unifying hypothesis: the trigeminovascular system)

The trigeminovascular system (TGVS) is composed of the pain sensitive intracranial structures (i.e. meninges and blood vessels), the nociceptive pain sensory afferent fibres of the ophthalmic division of the trigeminal nerve and its central projections into the trigeminocervical complex (i.e. the trigeminal nucleus caudalis and the C1-C2 superficial dorsal horns). Nociceptive signals are subsequently carried upwards to brain structures involved in the perception of pain (thalamus and cortical areas) while projections into the nucleus tractus solitarius mediate autonomic symptoms including nausea and vomiting (54). The TGVS is depicted schematically in Figure 1 below.

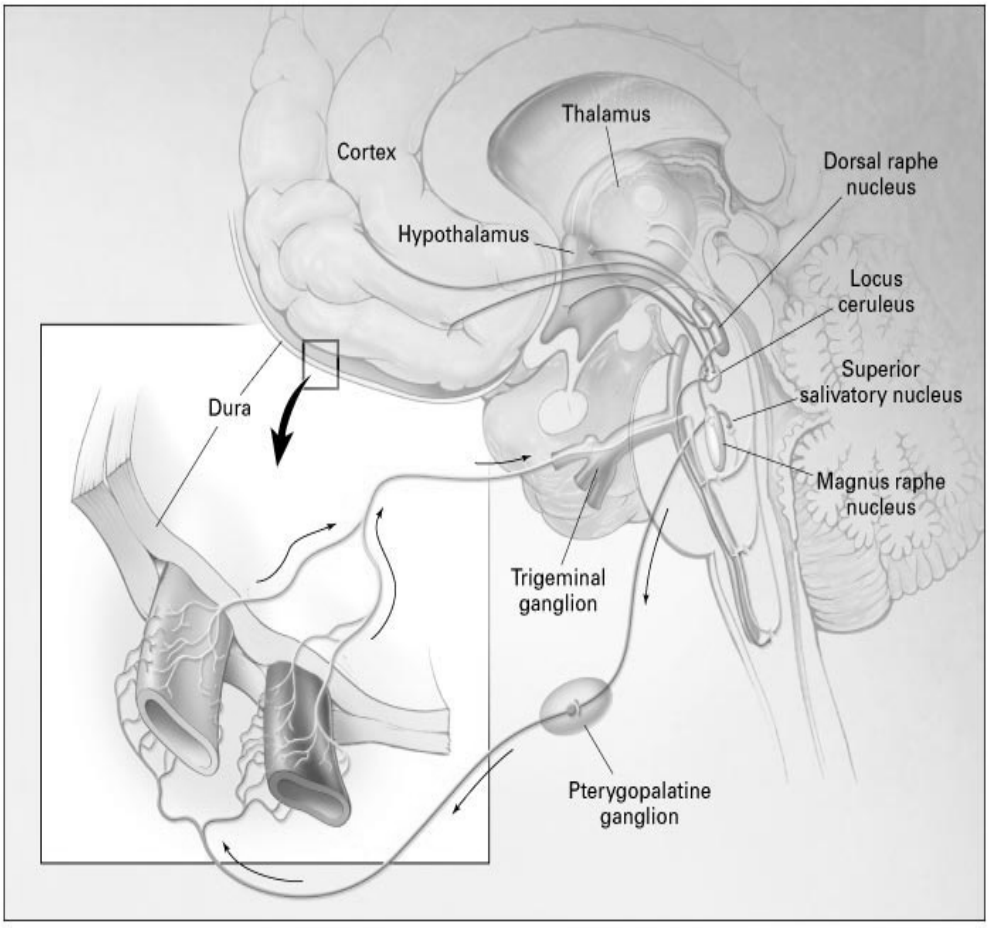


Figure 1: Pathophysiology of migraine and trigeminovascular system
(Reproduced from Olesen J. et al. N Engl J Med 350: 1104-1110, 2004)

Activation of the TGVS is considered a key event in the pathophysiology of migraine (3, 55). The observation that plasma levels of neuropeptides contained within perivascular trigeminal afferent nerve endings (e.g. CGRP) increase during a migraine attack, is considered to reflect the activation of the peripheral TGVS (56-58)}. Nevertheless, caution is warranted as these findings remain controversial (59, 60). In addition, it is not clear how the release of these mediators generates pain. Animal studies suggest that the release of neuropeptides results in sterile neurogenic inflammation in

the dura mater consisting of vasodilation, plasma protein extravasation, platelet aggregation and mast cell degranulation (61-63). Although this mechanism has not yet been shown to constitute an important component of the pathophysiology of migraine in humans, the success of recently developed CGRP receptor antagonists in treating acute migraine substantiates a role for neurogenic inflammation in the development of head pain (64-67).

Migraine headache is also accompanied by changes in pain modulation referred to as sensitization (68). Sensitization of the first-order trigeminovascular nerves, referred to as peripheral sensitization, is believed to result in altered perception of craniovascular input that is usually not painful which translates in the typical throbbing quality of migraine headache (69). Sensitization of second- and third-order trigeminal neurons, referred to as central sensitization, leads on the other hand to cutaneous allodynia during a migraine attack (68). When cutaneous allodynia develops a previously non-painful, innocuous stimulus to normal skin is perceived as painful. Interestingly, the presence of cutaneous allodynia decreases the probability of therapeutic success of triptans suggesting that central sensitization is a key player in maintaining headache (70).

Migraine headache thus probably results from a combination of neurogenic inflammation and both peripheral and central sensitization. The interaction between those components can change over time explaining why migraine is a spectrum disorder, in which the clinical and pathophysiological features of migraine may progress over time. It is believed that changes in nociceptive threshold as a result of central sensitization caused by recurrent migraine leads to the progression of migraine from an episodic to chronic headache disorder in susceptible individuals. As chronic migraine represents an important burden to both the individual and society, a better understanding of the mechanisms of chronification might greatly contribute to lift the burden of headache.

The trigger initiating migraine

Migraine should be considered as a paroxysmal neurovascular disorder. Any subject can experience a migraine attack, but it is the repeated occurrence that is considered abnormal and defines migraine as a disease (71). In view of the recent genetic findings concerning FHM, it is most tempting to speculate that this “low migraine threshold” results from a “cerebral chanellopathy” (71). In addition, the bi-directional relation between migraine and epilepsy also seems to suggest ion transporter dysfunction as a common etiological mechanism for both diseases (23, 24, 72). Ion channel mutations may render the migraine brain more susceptible to CSD (73). CSD was first described by Leão in the rabbit cerebral cortex and is characterized by a short-lasting neuronal depolarization wave that moves across the cortex at a rate of 3–5 mm/min. The wave generates a brief phase of excitation that is immediately followed by nerve cell depression and a failure of brain ion homeostasis (74, 75). Partly as a result of neurovascular coupling, CSD is accompanied by regional cerebral blood flow changes that lead to hypoperfusion in the same brain region as the depolarisation (75, 76). It is generally accepted that CSD is the animal experimental equivalent of migraine aura (77). Migraine aura is associated with an initial hyperaemic phase followed by reduced cortical blood flow, which moves across the cortex (spreading oligaemia) (78). Controversy surrounds the relationship between aura and headache,

although CSD has been demonstrated to activate trigeminovascular afferents and to evoke a series of cortical meningeal and brain stem events consistent with the development of headache (79).

An alternative view suggests that the primary cause of migraine headache is an episodic dysfunction of brain stem neurons involved in the central control of nociception (3). Indeed, several studies detect activation in several brain stem areas that might be involved in the central modulation of pain, in particular in pain inhibition, during migraine attacks (45, 80). Brainstem activation could either trigger the migraine headache (brainstem generator of migraine) or contribute to hyperexcitability of the TGVS.

Finally, it is important to remember that migraine is a complex disorder for which a unitary molecular explanation seems to be a long way off. Triggers of migraine are not only to be found within the migrainous brain itself. Many environmental factors are reported to precipitate migraine (81). Migraine patients may thus also be exposed to more frequent and/or strong migraine-inducing triggers. An example of the latter is the female preponderance in migraine, which is assumed to be associated with the additional trigger of fluctuating sex hormones during the menstrual cycle (82, 83).

The treatment of migraine

Traditionally, treatment for migraine is divided into acute and preventive approaches. Acute treatment is aimed at stopping the attack, whereas preventive treatment is designed to reduce attack frequency and the duration and/or severity of attacks. It remains unclear whether the divide between acute and prophylactic treatment is absolute or provides any mechanistic insight into the disorder (84). A wide range of anti-migraine drugs currently exists and the choice of treatment depends on the severity and frequency of the attacks, associated symptoms, coexisting disorders, previous treatment response, the drug's efficacy, potential for overuse and adverse events (85). Due to a lack of clinical efficacy in a number of patients, troublesome side-effects and safety concerns concerning available drugs, there remains a need for new therapies with improved efficacy, tolerability and safety.

Preventive approaches to migraine

Preventive strategies in migraine treatment are not currently exploited to their full potential (86). Preventive treatment should be considered when migraine attacks significantly interfere with daily routine despite appropriate acute treatment, when attacks are frequent (more than four attacks per month) or when they are accompanied by long, uncomfortable auras or uncommon migraine conditions that carry risk of neurological damage (e.g. hemiplegic migraine, basilar migraine and migraine with prolonged aura or migrainous infarction). Other factors that should warrant preventive treatment include contra-indication to, failure with, overuse of or intolerance to acute therapies. Patients' preference should also be taken into account. Many different medication groups are used for preventive treatment, including beta-blockers, calcium channel blockers, antidepressants and antiepileptic drugs. Although their mechanism of action remains a matter of debate, they are believed to include the following: raising the threshold to migraine activation, enhancing antinociception, inhibiting CSD, inhibiting peripheral and central sensitization, blocking neurogenic inflammation and modulating sympathetic, parasympathetic or serotonergic tone (87). The efficacy of prophylactic

migraine medication remains limited with only 50% of patients reporting a reduction of at least 50% in headache frequency (88). A better understanding of migraine pathogenesis should lead to improved prophylactic drugs.

Non specific acute drugs

Non specific acute headache medications include paracetamol (acetaminophen), aspirin, nonsteroidal anti-inflammatory drugs, antiemetics, opioids, corticosteroids, dopamine antagonists and combination analgesics (88). Although few comparative trials have been published, several non specific drugs have been shown to be equally or even more effective compared to specific anti-migraine drugs (89, 90). However the probability of successful treatment with non specific drug regimens seems to decrease with increasing headache related disability (91). Therefore, it is recommended to use non-specific analgesics as first line drugs only for mild or moderate headaches, while triptans should be used as first-line drugs for severe attacks (88).

Specific acute drugs

Ergot alkaloids

The ergot alkaloids were the first specific compounds reported to be effective in the treatment of acute migraine (92). Ergotamine tartrate was originally derived from a rye fungus (*Claviceps purpurae*). The dihydrogenated derivative, dihydroergotamine, became available in 1945 and only differs from ergotamine in that it is less toxic and less emetic (93). The ergot alkaloids are non-selective pharmacological compounds interacting with serotonergic (5 HT_1 and 5 HT_2 receptors), adrenergic (α_1 and α_2 receptors) and dopaminergic receptors (D_2-receptors) (93, 94). The anti-migraine activity of the ergot alkaloids is likely to result from cranial vasoconstriction, inhibition of neurogenic inflammation and inhibition of pain transmission within the TGVS and is thus comparable to that of triptans. Nonetheless, ergot alkaloids have major disadvantages. First, they have a very low oral and rectal bioavailability and exhibit very variable interindividual pharmacokinetics (95, 96). Second, since they are non-selective, they have multiple side-effects. Third, in particular for ergotamine, only limited placebo-controlled data are available on clinical efficacy (97). Finally, ergot alkaloids have potent and sustained generalized vasoconstrictor effects, which are associated with adverse vascular events, and carry a high risk of overuse syndromes and rebound headaches (98-100). Today, the use of ergot alkaloids should only be considered when cost precludes treatment with triptans (88).

Triptans

In the late 1980's attention focused on the role of the disturbance in 5-hydroxytryptamine (5-HT, serotonin) metabolism in migraine patients (101). Improved understanding of the neurobiology of migraine and 5-HT receptors eventually led to a new class of selective 5-HT receptor agonists known as the triptans (48). The first of the class, sumatriptan, was soon followed by the newer triptans (zolmitriptan, naratriptan, rizatriptan, almotriptan, frovatriptan and eletriptan), which were all developed to display high agonist activity at mainly the serotonin 5-HT_{1B} and 5-HT_{1D} receptor subtypes (102). The pharmacodynamic differences between the different triptans are only minor, although small difference

in bioavailibility, lipophilicity, half-life, metabolism and 5 HT_1 receptor binding properties exist. A large meta-analysis of randomized controlled trials looking at efficacy of oral triptans in migraine, concluded that all marketed oral doses of triptans were effective and well tolerated (103). However, since the oral absorption of drugs may be delayed during a migraine attack, non-oral administration (intranasal, rectal and subcutaneous) should be preferred (104). This explains why the “golden standard” in migraine treatment remains subcutaneous sumatriptan at a dose of 6 mg, which has been shown to have the best pharmacokinetic profile, clinical efficacy and intrapatient consistency over multiple attacks (102, 105). Studies with oral triptans have also shown that early treatment, i.e. before the headache reaches moderate intensity, increases their effectiveness (106, 107). This is probably due to changes in the pharmacokinetic profile of orally administered drugs during a migraine attack since it was not seen with subcutaneously injected sumatriptan (108).

Three main mechanisms of action of the triptans have been proposed: cranial vasoconstriction, peripheral trigeminal neuronal inhibition and inhibition of pain transmission through the TGVS (48, 109, 110). The relative importance of these mechanisms remains a matter of debate (49).

The development rationale of triptans however dates from the time when cranial vasoconstriction was seen as the key pharmacodynamic property for their efficacy. The vasoconstrictor properties of triptans were thus acknowledged from the very beginning and have always caused concern among patients and doctors alike (111). Triptan-induced vasoconstriction of human blood vessels is mediated through vascular 5-HT_{1B} receptors situated on vascular smooth muscle cells (112). Though triptans exhibit a cranio-vascular selectivity, i.e. a higher vasoconstrictor potency in cranial compared to extracranial arteries, extracranial vasoconstriction is a potential, unwarranted class effect (113-117). Due to the coronary vasoconstrictor activity of triptans, their cardiovascular safety remains a matter of debate (112, 118-121). Serious cardiovascular events after triptan administration have been reported in the literature, mostly in patients with established cardiovascular disease or cardiovascular risk factors (122-126). Though two large pharmaco-epidemiological studies have shown that triptans do not increase the risk of stroke, myocardial infarction, cardiovascular death, ischaemic heart disease or mortality, these findings cannot be extrapolated to patients at increased cardiovascular risk as triptans are generally prescribed to patients with low cardiovascular risk factors (127, 128). Very few studies have been conducted to assess the cardiovascular effects of therapeutic doses of a triptan in patients with cardiovascular disease or risk factors explaining the controversy on whether triptans can be administered safely in this particular patient population (129-132). Current consensus favors the use of triptans in patients at low risk of cardiovascular disease, but clearly emphasizes that their use is contra-indicated in patients with cardiovascular disease or at high cardiovascular risk (133).

Although the triptans revolutionized the treatment of migraine, they still have some shortcomings: incomplete and inconsistent pain relief, the recurrence of the headache and failure to alleviate headache when taken during the aura phase (134). Typically, about 40 percent of patients do not respond to oral triptans (135). In addition, their use is restricted to patients without cardiovascular diseases. Therefore, large efforts have been made in the last decade to develop novel approaches to treat migraine based on sound scientific bases and new insights in the pathophysiology of migraine.

Calcitonin gene-related peptide antagonists

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide belonging to a family of peptides which encompasses calcitonin, adrenomedullin, amylin and the recently cloned calcitonin receptor-stimulating peptides (136, 137). CGRP binds to the CGRP receptor, of which different subtypes have been suggested, although thus far only one receptor has been indentified molecularly (136, 138-142). The receptor is known as a calcitonin-like receptor (CLR), belonging to the family of G-protein-coupled receptors (GPCRs). The GPCR forms a heterodimer with a single transmembrane domain protein, termed receptor activity-modifying protein (RAMP). Co-expression of CLR with RAMP1 forms the CGRP receptor (143). The CGRP-induced response following activation of the receptor involves cyclic adenosine monophosphate (cAMP), nitric oxide (NO), cyclic guanosine monophosphate (cGMP) and the K^+ channel as second messengers pathways. All these cascades have been shown to translate CGRP binding into a physiological response – for example, vasodilation of blood vessels (Figure 2) (144).

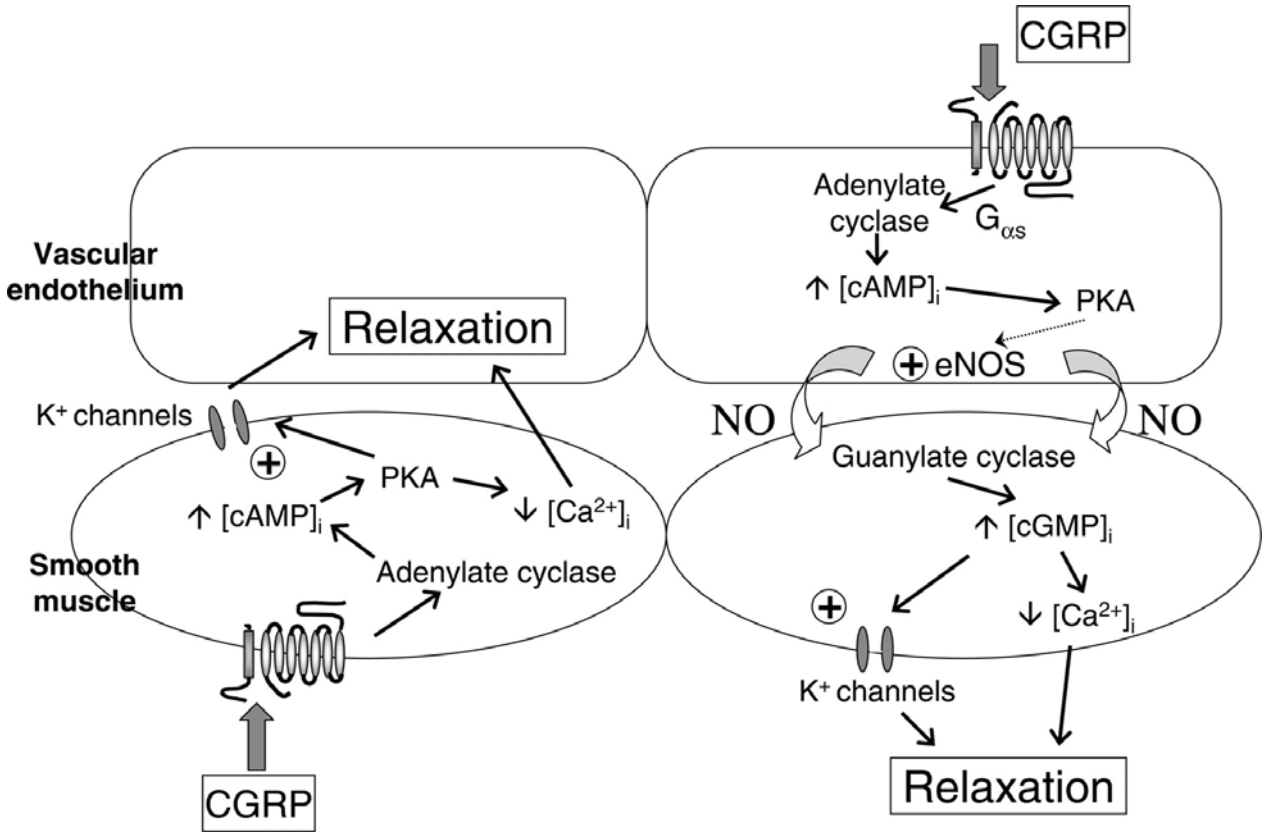


Figure 2: The cellular mechanisms of vasodilatation to CGRP (Reproduced from Brain, S. D. et al. Physiol. Rev. 84: 903-934 2004).

The concept that CGRP is a key player in migraine pathophysiology dates to the late 1980's (145, 146). Several studies demonstrated a rise in plasma CGRP both during spontaneous or nitroglycerin-induced migraine attacks (56-58, 147-149). However, Tvedskov and colleagues could not replicate similar elevations of CGRP levels in the human external jugular vein during migraine and suggested several reasons for this discrepancy (59). In addition, it is still uncertain whether CGRP levels are increased between migraine attacks (147, 150). Nonetheless, CGRP elevation during migraine does not seem to be spurious since in a small open-label study, relief of migraine by a triptan was demonstrated to be accompanied by a normalization of CGRP plasma levels and intravenous administration of CGRP causes headache and migraine attacks in migraine patients (36, 58, 151, 152). Consequently, small molecule CGRP receptor antagonists have been developed as a novel therapeutic approach in the treatment of migraine (153, 154). Given the positive phase II trials on 2 different CGRP receptor antagonists (i.e. olcegepant and telcagepant) and the recently published positive phase III data on telcagepant, CGRP receptor antagonism now strongly stands as a promising target for the treatment of acute migraine headache (65-67).

Furthermore, CGRP receptor antagonists are well tolerated and seem devoid of direct vasoconstrictor activity which makes them an attractive alternative and safe treatment for migraineurs at cardiovascular risk or with cardiovascular diseases (155-157).

Much debate still surrounds the site of action of the CGRP receptor antagonists. There are three potential targets: (i) the intracranial blood vessels; (ii) parts of the trigeminal nerve, either at the peripheral or central ends; or (iii) the central nervous system, in several areas that include the trigeminal nucleus caudalis, periaqueductal grey matter, tractus solitarius, amygdala and the colliculi (158, 159). Animal studies support a central site of action for CGRP in promoting migraine, as well as the anti-migraine effect of CGRP receptor antagonists (160-162). In humans, the effective dose of the CGRP receptor antagonist telcagepant suggests a central action. Indeed, small dosages of telcagepant, sufficient to block peripheral CGRP receptors did not work clinically, while larger, presumably central penetrant dosages did work (66, 163). A central action of CGRP-receptor antagonists is plausible as CGRP receptors in animals are localized in virtually every nucleus of the brain stem implicated in migraine genesis (161, 164-166). However, it is unlikely that CGRP or its receptor antagonists can penetrate the blood-brain barrier, given their hydrophilic nature and large molecular size (167). Thus, the mechanism and site of action of CGRP receptor antagonists is currently a matter of debate. It is nevertheless tempting to speculate that if CGRP receptor antagonists work centrally, they might have beneficial effects on other pain syndromes mediated by the trigeminovascular system, where CGRP receptors are numerous. CGRP receptor antagonists could also be valuable for treating other neurovascular pain syndromes where CGRP is released, for example complex regional pain syndrome (168).

Nitric oxide inhibitors

The link between NO and migraine has been the subject of considerable research (169, 170). Indeed, exogenously administered NO can induce a delayed headache that fulfills the diagnostic criteria for migraine (171). Whereas, it was once considered that NO triggered migraine by dilation of cranial

vessels, it is now apparent that the action of NO probably relies on a neural mechanism (33). Three recent observations suggest that the vasodilation is an epiphenomenon. First, nitroglycerin not only triggers delayed migraine headache in migraine patients, but also premonitory symptoms such as yawning, tiredness, irritability, neck stiffness, frequency of urination, hunger and low mood (172). These symptoms are similar to those reported in spontaneous migraine attacks and they occur well before any vascular change would have occurred (173). Second, sildenafil can induce migraine without any change in middle cerebral artery diameter through activation of the cGMP pathway, which is part of the NO-mediated signaling cascade (174). Finally, other vasodilators such as papaverine or VIP do not trigger migraine in migraine patients, suggesting that it is not the vasidilation *per se* that provokes a delayed migraine attack (33-39).

Nonvascular mechanisms have been suggested and do offer the possibility of a nonvascular approach to migraine treatment using nitric oxide synthase (NOS) inhibitors. Three isoforms of NOS have been indentified: the endothelial isoform (eNOS), neuronal isoform (nNOS) and inducible isoform (iNOS) (175). NOS inhibition using the nonselective inhibitor N^G-monomethyl-L-arginine monoacetate (L-NMMA) has been shown to abort acute migraine, but through eNOS inhibition also has unwanted vasoconstrictive effects (176, 177). iNOS, however, is believed to be involved in the pathophysiology of migraine through the development of inflammation of the dura mater and iNOS inhibition with GW274150, which has an analgesic effect in rat models, is devoid of vascular effects (178, 179). Inhibition of iNOS thus seems to be an attractive target for treating migraine, although it has recently fallen short of expectations (180-182). The potential of nNOS inhibition as a nonvascular target in migraine treatment remains largely unexplored, but offers promises that should be explored (183, 184).

Endocannabinoid system

Cannabinoids have been used for the treatment of migraine for many centuries but proof of their efficacy is obscured by the unreliability and anecdotal nature of reporting (185). Recently, important advances in its pharmacological characterization and role in pain modulation have drawn attention to the possible involvement of the endocannabinoid system in the pathogenesis of migraine (186, 187). Recent experimental findings suggest that anandamide, the endogenous ligand of the type 1 and type 2 cannabinoid receptor (CB1R and CB2R), is tonically released to play a modulatory role in the trigeminovascular system. In an animal model, anandamide was able to attenuate neurogenic, CGRP and NO-induced dural vessel dilation (186). In addition, anandamide seems to act presynaptically to prevent CGRP release from trigeminal sensory fibers. In humans, cannabinoid receptor activation has been reported to have antimigraine effects and to alleviate headache in idiopathic intracranial hypertension (185, 188). In addition, female migraine patients exhibit increased degradation of anandamide in platelets compared with controls, consistent with lower endocannabinoid tone (189). Whereas the use of cannabinoid receptor agonists for the treatment of migraine in the clinic is greatly limited by their known psychoactive effects, manipulating the transport reuptake of tonically released ananamide in the trigeminovascular system would largely circumvent this problem. Nonetheless, it remains largely unknown whether pharmacological intervention in the endocannabinoid system will prove successful in the treatment of migraine.

Other targets

Drugs aimed at various targets including the 5-HT_{1F} receptor, nociceptin receptor, orexin receptor, vanilloid receptor, $adenosine_{A1}$ receptor, glutamate receptor, adrenoreceptor and voltage-gated calcium channels have all been studied in preclinical migraine models (190-214). All were developed as migraine-specific medication without vasoconstrictive or vascular side effects. Some of them have undergone clinical testing or are the subject of current testing.

Non-pharmacological treatment

Ample data suggest the effectiveness of non-pharmacological treatment in the management of migraine patients. A wide variety of treatments is available ranging from dietary measures or lifestyle changes to acupuncture or cognitive behavioural therapy. These therapeutic tools carry the benefit of having no pharmacological side-effects and or thus safer than drug intervention.

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Chapter 2

Surrogates and models in the development of new anti-migraine drugs

Due to increasing costs and increased market competitiveness, the process of clinical drug development has been changing. The principal aim remains the safe and swift introduction of novel compounds into daily clinical practice. However, at the same time, there is an increased use of tools by which to assess at the earliest clinical stage possible whether a new target or drug will be successful. This approach allows less effective drugs to be discarded earlier and facilitates greater focus on potentially more beneficial drugs, both from the point of view of efficacy and safety.

Traditionally, clinical drug development was divided into three phases (1). In phase I, kinetics, tolerability and safety of single doses of a new compound are assessed by administration to a small number of healthy volunteers or patients. Frequently, the “maximum tolerated dose” was sought. In phase II, efficacy evaluation is the primary aim and dose-response relationships are sought in highly selected patient groups. In phase III confirmatory efficacy and safety are further assessed in larger trials. This approach results in considerable time loss between first-in-man studies and preliminary efficacy assessments. Importantly, such trails are also responsible for excessive dosing with new drugs in initial efficacy studies. Indeed, phase I studies can often be considered as little more than human toxicity studies in which the effects of dose regimens far in excess of the clinically useful dose are examined. As newer compounds are designed to target only key molecules specific to a disease condition or pathology, the risk of testing a compound in phase I at very large multiples of the pharmacologically effective dose becomes an increasing reality. In addition, the concept of the “maximum tolerated dose” is difficult to define and often poorly assessed in the context of a phase I trial (2). For example, an important adverse effect occurring at a frequency of 1 in 20 at a given dose is likely to be unacceptable in clinical practice but is unlikely to be detected and related to the study drug in a typical phase I study of healthy volunteers (3).

Selecting the dose regimens for clinical trials and the marketing of a drug based on the “maximum tolerated dose” approach or fractions thereof thus often leads to the dismissal of an otherwise invaluable drug due to the apparent unacceptable safety profile resulting from overdosing. Sumatriptan, for example, was initially marketed at an oral dose of 100 mg, whereas 50 and 25 mg have been shown to be equally effective and more tolerable as starting doses (4).

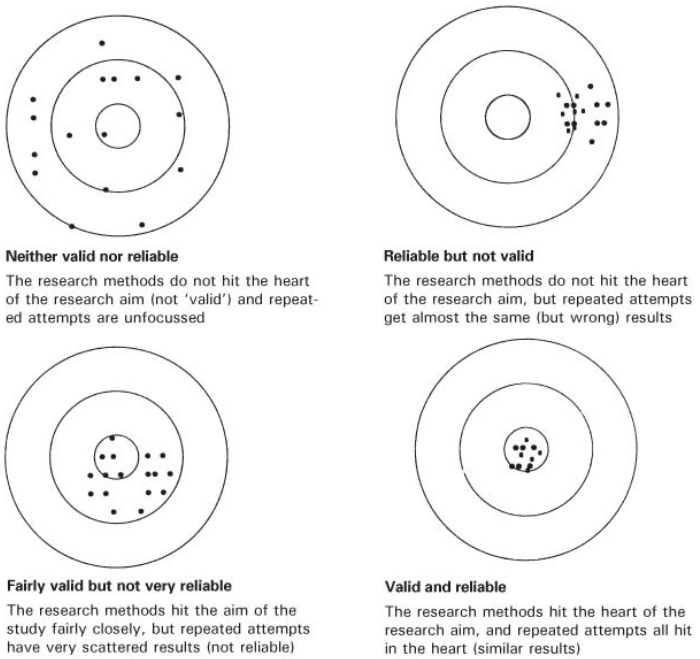
Clinical pharmacology surrogates and models

In order to provide the possibility of establishing a sound foundation for dose selection at the earliest possible opportunity, clinical pharmacologists have been developing surrogates and models. Whilst for some drugs, the desired therapeutic effect can be observed directly (e.g. antihypertensives), for most drugs no such direct observation is available and a surrogate or model is needed. The same applies to undesired side-effects that might not be immediately apparent (e.g. mild vasoconstriction), but are nevertheless detectable using the appropriate tools.

A *surrogate* may be defined as a clinical measurement known to be statistically associated with and believed to be pathophysiologically related to a clinical outcome (5, 6). A *model*, on the other hand, is an experimental system or paradigm, used in drug development to simulate some aspects of the disease studied in which the effects of a drug can be examined (3). The difference between a surrogate and a model is thus largely operational and not conceptual.

In many ways, surrogates and models can be used to obtain so-called “proof of concept” at an early stage of the clinical development of a drug. In a “proof of concept” approach, one attempts to demonstrate, before launching large patient studies, that a chosen dose of a drug does what it is supposed to do or is devoid of unwanted side-effects.

In order to infer any clinical consequence from a given surrogate or model, it is crucial to consider its *validity*. A model or surrogate can be considered valid if the obtained results are relevant and predictive of the clinical outcome studied (7). The meaning of validity is closely related to but distinct from that of reliability. *Reliability* is the extent to which a measurement yields the same results when it is repeated either by the same observer (intra-observer reliability) or by different observers (inter-observer reliability). Reliability relates to the reproducibility of measurements, whereas validity deals with the accuracy (correctness) of inferences drawn from such measurements. The relationship between validity and reliability is best illustrated by analogy to target shooting (8).



The fourth target this figure clearly indicates that to obtain a high degree of validity, reliability of measurements is largely presupposed. The first component of validation of a surrogate or model should thus comprise statistical analysis relating to the reproducibility of the measurements, in other words, to their reliability. Nevertheless, reliability in measuring does not automatically imply validity. The next step for the thorough validation of a surrogate or model

relates to the fact that the intervention with the drug should induce changes to the surrogate or measurements made in the model that correlate closely with the effect of the drug on clinical outcome. This aspect of validation is referred to as *criterion validity* and is established by reference to existing clinical data (7). The need for an established correlation between the surrogate or model and clinical outcome highlights the greatest limiting factor to the validation of surrogates and models in the exploratory development of innovative therapies because clinical data are simply not yet available. In such context validity can merely be based on the assumption that the surrogate or model shares a causal mechanism with the ultimate clinical outcome. This type of validity is referred to as *construct validity*. At the beginning of the validation of a surrogate or model, construct validity is solely based on theoretic assumptions. Only later, when clinical data become available, can criterion validity of the surrogate or model be verified. By integrating the imperfectly validated surrogate or model in efficacy trials, retrospective validation can thus be obtained. As it is rare that only one compound of a therapeutic class is in development, the fully validated surrogate or model can be used in the development of subsequent compounds, resulting in major time savings and better dose settings (9).

When developing surrogates or models for innovative therapies or diseases where no well-validated surrogates or models exist, the likelihood of success greatly depends on the careful exploration of potential candidates well before the clinical development of the new compound. Only then can founded construct validity of a surrogate or model be assured. In the preclinical development of CGRP receptor antagonists for example, pharmacodynamic models had already been developed in different animal species (10). When these models were translated into a human pharmacodynamic model used in early clinical development they proved to be indicative of clinical efficacy in the treatment of migraine (11-14).

Need for surrogates and pharmacodynamic modelling in the development of anti-migraine drugs

Migraine headache is by its nature a human experience. Whereas in animal models it is possible to study the effect of a drug on mechanisms that are believed to play a role in the pathophysiology of migraine (e.g. vascular responses, neurogenic inflammation, etc.), no direct inferences can be made concerning the effect on the actual headache (15). Moreover, the actual pathohysiology of migraine remains unsolved and even within the putative mediators of migraine, there appears to be enormous variability between species (10). In addition, migraine is a highly heterogeneous syndrome with an attack-wise appearance which complicates recruitment of patients into early efficacy studies. For all of these reasons, the need for experimental modelling in humans is obvious. The main goal is to prevent undertaking long, expensive efficacy trials without any proof of concept or using inappropriate dose regimens.

A first approach to increase efficacy of drug development for migraine consists of provoking a core mechanism of migraine pathohysiology in healthy volunteers. Healthy volunteers are easy to recruit and they make a more homogenous population than patients as homogeneity can be improved through specific selection criteria. The evidence that CGRP is a key molecule in the pathogenesis of migraine headache is strong and triggered the development of CGRP receptor antagonists which proofed effective for the treatment of acute migraine (13, 14, 16). Our group developed a human pharmacodynamic model using venous occlusion plethysmography combined with intra-arterial administration of CGRP to allow early clinical assessment of the pharmacodynamic efficacy of CGRP receptor antagonists in healthy volunteers (17). One of the primary aims of the present thesis is to propose and evaluate a less complicated, non-invasive pharmacodynamic model in humans with rapid and objective end-points to guide the finding of the clinical effective dose of newly developed CGRP receptor antagonists (**Chapter 3 and 4**). In addition we provide tools of clinical pharmacology to assess the cardiovascular safety of CGRP-receptor antagonist in the early stage of their clinical development (**Chapter 5**).

A second approach consists of identifying possible drug targets in migraineurs. It was by studying migraine patients during an attack, that the involvement of CGRP in migraine pathophysiology was suspected (18, 19).

The link between NO and migraine has also been the subject of considerable research and exogenously administered NO can induce a delayed headache that fulfills the diagnostic criteria for migraine (20-23). Nevertheless, the question whether migraine patients display a chronic NOS hyperactivity remains unsolved. Our aim is to develop methods allowing the evaluation of endogenous NO production in migraine patients and healthy volunteers (**Chapter 6**). In addition to providing indications of the involvement of NOS in the pathogenesis of migraine, measuring systemic NO production also permit the pharmacodynamic assessment of NOS inhibitors (**Chapter 7**).

Recent findings suggest that a failure of the endogenous cannabinoid system might also play a role in the pathophysiology of migraine (24, 25). Advances in neuroimaging allow us to study the type 1 cannabinoid receptor availibility (CB1R) in vivo and we are thus able to compare CB1R availability between migraineurs and healthy controls (**Chapter 8)**. This might provide an indication of

dysregulation of the endocannabinoid system in migraineurs and lead to the identification of the endocannabinoid system as a potential treatment target for migraine.

A third approach consists of inducing an experimental headache in migraine patients. This approach is especially useful as recruitment of migraine patients during an attack, necessary for assessing the efficacy of acute anti-migraine drugs, is often bothersome and slow. In addition, it may provide information on key molecules involved in the pathogenesis of migraine (21, 22). Nonetheless, provoking an acute headache is not attractive to those who participate in clinical studies. In addition, it remains debatable whether these “provoked” headaches are identical to spontaneous migraine headaches. Therefore, we did not primarily aim to induce so-called “experimental” headaches.

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OUTLINE OF THE THESIS

The present thesis comprises studies performed in healthy volunteers and migraine patients. The overall aim was to develop *in vivo* tools for investigating putative targets for migraine and speed up the clinical development of new compounds that engage them.
Based on new insights in the pathophysiology of migraine, three new treatment strategies are investigated: calcitonin gene-related peptide (CGRP) receptor antagonism **(Part I)**, nitric oxide synthase (NOS) inhibition **(Part II)** and intervention in the endocannabinoid system **(Part III)**.

In **Part I** of the thesis, a human pharmacodynamic model is developed in healthy volunteers using capsaicin-induced dermal vasodilation to evaluate potency and dosing of CGRP receptor antagonists. We assess the model's reproducibility (**Chapter 3**) and evaluate the model's validity using the CGRP receptor antagonist $CGRP_{8-37}$ (**Chapter 4**). In addition, the involvement of prostaglandins, nitric oxide (NO) and substance P (SP) in capasaicin-induced dermal vasodilation is assessed.
Whereas existing specific anti-migraine drugs were developed as cranial vasoconstrictors (i.e. triptans and ergot alkaloids), CGRP receptor antagonists seem devoid of direct vasoconstrictor activity. This renders CGRP receptor antagonists a more favourable cardiovascular safety profile and affirms their potential as an alternative safe treatment in migraine patients with cardiovascular risks/disease. Nevertheless, CGRP is believed to be released following the administration of nitroglycerin (NTG), which is used in the treatment in cardiac ischemic pain (angina pectoris). We characterize the vascular effects of NTG and the potent CGRP receptor antagonist telcagepant in healthy volunteers by the use of oscillometric blood pressure measurement, ultrasound measurement of the brachial artery diameter and systolic pulse contour analysis. By administrating NTG following a supra-therapeutic dose of telcagepant, we are also able to assess the degree to which the vasodilatory properties of NTG depend on the release of CGRP (**Chapter 5**).

In **Part II** of the thesis, we focus on the likely involvement of NOS hyperactivity in the pathogenesis of migraine. First, the reproducibility of the increase in endogenous NO production following a loading dose of the amino-acid L-arginine is assessed in healthy volunteers using the following biomarkers associated with the L-arginine-NO pathway: exhaled/nasal NO (eNO, nNO), plasma L-citrulline and L-arginine and urinary excretion of nitrite/nitrate and cGMP. Subsequently, we compare the endogenous NO production following L-arginine infusion between migraine patients (interictally) and matched healthy control subjects. Meanwhile, we evaluated whether the increase in NO production following L-arginine triggered an acute migraine headache in migraineurs (**Chapter 6**). In a second study conducted in 15 migraine patients, the effects of an acute migraine headache on the pharmacokinetics and pharmacodynamics of GW273629, a potent inhibitor of inducible NOS (iNOS), was evaluated. This allowed the concomitant assessment of the effects of migraine and selective iNOS inhibition on systemic NO production. In addition preliminary evidence was obtained concerning the efficacy of iNOS inhibition for treating acute migraine (**Chapter 7**).

The final part (**Part III**) of the thesis addresses the putative involvement of the endocannabinoid system in the pathophysiology of migraine. Recent findings suggest that a failure of the endogenous cannabinoid system might play a role in the pathophysiology of migraine and its tendency to progress to chronic migraine. Using [^{18}F]MK-9470, a positron emission tomography radioligand of the type 1 cannabinoid receptor (CB1R), we compared the cerebral CB1R availability between female migraine patients and matched healthy women (**Chapter 8)**.

Part I

Calcitonin gene-related peptide as a target for migraine treatment:

Chapter 3 and 4:

Development of a pharmacodynamic model to assess CGRP receptor antagonists *in vivo.*

Chapter 5

Assessment of the vascular effects of CGRP receptor antagonists

Chapter 3

Reproducibility of the capsaicin-induced dermal blood flow response as assessed by laser doppler perfusion imaging

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Abstract

Aims

Part I: To establish the dose and appropriate application site of capsaicin on the human forearm in order to produce a robust and reproducible dermal blood flow (DBF) response. Part II: To evaluate the within-subject arm-to-arm and period-to-period reproducibility.

Methods

Both parts consisted of two study visits. In part I, placebo and 100, 300 and 1000 µg capsaicin were applied at 4 predefined sites on the volar surface of both forearms. Placebo and capsaicin doses were randomized and balanced by site between subjects. Changes in DBF were assessed by laser Doppler perfusion imaging up to 60 minutes after capsaicin application. In part II, only 1000 µg capsaicin was applied on the proximal forearm and changes in DBF assessed up to 30 minutes (t_{30}). DBF response was expressed as percent change from baseline ± SD and the corresponding $AUC_{0\text{-}30}$. Reproducibility assessment included calculation of the concordance correlation coefficient (CCC).

Results

Part I (n=12 subjects): compared to placebo, 300 and 1000 µg capsaicin increased DBF ($p<0.05$) at all time-points except at 10 minutes. This increase was reproducible at the two most proximal sites from the 30 minutes time-point onwards when compared between arms (CCC ≥ 0.8, i.e. substantial to almost perfect reproducibility). In part II (n=11), t_{30} averaged 390±120 % and arm-to-arm reproducibility was almost perfect (CCC = 0.91) for $AUC_{0\text{-}30}$.

Conclusions

Capsaicin induces a reproducible within-subject arm-to-arm increase in DBF. We provide a non-invasive pharmacodynamic model in humans to test antagonists of mediators involved in capsaicin-induced dermal vasodilation, including CGRP-antagonists.

Introduction

Capsaicin is a vanilloid responsible for the pungent taste of hot peppers. Högyes was the first to state that the pungent and irritant nature of capsaicin is mediated by sensory nerves (1, 2). This was confirmed in the second half of the twentieth century mainly by the work of Hungarian scientists (3). More recently, it emerged that the selectivity of capsaicin for the thin afferent neurons of mammalian species is due to the existence of the transient receptor potential vanilloid type 1 receptor (TRPV1) (4-8). This receptor is expressed on a subpopulation of primary sensory neurons consisting of Aδ- and C-fibre nociceptors. Binding of capsaicin to the TRPV1 receptor provokes an inward non-selective cationic current depolarizing the neuron (5, 9). This activation results in the release of bioactive substances which in turn act on target cells in the surrounding tissue including mast cells, immune cells and vascular smooth muscle cells. The resulting response is characterized by redness and warmth (secondary to vasodilatation), swelling (secondary to plasma extravasation) and hypersensitivity (secondary to alterations in the excitability of primary sensory neurons). Collectively, this response is referred to as “neurogenic inflammation” (10).

Capsaicin has been used extensively in human pain models to induce experimental pain (11-13). When capsaicin is locally administered to the human skin, by topical application or intradermal injection, it rapidly produces a local neurogenic inflammation by binding to the TRPV1 receptor present on sensory nerve endings in the skin (10, 14). Most evidence indicates that the release of calcitonin gene-related peptide (CGRP) is a major initiator of this response (15, 16). Other putative bioactive mediators are substance P (SP), neurokinin A (NKA), somatostatin, nitric oxide (NO), histamine and prostaglandins, though their role in capsaicin-induced neurogenic inflammation in the normal human skin is less well established (17-21)

Capsaicin-induced neurogenic inflammation shows striking similarities with the neurogenic inflammation of the trigeminovascular system, which is thought to be of central importance during a migraine attack (22). The cranial release of CGRP for instance, is suggested to be pivotal in the pathogenesis of migraine as confirmed by the fact that the CGRP receptor antagonist BIBN4096BS is efficacious in the treatment of migraine (23). It is tempting to speculate that capsaicin-induced dermal neurogenic inflammation might serve as a model for neurogenic inflammation elsewhere in the body. Therefore, a better knowledge of the cascade of antidromic effects of dermal sensory neuron stimulation may improve our understanding of the pathophysiology of migraine and the ways to prevent and/or abort a migraine attack.

Helme et al. extensively studied the dermal neurogenic inflammation that occurs after the topical application of capsaicin onto the human skin. They assessed both the size of the flare response (i.e. erythema due to an increase in blood flow) and allodynia (i.e. hypersensitivity to heat and touch) (10). The advent of an X-Y scanning Laser probe now allows a more reliable method for characterizing the microvascular changes leading to the flare reaction (24). Laser Doppler perfusion imaging has been used to study the influence of topically applied capsaicin on blood flow responses in the human skin

(25). Furthermore, Hershey et al. used topical application of capsaicin on the skin of nonhuman primates and rats to stimulate CGRP-mediated vasodilation which was measured by laser Doppler perfusion imaging (26). They also demonstrated that this capsaicin-induced vasodilation was inhibited by a novel potent and selective CGRP antagonist, thus validating a non-invasive pharmacodynamic assay in animals that provides a rapid evaluation of CGRP antagonists.

The overall aim of the present study was to evaluate whether this pharmacodynamic model could be translated in healthy volunteers which would allow rapid and objective readouts of CGRP antagonist activity directly in the human species itself. In order to develop a useful model for human studies, we first needed to address two major issues, for which we designed a two part study.
In the first part, we identified the *dose* of capsaicin needed to produce a robust and reproducible dermal blood flow (DBF) response after topical application to the human forearm. Simultaneously, the influence of the forearm *location* on the capsaicin response was assessed.
In the second part, the within-subject arm-to-arm and period-to-period reproducibility of the forearm DBF response to capsaicin were evaluated using the optimal dose of capsaicin and the most appropriate forearm location as assessed in part I. In addition, different ways of expressing the DBF response were compared and sample size calculations performed.

Methods

Subjects

After approval by the ethics committee of the University Hospital, written informed consent was obtained from all subjects during a screening visit. Twelve men were recruited for participation in the first part of the study; 21 in the second part. All subjects were Caucasian, non-smoking, healthy males between 18 and 45 years of age.

Study design

Subjects were instructed to abstain from any drugs during three days and from chocolate-, alcohol- and caffeine-containing beverages and food during 12 hours preceding each study period. Both study parts (part I and part II) were open-label and consisted of two periods separated by at least one week. Subjects fasted for at least 4 hours before each study period. All measurements were performed while the subjects rested in a semi-recumbent position on a comfortable bed in a quiet, temperature controlled room (ambient temperature of 24°C $\pm$ 1 °C).
During each period, 10 mm rubber O-rings (McMaster-Carr, New Brunswick, US; 8 mm inner diameter) were placed onto the skin at 4 equally spaced sites on the volar surface of both forearms. The rings were positioned so that their distal edges were 10, 14, 18 and 22 cm proximal to the wrist crease, within approximately 1 cm of the midline and avoiding visible veins. The proximal ring (i.e. closest to the antecubital crease) is referred to as site 1, the distal ring (i.e. closest to the wrist crease) as site 4. After placement of the O-rings a laser Doppler imager was used to obtain baseline scans of the dermal blood flow in the areas defined by the rings. Subsequently, these O-rings served as reservoirs to contain the topically applied 20µL capsaicin or placebo solutions.

Capsaicin powder was obtained from Sigma-Aldrich N.V. (Bornem, Belgium) and was dissolved in a 3:3:4 mixture of ethanol 100%, Tween-20 and distilled water. Capsaicin was diluted such that 20 µL of the mixture contained 100, 300 or 1000 µg capsaicin. The placebo solution corresponded to the same 3:3:4 mixture of ethanol 100%, Tween-20 and distilled water without capsaicin.

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Part I: dose finding and assessment of the influence of the forearm location

After obtaining baseline scans, subjects received single topical doses of 100, 300 and 1000 µg capsaicin/20µL vehicle and placebo (i.e. 20 µL of vehicle) in the 4 rings at each forearm. Applications were randomized and balanced by site between the different subjects. Each subject received the same treatment on the left and right forearms and during both study periods. The time course of the blood flow response to each dose of capsaicin/placebo was measured by performing laser Doppler scans of the area within each ring at 10, 20, 30, 45 and 60 minutes post-capsaicin application.

Part II: within subject arm-to-arm and period-to-period reproducibility

After obtaining baseline scans subjects received on both forearms a topical dose of 1000 µg capsaicin/20µL vehicle in the two proximal O-rings; placebo in the two distal O-rings. Subsequently, Laser Doppler scans were performed at 10, 20 and 30 minutes post-capsaicin application. Only subjects with an increase in forearm blood flow from baseline of 100% or more in both proximal sites of both arms and during both study periods were allowed to participate. If this DBF increase threshold was not attained, subjects were excluded from the study.

Assessment of forearm dermal blood flow response to capsaicin

The skin perfusion of the test sites, i.e. the region delimited by the rubber O-rings, was mapped using a High Resolution Laser Doppler Perfusion Imager (HR-LDPI system, PeriScan PIM II®, Perimed, Sweden). With the X-Y scanning laser probe, laser Doppler perfusion imaging allows the two-dimensional mapping of the blood flow variability over an extended skin surface. The method is non-contact, the laser beam being controlled by the computer-controlled rotation of a mirror about two perpendicular axes. The red light of a 633 nm helium-neon laser penetrates the skin variably to a depth of about 0.6 mm. The vasculature within these layers comprises vessels of variable size, orientation and function. For this reason, it is not meaningful to express the cutaneous flow in absolute terms, but rather as arbitrary 'perfusion units'. The estimation of the average skin perfusion at the test sites was based on the measurement of perfusion values of approximately 60 individual sampling sites. These calculations were performed using the built-in statistical function of the HR-LDPI system (LDPIwin, Perimed, Sweden). The methodology is fully described by Fullerton et al. (24).

Data analysis and statistics

The change in DBF in response to capsaicin was expressed as the percent change from baseline. At each time-point the mean of observations with the 90% two-sided confidence interval (CI) is given. In

addition a paired Student's *t*-test was performed to compare the DBF percent change from baseline to placebo.

In *part I* of the study, we first calculated the means of the DBF responses in both arms and during both periods for every dose of capsaicin or placebo, at each time-point regardless the site of application on the forearm. A second analysis was made in which the means of the increases in DBF in both arms and periods were assessed at each site tested for each dose of capsaicin or placebo. For each subject, the DBF percent change from baseline was then compared between both arms (i.e. arm-to-arm reproducibility) and between both study periods (i.e. period-to-period reproducibility). To assess the test-retest reproducibility of the DBF increase, the Bradley-Blackwood procedure, which corresponds with the graphical method of Altman and Bland, was used (27, 28). Practically, the individual means of the increases of DBF for each dose, at each time-point and test site were plotted versus the individual differences in DBF response between the right and left forearm (arm-to-arm reproducibility) or first and second study period (period-to-period reproducibility). For the arm-to-arm comparison, the observations from the first and second study period were used, whereas for the period-to-period comparison, the observations from both arms were included in the analysis. Using the Bradley-Blackwood procedure, we then tested site-by-site whether at any given time-point the regression coefficient of this regression analysis was significantly different from zero. A regression coefficient significantly different from zero suggest that the test-retest reproducibility or model assumptions may be violated. The Bradley-Blackwood test was performed at the 0.05 significance level. As an exploratory assessment for test-retest reproducibility, the concordance correlation coefficient (CCC) was calculated for each time-point and each site (29-31). A CCC value closer to 1 indicates a more reproducible response.

In *part II* of the study, only subjects with an increase in forearm blood flow from baseline of 100% or more (i.e. responders) in both proximal sites of both arms and during both study periods were included. The means of the DBF percent change from baseline were calculated for the two proximal test sites (i.e. the rings containing the 1000 µg capsaicin dose) and for the two distal test sites.(i.e. the rings containing placebo). As in part I we used the observations from the first and second study period for the arm-to-arm-comparison, whereas for the period-to-period comparison the observations from both arms were included in the analysis. In addition the area under the curve of the percent change from baseline up to 30 minutes after capsaicin application ($AUC_{0\text{-}30}$) was calculated as a summary measure. To assess test-retest reproducibility, the mean difference and the repeatability coefficient (RC), i.e. 1.96 times the standard deviation of the differences, were calculated according to Bland and Altman (32). Secondly, the mean within-subject coefficient of variation (WCV) was calculated by dividing the within-subject standard deviation by the mean and expressing it as a percentage. The within-subject standard deviation was calculated as the square root of the residual mean square using two-way analysis of variance (ANOVA) (33). A two sided 95% CI was calculated for the mean difference and WCV. Finally, the CCC was calculated.

Sample size calculations for a paired study design with continuous response measures were performed using the mean response of period 1 and 2 and the standard deviation of the difference in response between period 1 and 2 and between the dominant and non-dominant arm (34). Sample sizes required to detect a predetermined difference of 10, 20 and 50% in DBF response between treatment periods and arms given a type I error probability (α) of 0.05 and a power of 80% were calculated. $P < 0.05$ was considered statistically significant.

Results

Capsaicin application was well tolerated by all subjects in both study parts and no adverse events of note were reported. In most of the subjects, capsaicin provoked a local flare and stinging sensation that disappeared within 2-6 hours after application. Two subjects experienced a stinging sensation at the exact spot of capsaicin application whilst a sleep. whereas another healthy volunteer noted an aggravation of the residual stinging whilst taking a hot bath.

Part I: dose finding and assessment of the influence of the forearm location

The study was completed successfully by all 12 subjects. Mean ± SD (range) for age, weight and height was 24 ± 3 (20-30) years, 81 ± 12 (67-115) kg and 184 ± 7 (169-196) cm, respectively.

When calculating the means of the DBF responses in both arms and during both periods for each dose of capsaicin or placebo, at each time-point regardless the site of application on the forearm, a gradual increase of the DBF after both the 300 µg and 1000 µg capsaicin applications became apparent (Figure 1). This increase was statistically significant compared to placebo at all time-points except at 10 minutes post-dose. The 100 µg dose failed to increase DBF significantly at any time-point.

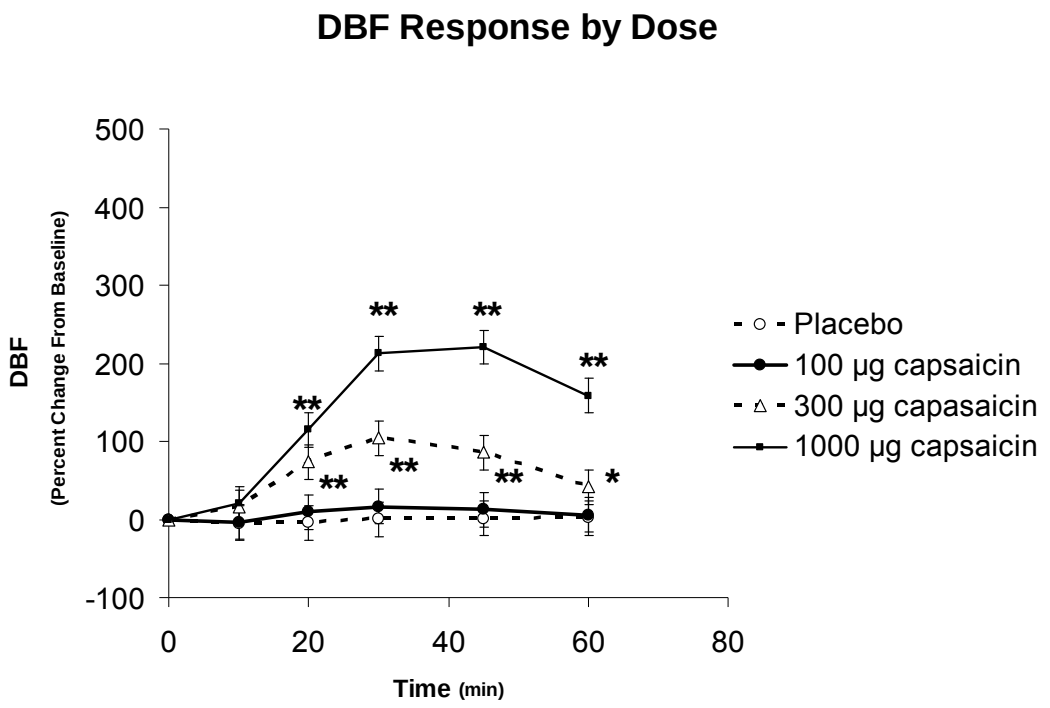


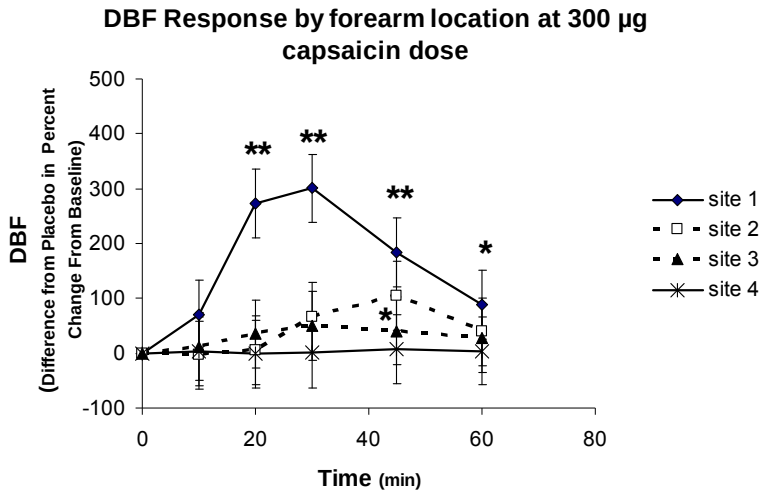
Figure 1: DBF response (percent change from baseline) after application of placebo, 100, 300 and 1000 µg capsaicin. The mean of the DBF responses in both arms and during both periods for each dose of capsaicin or placebo was calculated for each subject.

number of subjects = 12, number of observations per dose/placebo = 48

* $p < 0.05$, ** $p < 0.001$ (paired Student's t-test comparing the percent change from baseline to placebo). Data are mean of observations ± 90% confidence interval.

Data were also analysed as a function of the forearm location for each dose of capsaicin or placebo. At all time-points and at all sites, the 100 µg dose failed to raise DBF compared to placebo (data not shown). At the 300 µg dose, only site 1 (i.e. most proximal) demonstrated a significant increase compared to placebo at every time-point except the 10 minute time-point. At the 1000 µg dose, sites 1, 2 and 4 showed significant increases of DBF compared to placebo, again at all time-points except at 10 minutes (Figure 2).

A



B

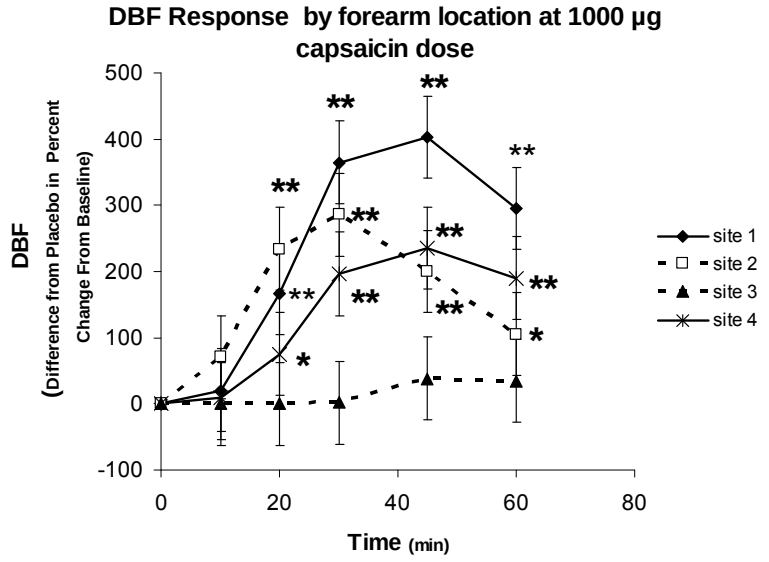


Figure 2: DBF response (difference from placebo in the percent change from baseline) after application of 300µg (**Figure 2A**) and 1000 µg (**Figure 2B**) capsaicin. The mean of the DBF responses in both arms and during both periods was calculated for each subject. Site 1 is most proximal; site 4 most distal. Number of subjects receiving 300 µg capsaicin at given site = 3, number of observations per site = 12

* $p < 0.05$, ** $p < 0.001$ (paired Student's t-test comparing the percent change from baseline to placebo). Data are mean of observations ± 90% confidence interval.

Test-retest reproducibility was assessed by site from arm-to-arm and from period-to-period using the Bradley-Blackwood test (Table 1).

Table 1

Test-Retest Reproducibility for Dermal Blood Flow Response by Site

Site	Time	Arm-to-Arm Reproducibility		Period-to-Period Reproducibility	
		p-value*	CCC	p-value	CCC
1	**10 min**	**<0.001**	**0.269**	**0.0005**	**0.190**
	20 min	**0.0360**	**0.766**	0.7684	0.697
	30 min	0.2117	0.804	0.8034	0.780
	45 min	0.4483	0.790	0.7481	0.859
	60 min	0.9618	0.760	0.4384	0.715
2	10 min	0.0692	0.355	**0.0244**	**0.215**
	20 min	0.2384	0.951	**0.0009**	**0.836**
	30 min	0.2479	0.887	0.1039	0.714
	45 min	0.4876	0.932	0.4859	0.376
	60 min	0.1515	0.797	**0.0431**	**0.344**
3	10 min	0.4623	0.163	0.4421	0.423
	20 min	**0.0025**	**0.325**	**0.0006**	**0.163**
	30 min	0.1137	0.513	0.3259	0.117
	45 min	0.0552	0.372	0.5679	0.408
	60 min	0.1548	0.490	0.1614	0.245
4	**10 min**	**0.0448**	**0.236**	0.1160	0.050
	20 min	**0.0056**	**0.073**	**0.0001**	**0.367**
	30 min	**0.0028**	**0.623**	**0.0220**	**0.644**
	45 min	**0.0048**	**0.808**	0.8164	0.720
	60 min	0.4062	0.951	0.3193	0.788

number of subjects = 12, number of observations = 48

the proximal ring (i.e. closest to the antecubital crease) is referred to as site 1, the distal ring (i.e. closest to the wrist crease) as site 4.

* Based on Bradley-Blackwood Test: P<0.05 indicates evidence of unequal means or unequal variances from right arm to left arm or from period 1 to period 2.

CCC, Concordance correlation coefficient indicating strength-of-agreement; CCC > 0.9, almost perfect; CCC 0.8 - 0.9, substantial; CCC 0.65 - 0.8, moderate, CCC < 0.65, poor.

Apart from site 4, no sites violated arm-to-arm or period-to-period test-retest reproducibility from 30 minutes onwards. One exception was site 2 for which the period-to-period reproducibility was violated at 60 minutes post-dose.

The CCC showed substantial to almost perfect strength-of-agreement for test-retest arm-to-arm DBF measurements at the 30 minutes time-point at sites 1 and 2. The test-retest period-to-period reproducibility was moderate for the same sites at the same time-point.

Part II: within-subject arm-to-arm and period-to-period reproducibility

Of the 21 subjects enrolled in part II, 10 non-responders were excluded from further analysis (i.e. the increase in forearm blood flow was below 100% in one of the proximal sites during one of the two study periods). The data of the 11 remaining subjects were included in the reproducibility analysis. Mean ± SD (range) for age, weight and height of these 11 subjects was 25 ± 5 (20-37) years, 81 ± 7 (69-91) kg and 182 ± 5 (174-192) cm, respectively.

The gradual increase in the DBF after 1000 µg capsaicin application is shown by arm pooled over periods (Figure 3A) and by period pooled over arms (Figure 3B).

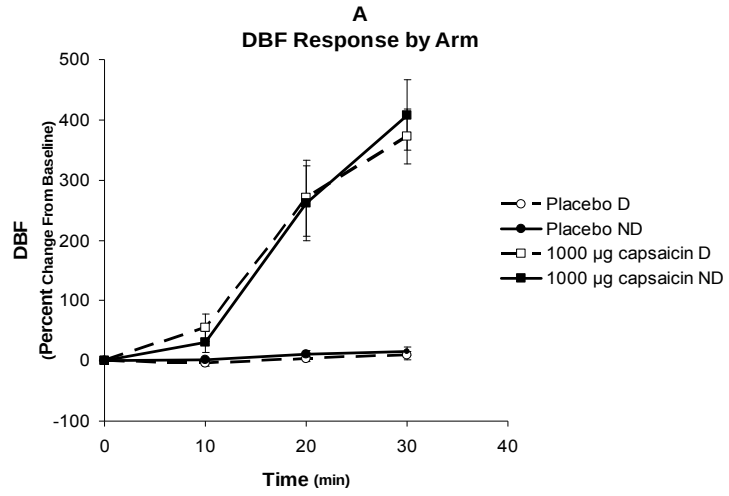


Figure 3A: DBF response (percent change from baseline) after application of placebo and 1000µg capsaicin. The mean of the DBF responses at the capsaicin or placebo application sites during both study periods was calculated. Number of subjects = 11, number of observations = 44D, dominant arm ; ND, non-dominant arm. Data are mean of observations ±90%

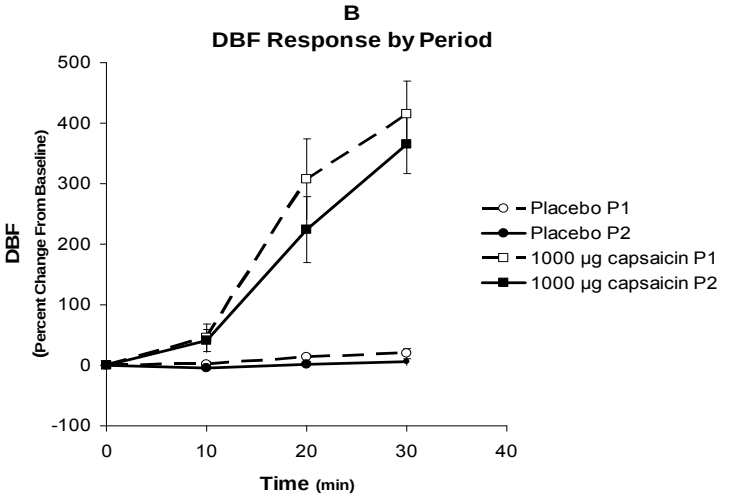


Figure 3B: DBF response (percent change from baseline) after application of placebo and 1000µg capsaicin. The mean of the DBF responses at the capsaicin or placebo application sites in both arms was calculated.

number of subjects = 11, number of observations = 44

P1, period 1; P2, period 2. Data are mean of observations ± 90% CI

The increase in DBF was statistically significant compared to placebo at all time-points. The increase in the DBF was however not significantly different when comparing the same time-points between arms or periods. Results for the reproducibility analyses for DBF responses are given in Table 2

Table 2: Test-Retest Reproducibility of Dermal Blood Flow Response and Sample Size Calculations

DBF response	Test-Retest	Mean difference (95% CI)	RC	WCV (%) (95% CI)	CCC	SSC 10% shift	SSC 20% shift	SSC 50% shift
t_{30} (%)	Period-Period	49 (-19.6, 118.0)	307	28 (19, 38)	0.40	129	34	7
	Arm-Arm	36 (-85.4, 14.4)	223	21 (14, 27)	0.68	69	19	5
AUC_{0-30} (%.min)	Period-Period	1119 (-239, 2476)	6058	43 (30, 57)	0.33	297	76	14
	Arm-Arm	139 (-340, 619)	2140	15 (11, 20)	0.91	39	11	4

number of subjects = 11, number of observations = 44, the mean DBF response in the two proximal rings was used in the test-retest analysis

Test-retest period-to-period and arm-to-arm reproducibility data for DBF response expressed as percent change from baseline at 30min (t_{30}) and as the area under the curve of the percent change from baseline (AUC_{0-30}).

DBF: dermal blood flow

RC: repeatability coefficient

WCV: within-subject coefficient of variation

CCC: concordance correlation coefficient

SSC: Sample size calculation

95% CI: 95% two-sided confidence interval

The test-retest reproducibility was better from arm-to-arm then when compared from period-to-period. This is reflected by the CCC for arm-to-arm test-retest reproducibility, which varies from moderate for DBF expressed at t_{30} to almost perfect for DBF expressed as AUC_{0-30}. As a consequence a smaller number of subjects is needed to detect a predetermined shift in DBF response between arms than between periods.

Discussion

The present study convincingly demonstrates that the increase in dermal blood flow following capsaicin application is adequately reproducible at the proximal forearm when the DBF response is sufficiently robust (i.e. defined as 100 % or more increase from baseline). The procedure was well tolerated and no adverse events of note were reported.

Exploration of the skin microcirculation by Laser Doppler technology has often been considered poorly reproducible (35). However, the introduction of laser Doppler perfusion imagers that allow the quasi-

simultaneous measurement of skin blood flow at a very large number of points, thus allowing the "averaging out" of spatial variations encountered within the explored area, improved the reproducibility of this technique considerably (24, 36, 37). Clear guidelines for the measurement of dermal blood flow by laser Doppler perfusion imaging are now available and it has become a regularly used technique for assessing mean cutaneous blood flow of both normal and irritated forearm skin (24).
Data from the first part of this study revealed a dose-dependent capsaicin-induced increase in DBF. At the 1000 µg capsaicin dose, a robust and reproducible response was present in both proximal sites at the 30 minute time-point. The maximum response occurred between 30 and 45 minutes after capsaicin application, with a clear decline at the 60 minutes time-point. The transient nature of the capsaicin-induced vasodilation was also seen in rats, rabbits and rhesus monkeys and may be attributed to the half-life and depletion of the endogenous mediators and/or the desensitization of the TRPV1 receptor (26, 38, 39).

Data from the first part of this study clearly indicated that, compared to the more distal sites, the two proximal sites of the human forearm showed the most reproducible and robust responses to capsaicin. This regional variation in response was also seen in a study in humans using transdermal iontophoretic application of acetylcholine to induce vasodilation in the forearm skin (36). The reasons for this regional variation remain uncertain. Heterogeneity in the density and function of the capsaicin-sensitive nociceptive nerve endings as well as of the dermal microcirculation seem the most plausible explanations. In addition, the transdermal transfer of the capsaicin containing solution might differ between the proximal and the distal forearm due to differences in the skin thickness. Whatever the explanation, the results indicate the importance of clearly defining application sites and of using the same sites on different occasions if one wishes to optimize the test-retest reproducibility of the DBF response.
From the first part of the study, it became apparent that although overall DBF increased significantly after both the 300 µg and 1000 µg capsaicin applications, there were large inter-individual differences.
In a large percentage of the volunteers, DBF failed to increase with more than 100 % when compared to baseline, even at the 1000 µg capsaicin dose. Helme et al. extensively investigated the wide variation in size and intensity of the capsaicin-induced flare response. They found that the major factors in flare response were body site and age, though they also emphasized that one must be guarded in the interpretation because of the large number of variables involved (10). Gazerani et al. recently found the capsaicin-induced sensory and vasomotor responses were also gender specific (40). As in the present study, our aim was to produce a robust and reproducible DBF response, we choose to include males only and decided, in part II of the study, to select only men with a DBF increase from baseline of at least 100 % following capsaicin application. This decision was based on our findings in part I, which suggested that in this way reproducibility would further increase.
In part II, we chose to report the mean difference and repeatability coefficient (32), the within subject coefficient of variation (33) and the concordance correlation coefficient (29-31) to characterize the test-retest reproducibility of the DBF response to capsaicin application. Comparing reproducibility data between studies is often a daunting experience, as various measures of reproducibility are reported in

medical literature and measurement protocols often differ between studies. The majority of studies that used laser Doppler perfusion imaging and for which reproducibility data are available assessed the dermal blood flow response to acetylcholine and sodium nitroprusside (41-43). These studies differ strongly in design, population and even drug administration which makes comparison of reproducibility with our current study rather meaningless. Furthermore, there is general lack of detailed information on the exact conditions under which reproducibility was tested in most of these studies.

In our study we used the AUC_{0-30} as a summary measure. The use of summary measures to analyse serial measurements is a useful and simple tool in medical research (44). Summary measurements are considered to be more clinically relevant than data obtained on discrete points in time and allow the presentation of a response over time as a single value. It is also believed that the use of summary measures may reduce variability and thus increase reproducibility. In the present study, reproducibility of the summary response AUC_{0-30} was not consistently better compared to reproducibility of the single time point measure t_{30}. This is in agreement with previous findings of our group (45). Here the use of the AUC as a summary measure accurately accentuates the inferiority of the period-to-period reproducibility compared to the arm-to-arm reproducibility.

In rats and rabbits, CGRP has been identified as one of the major mediators of the capsaicin-induced vasodilation (46, 47). This was confirmed by Hershey et al. who developed and validated a non-invasive pharmacodynamic model using capsaicin in rats and nonhuman primates to assess CGRP antagonist activity in vivo (26). They demonstrated that capsaicin induces a dose- and time-dependent vasodilation that could be readily detected and quantified by laser Doppler perfusion imaging. They also showed that a CGRP antagonist inhibited the capsaicin-induced increase in dermal blood flow but at doses that were different between rhesus monkeys and rats. This difference in inhibitory dose serves as an illustration of the molecular differences in CGRP receptor pharmacology between species and was one of the prime motives for the translation of this model for CGRP release to the human species itself.

In addition to CGRP, capsaicin-sensitive nociceptors in the skin contain a number of other mediators that might play a role in the capsaicin-induced vasodilation. The involvement of SP in capsaicin induced vasodilation has been proven in several animal species (16, 48). In the human skin, SP was shown to induce vasodilation in the absence of neuropeptide-induced activation of nociceptors (49). There is however so far no conclusive evidence for the involvement of SP in capsaicin-induced vasodilation in the human skin (17, 50). Additionally, there are to our knowledge no reports on the influence of NK1 receptor antagonists on capsaicin-induced flare in vivo in humans. The putative role of pro-inflammatory prostaglandins and NO in capsaicin-induced neurogenic inflammation also remains a much debated issue (21, 51, 52). Therefore, a further characterisation and understanding of the (patho)physiologic process following dermal capsaicin application is warranted.

The pathophysiology of migraine is complex and incompletely understood but vasodilation of cranial blood vessels and the activation of the trigeminal vascular system probably play a significant role (53). Observations in animal and human studies suggest that CGRP, released from perivascular nerve terminals, is a key player involved in the activation of the trigeminovascular system. Therefore, the inhibition of CGRP-driven pathophysiological processes may yield a novel therapeutic approach in

migraine as confirmed by the experience with BIBN4096BS, the first highly selective and potent CGRP receptor antagonist proven to be efficacious in the acute treatment of migraine headache (23). Given the species dependent affinity of CGRP receptor antagonists for the CGRP receptor (26), different pharmacodynamic assays have been developed in several clinically relevant animal models to predict the efficacy of CGRP receptor antagonists in vivo. One of our primary goals was to develop a noninvasive pharmacodynamic model in healthy volunteers to provide a rapid assessment of CGRP antagonist activity and/or CGRP release inhibition in humans. Nevertheless, depending on the further characterization of the dermal capsaicin response, the model might also be of value when studying drugs that influence SP, prostaglandins or NO release.

Although we are fully aware of the gender specific response to capsaicin, we solely used our model in male healthy volunteers on account of standardization concerns (40). Future gender specific investigations using capsaicin-induced neurogenic inflammation could nevertheless greatly improve our understanding of gender related (patho)physiological processes, such as migraine related trigeminal sensitization. Finally, it should be noted that no pharmacodynamic model, including the one presented here, completely mimics the pathophysiological processes one wants to study. The effect of a drug on capsaicin-induced dermal neurogenic inflammation is only indicative for its efficacy in treating the neurogenic inflammation within the meningeal vasculature during a migraine attack. Nevertheless, the use of such a model can have great advantages in dose finding and proof of concept studies.

In summary, we developed a pharmacodynamic model in humans which is non-invasive, technically uncomplicated and has a rapid and objective endpoint. This pharmacodynamic model allows for repeated measurements to be performed which we have shown to be adequately reproducible. This model might therefore facilitate the early clinical evaluation of antagonists of mediators involved in neurogenic inflammation, including CGRP, TRPV1 and possibly SP antagonists.

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Chapter 4

$CGRP_{8-37}$ antagonizes capsaicin-induced vasodilation in the skin: evaluation of a human *in vivo* pharmacodynamic model.

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Abstract

Objectives To identify the mediators involved in capsaicin-induced vasodilation in the human skin and to evaluate a pharmacodynamic model for the early clinical evaluation of CGRP receptor antagonists. **Methods** Dermal blood flow (DBF) response of the forearm skin to topically applied capsaicin was measured using laser Doppler perfusion imaging in 22 subjects. The effect of intra-arterially administered $CGRP_{8-37}$ (1,200 $ng.min^{-1}.dL^{-1}$forearm), indomethacin (5 $\mu g.min^{-1}.dL^{-1}$forearm), N^G-monomethyl-L-arginine (L-NMMA, 0.2 $mg.min^{-1}.dL^{-1}$forerarm) and orally administered aprepitant (375 mg) on capsaicin-induced dermal vasodilation was assessed. Furthermore, the diurnal variation of the DBF response to capsaicin was studied. **Results** $CGRP_{8-37}$ inhibited the capsaicin-induced DBF increase: 217(145, 290)% in infused versus 370(254,486)% in the non-infused arm (mean (95% CI); p=0.004). In contrast, indomethacin, L-NMMA, aprepitant and the time of assessment did not effect the DBF response to capsaicin. **Conclusions** Capsaicin-induced vasodilation in the human forearm skin is largely mediated by CGRP, but not by vasodilating prostaglandins, nitric oxide or substance P. The response to capsaicin does not display a circadian rhythm. A pharmacodynamic model is proposed to evaluate CGRP receptor antagonists in humans *in vivo*.

Introduction

Neurogenic inflammation results from the release of bioactive substances from a subpopulation of primary sensory neurons consisting of Aδ- and C-fibre nociceptors. It is a known phenomenon within the skin, joints, gut, urinary and respiratory tracts (1-4). In addition, there is growing evidence that in the pathophysiology of migraine, headache develops at least in part as a result of sterile neurogenic inflammation of large intracranial blood vessels (5). These blood vessels are innervated by a dense supply of sensory C-fibres originating from the trigeminal ganglion, which contains several neuropeptides including Substance P (SP), neurokinin A and calcitonin gene-related peptide (CGRP) (6). The release of these vasoactive neuropeptides is believed to result in a sustained neurogenic inflammation within the cephalic tissue, which causes pain through the activation of nociceptors. The efficacy of the CGRP-receptor antagonists BIBN4096BS and telcagepant in the treatment of acute migraine confirmed that compounds interfering with the mediators of neurogenic inflammation holds promising prospects (7-9).

We previously studied dermal vasodilation elicited by the topical application of capsaicin to the forearm skin of healthy male subjects (10). Capsaicin is the pungent ingredient in hot chilli peppers and activates the transient receptor potential vanilloid type 1 receptor on Aδ- and C-fibre nociceptors (11). Binding of capsaicin to the vanilloid type 1 receptor provokes neurogenic inflammation through depolarisation of neurons leading to the release of bioactive mediators. CGRP, SP, Neurokinin A, nitric oxide (NO) and prostaglandins are amongst the mediators believed to play a role in neurogenic inflammation in healthy human skin (12). Although dermal vasodilation is only one component of capsaicin-induced neurogenic inflammation, the easy and objective assessment of it by laser Doppler perfusion imaging makes it an attractive parameter for evaluating neurogenic inflammation (10). Furthermore, most putative mediators of neurogenic inflammation have vasodilatory properties, which makes it reasonable to assume that, if these mediators are major contributors to capsaicin-induced neurogenic inflammation, antagonizing them will affect the dermal blood flow response.

The aims of this study were (1) to characterize and identify the mediators involved in the dermal vasodilation induced by capsaicin application to the human skin and (2) to evaluate a human in vivo pharmacodynamic model for its usefulness in the early clinical development of CGRP antagonists. As CGRP-receptor antagonists, $CGRP_{8-37,}$ a C-terminal fragment of the CGRP peptide, which has been shown to block CGRP-induced vasodilation in a competitive manner in both animal and human studies was used (13, 14). $CGRP_{8-37}$ has limited potency and cannot be administered systemically in humans to inhibit CGRP-induced vasodilation. This is circumvented when $CGRP_{8-37}$ is administered into the brachial artery resulting in sufficiently high local concentrations within the forearm without causing any systemic effects (14). In this way, the contra-lateral arm or non-infused arm is not treated and serves as the control arm. The effect of the cyclo-oxygenase inhibitor indomethacin and the non-selective nitric oxide synthase inhibitor N^G-monomethyl-L-arginine monoacetate (L-NMMA) on capsaicin-induced dermal vasodilation were also assessed by infusing dosages in the human forearm which have previously been shown to be effective in antagonising vasodilating prostaglandins and NO, respectively (15, 16). As there is no infusible formulation of a SP antagonists available, the potent SP antagonist aprepitant was given orally and capsaicin-induced vasodilation was measured on the left

arm pre-aprepitant and on the right arm at 4 hours post-aprepitant (T_{max}) (17, 18). As in the latter part of the study protocol, measurements of dermal perfusion on both arms were not simultaneously performed, the diurnal reproducibility of the capsaicin-induced DBF response was evaluated by adding a control period in which no treatment was given, but only capsaicin was applied once in the morning on the left arm and 4 hours later, in the afternoon, on the right arm.

Methods

Subjects

After approval by the ethics committee of the University Hospital of Leuven, written informed consent was obtained from all subjects during a screening visit. In total, 44 subjects were recruited for participation in at least one part of the study. All subjects were White, non-smoking, healthy males between 18 and 45 years old.

Study design

Subjects were instructed to abstain from any drugs during three days and from chocolate-, alcohol- and caffeine-containing beverages and food during 12 hours preceding the screening visit and each study period. All measurements were performed while the subjects rested in a supine position on a comfortable bed in a quiet, temperature controlled room (ambient temperature of 24°C ± 1 °C). During each visit (screening and study periods) 10 mm rubber O-rings (McMaster-Carr, New Brunswick, US; 8 mm inner diameter) were placed at 4 equally spaced sites on the volar surface of the forearms. The rings were positioned so that their distal edges were 10, 14, 18 and 22 cm proximal to the wrist crease, within approximately 1 cm of the midline and avoiding visible veins. The proximal ring (i.e. closest to the antecubital crease) is referred to as site 1, the distal ring (i.e. closest to the wrist crease) as site 4. After placement of the O-rings, a laser Doppler perfusion imager (HR-LDPI system, Periscan PIMII®; Perimed, Järfälla, Sweden) was used to obtain baseline scans of the DBF of the areas defined by the rings. Subsequently, these O-rings served as reservoirs to contain the topically applied 20-µl capsaicin or placebo solutions.

Capsaicin powder was obtained from Sigma-Aldrich N.V. (Bornem, Belgium) and was dissolved in a 3:3:4 mixture of ethanol 100%, Tween-20 and distilled water. Capsaicin was diluted so that 20 µl of the mixture contained 1000 µg capsaicin. The placebo solution corresponded to the same 3 : 3 : 4 mixture of ethanol 100%, Tween-20 and distilled water without capsaicin. We fully described the methodology previously (10).

Study 1: Effect of intra-arterial infusion of $CGRP_{8-37}$, L-NMMA and indomethacin on capsaicin-induced dermal vasodilation

Twenty-one subjects were screened for this study. During the screening visit subjects were evaluated as being responders or non-responders. To that end all subjects received on both forearms a topical dose of 1000 µg capsaicin per 20 µl vehicle at the two proximal sites and placebo (i.e. 20 µl of vehicle) at the two distal sites. The application of the capsaicin solution always started at site 1 of the dominant arm. Capsaicin or placebo solutions were then applied with 1 minute intervals to the remaining sites of the dominant arm. Two minutes after the last application on the dominant arm, application started at site 1 of the non-dominant arm and, respecting the 1 minute time interval between applications, to all remaining sites. Subsequently, Laser Doppler scans were performed at precisely 10, 20 and 30 minutes post-capsaicin or placebo application at each site starting from site 1 on the dominant arm to site 4 of the non-dominant arm respecting the same time-intervals as during application. Scanning of all sites thus required approximately 8 minutes at each time-point. Only responders, defined as

subjects with a capsaicin-induced increase in DBF of ≥ 100% in both proximal sites of both arms were included. This criterion was fulfilled by 11 out of the 21 screened subjects.

The 11 included subjects participated in a randomised, single-blind, 3-way, cross-over study *(flow chart, figure 1A)*. In each of the treatment periods, after insertion of a 27 gauge mounted needle (Sterican®, B Braun, Melsungen, Germany) into the brachial artery of the non-dominant arm, first 0.9% saline (B Braun, Melsungen, Germany) was infused at 100 $\mu L.min^{-1}.dL^{-1}$ forearm using automated infusion pumps (Ivac® P1000, Ivac Medical Systems, Brussels, Belgium) for about 20 minutes for equilibration. Subsequently, baseline DBF was measured at both arms. Thereafter, capsaicin and placebo were applied to the skin of the dominant arm (i.e. the non-infused arm) as described above. Two minutes prior to capsaicin application on the non-dominant (i.e. infused arm) infusion with saline 0.9% was substituted by one of the following antagonists:

1) $CGRP_{8-37}$ at a 1,200 $ng.min^{-1}.dL^{-1}$ forearm dose which has previously been shown to inhibit CGRP-induced vasodilation in the human forearm (14).
2) Indomethacin at a 5 $\mu g.min^{-1}.dL^{-1}$ forearm dose known to suppress tromboxane B2 formation, a marker for cyclo-oxygenase activity, when infused in the human forearm (15, 19).
3) L-NMMA at 0.2 $mg.min^{-1}.dL^{-1}$ forearm as a non-selective nitric oxide synthase inhibitor. Together with L-NMMA, sodium nitroprusside (exogenous NO-donor, 0.2 $\mu g.min^{-1}.dL^{-1}$ forearm) was co-infused intra-arterially to correct for L-NMMA-induced vasoconstriction which results from inhibition of basal endothelial NO release (i.e. “NO-clamp” technique) (16).

All antagonists and sodium nitroprusside were dissolved in 0.9% saline immediately before each experiment and doses were normalized to forearm volume (measured by water displacement) in order to keep the rate of all intra-arterial infusions constant at 100 $\mu L.min^{-1}.dL^{-1}$ forearm. The total duration of antagonist infusion was 37 minutes as it was started after placebo application at site 4 of the dominant arm and prior to capsaicin application to site 1 of the non-dominant arm and was stopped when the last laser Doppler measurement at site 4 of the non-dominant arm was performed.

Study 2: Effect of oral administration of aprepitant on capsaicin-induced dermal vasodilation

For this study, another 23 subjects were screened during a morning visit. After 20 minutes of acclimatisation, baseline DBF was measured in the left arm. Subsequently, capsaicin and placebo were applied to the skin of the left forearm as described above and Laser Doppler scans were performed at precisely 10, 20 and 30 minutes post-capsaicin or placebo application. After this evaluation, 11 responders (defined as above) were included for participation in a randomised, open-label, 2-way, cross-over study *(flow chart, figure 1B)*.

In each of the two study periods, the capsaicin-induced DBF response was evaluated twice: once in the morning at the left arm and once in the afternoon at the right arm. During one study period (i.e. the treatment period), subjects received a supra-therapeutic dose of 375 mg aprepitant after the morning session (T_{max}=4h) (18, 20) which is known to achieve a level of ≥90% neurokinin-1 receptor occupancy in the central nervous system of humans (18, 20, 21). During the other period (i.e. control period) no treatment was given. The DBF responses to capsaicin on the right forearm were evaluated at either 4 hours post-aprepitant during the treatment period or at the corresponding time during the control

period. By comparing the morning responses to capsaicin with the afternoon responses, the contribution of SP to capsaicin-induced vasodilation was evaluated during the treatment period. During the control period a diurnal variation in capsaicin-induced vasodilation was excluded.

In both studies, the sequence in which the treatments were administered over the course of periods was randomly allocated. In each case, study periods were separated by wash-out periods of at least 1 week.

Figure 1

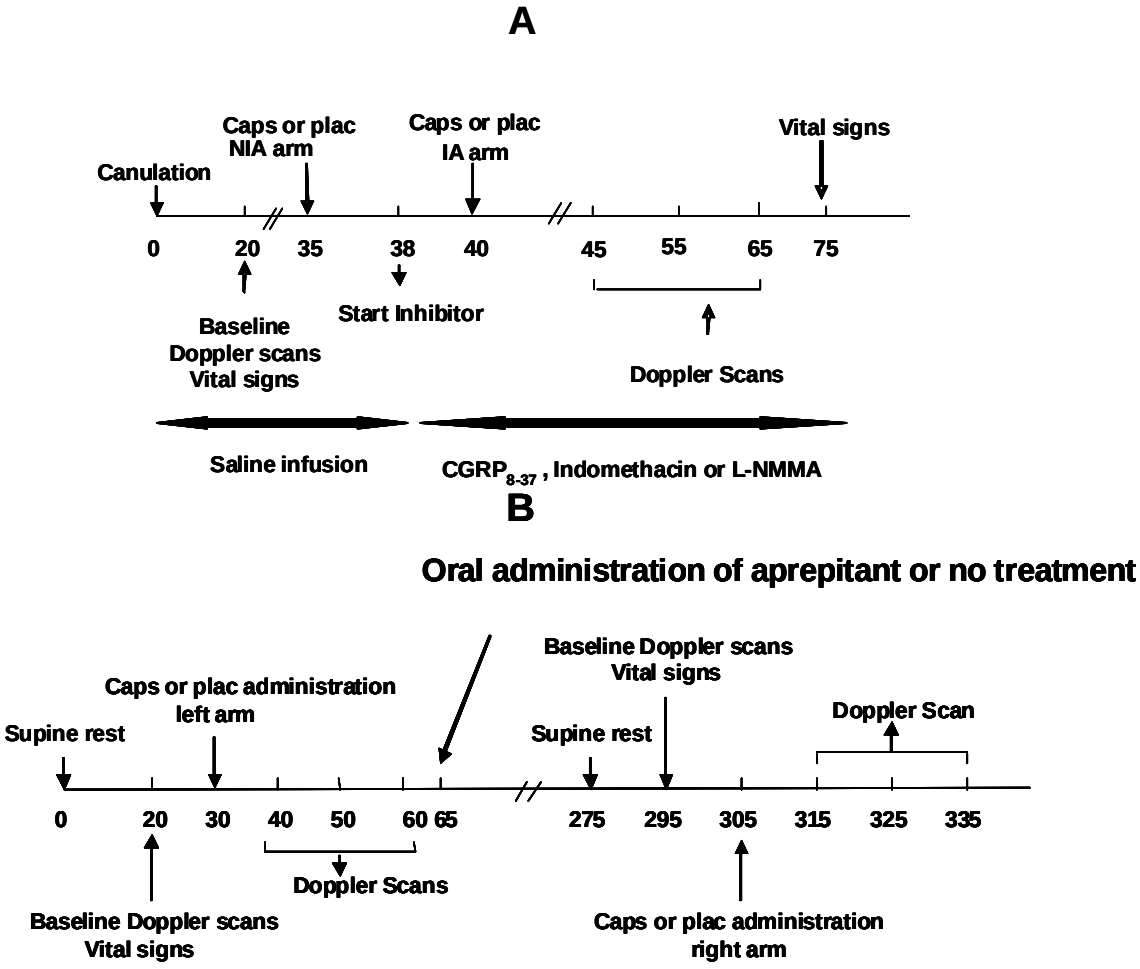


Figure 1A: Study 1: flow chart.

NIA, non-infused arm (i.e. dominant arm); IA, infused arm (i.e. non-dominant arm)

Figure 1B: Study 2: flow chart

Measurements

Supine systolic blood pressure, diastolic blood pressure and heart rate were measured in the dominant arm with a validated semi-automated oscillometric device (Omron HEM-705CP; Omron Healthcare, Hamburg, Germany). DBF was determined as previously described (10).

Data analysis and statistics

Based on the within-subject standard deviation observed in the reproducibility study and given a type I error probability (α) of 0.05, a sample size of 11 subjects provides 80% power for detecting a difference in DBF increase following capsaicin application of 20% between arms (10). The baseline DBF was expressed in arbitrary perfusion units (AU) (22). The change in DBF in response to capsaicin was expressed as the percent change from baseline. This percent change was compared with the percent change of DBF at the placebo sites in all three treatment periods and both arms using ANOVA for repeated measures. In addition, the area under the curve of the percent change from baseline up to 30 minutes after capsaicin application ($AUC_{0\text{-}30}$) was calculated as a summary measure. At each time-point the mean of observations with the 95% two-sided confidence interval (95% CI) are given. The normality of the distribution of the data was assessed and Wilcoxon's matched-pairs signed-rank tests or paired Student's t-tests were performed accordingly to compare the DBF percent change at the 30 minutes time-point (t_{30}) and the $AUC_{0\text{-}30}$ between the subjects forearms within the same study period.

Blood pressure and heart rate were compared between baseline and the end of the infusion (Study 1) and between pre and 4 hours post aprepitant administration (Study 2) by Wilcoxon's matched-pairs signed-rank test.

Unless stated otherwise, data are expressed as mean ± 95% CI. $P < 0.05$ was considered statistically significant.

Results

Capsaicin application was well tolerated by all subjects and no adverse events of note were reported. In most subjects, capsaicin provoked a local flare and stinging sensation that disappeared within 2-6 hours after application. All included subjects completed the study.

Study 1: Effect of intra-arterial infusion of $CGRP_{8-37}$, L-NMMA and indomethacin on capsaicin-induced dermal vasodilation

Mean ± SD (range) for age, weight and height of the 11 included subjects was 25 ± 5 (20-37) years, 81 ± 7 (69-91) kg and 182 ± 5 (174-192) cm, respectively. Compared with baseline a small increase ($P < 0.05$) in diastolic blood pressure was seen at the end of all three treatment periods. Blood pressure data are given in table 1.

Table 1

Haemodynamic parameters

Parameter	Time	L-NMMA	$CGRP_{8-37}$	Indomethacin
SBP (mmHg)	**Baseline**	123 (119, 127)	120 (116, 125)	126 (119, 132)
	40 min	125 (120, 129)	123 (119, 126)	128 (120, 136)
DBP (mmHg)	**Baseline**	67 (43, 92)	66 (42, 91)	69 (44, 93)
	40 min	72[a] (46, 96)	71[a] (46, 95)	72[a] (48, 97)
HR (bpm)	**Baseline**	60 (35, 84)	55 (30, 79)	58 (34, 83)
	40 min	59 (35, 84)	57 (33, 82)	59 (34, 83)

Data presented as mean ± 95% confidence interval; number of subjects = 11

SBP, DBP and HR: systolic blood pressure, diastolic blood pressure and heart rate.

[a] $p < 0.05$ versus baseline assessed by Wilcoxon matched-pairs signed-rank test.

DBF at baseline and post capsaicin or placebo are given in table 2.

Table 2

Comparison of dermal blood flow responses between arms

	Parameter	Arm	$CGRP_{8-37}$	Indomethacin	L-NMMA
Baseline (all sites)	**DBF (AU)**	**NIA**	0.44 (0.42, 0.46)	0.51 (0.48, 0.54)	0.48 (0.46, 0.49)
		IA	0.44 (0.41, 0.46)	0.49 (0.46, 0.52)	0.49 (0.46, 0.51)
1000 µg capsaicin	**T_{30} (%)**	**NIA**	370 (254, 486)	337 (256, 418)	309 (243, 375)
		IA	217 (145, 290)[a]	319 (247, 392)	314 (252, 375)
	AUC_{0-30} (%.min)	**NIA**	5,093 (3,211; 6,974)	4,392 (2829; 5,956)	3,717 (2,569; 4,866)
		IA	2,721 (1,929; 3,513)[b]	3,945 (2834; 5,056)	3,417 (2,526; 4,308)
placebo	**T_{30} (%)**	**NIA**	20 (7, 32)	10 (1, 19)	13 (2, 23)
		IA	11 (0, 22)	10 (0, 20)	6 (0, 12)
	AUC_{0-30} (%.min)	**NIA**	179 (-2, 361)	141 (-48, 330)	172 (3, 341)
		IA	134 (-71, 339)	68 (-74, 209)	1 (-71, 73)[c]

Data presented as mean ± 95% confidence interval; number of subjects = 11

t_{30}, DBF percent change 30 minutes post capsaicin or placebo; AUC_{0-30}, area under the curve of the DBF percent change up to 30 minutes post capsaicin or placebo. Number of observations at baseline, n = 44 for NIA and IA; number of observations after capsaicin/placebo, n = 22 for NIA and IA. IA, infused arm; NIA, non-infused arm

[a] p = 0.004 (Wilcoxon's matched-pairs signed-rank test comparing IA to NIA)

[b] p = 0.004 (paired Student's t-test comparing IA to NIA)

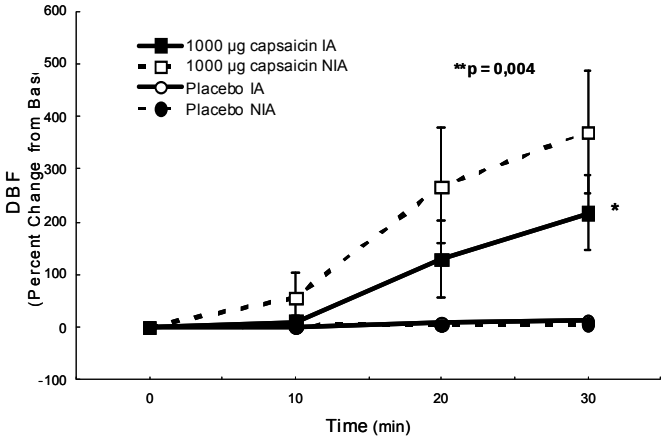
[c] p = 0.01 (paired Student's t-test comparing IA to NIA)

DBF at baseline did not differ between arms and increased following capsaicin application when compared to placebo in all three treatment periods and both arms (P < 0.0001, ANOVA for repeated measures; Figure 2). The capsaicin-induced DBF increase was partially blocked in the $CGRP_{8-37}$-infused arm when compared with the non-infused arm (Table 2 and Figure 2A). Indomethacin and L-NMMA did not effect the capsaicin-induced increase in DBF (Table 2 and Figures 2B and 2C).

The DBF changes at the placebo sites did not differ between the infused and non-infused arm during $CGRP_{8-37}$ and indomethacin infusion. In contrast, AUC_{0-30} of the DBF percent change from baseline at the placebo sites was smaller in the L-NMMA-infused than the non-infused arm (Table 2).

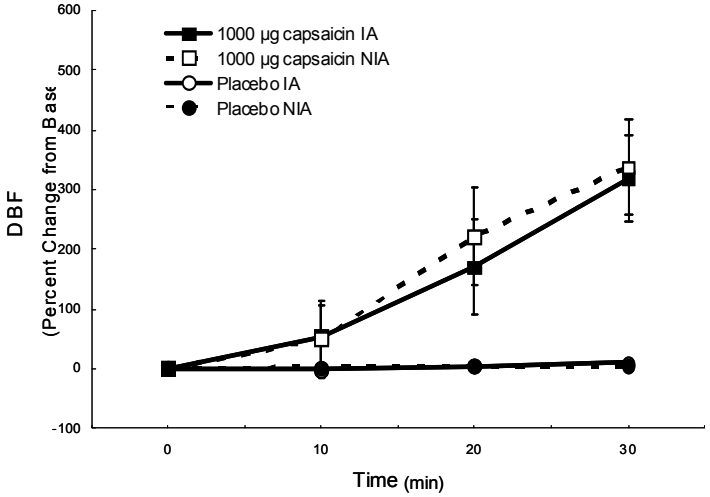
A

DBF Response by Arm during $CGRP_{8-37}$ infusion



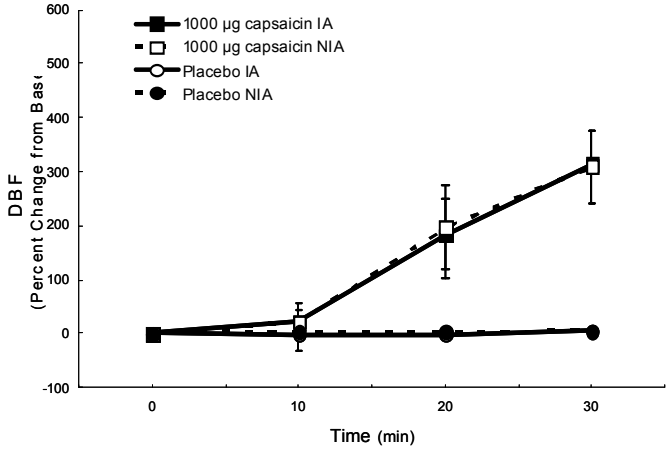
B

DBF Response by Arm during Indomethacin infusion



C

DBF Response by Arm during L-NMMA infusion



Study 1:

Figure 2A: Dermal Blood Flow (DBF) response (mean ± 95 % CI, number of subjects = 11, number of observations after capsaicin/placebo per arm = 22) during intra-arterial infusion of $CGRP_{8-37}$ (1,200 $ng.min^{-1}.dL^{-1}$ foreram). $CGRP_{8-37}$ inhibits DBF increase in the infused arm (IA) when compared to the non-infused arm (NIA) after application of 1000 µg capsaicin, whereas resting DBF (i.e. after placebo application) was comparable between both arms.

*p = 0.004 at t_{30} (Wilcoxon's matched-pairs signed-rank test).

** p = 0.004 for AUC_{0-30} (paired Student's t-test).

Figure 2B: Dermal Blood Flow (DBF) response (mean ± 95 % CI, number of subjects = 11, number of observations after capsaicin/placebo per arm = 22) during intra-arterial infusion of indomethacin (5 $µg.min^{-1}.dL^{-1}$ forearm). DBF increase was similar in the indomethacin infused arm (IA) and the non-infused arm (NIA) after application of 1000 µg capsaicin. Resting DBF (i.e. after placebo application) was unaffected by indomethacin infusion.

Figure 2C: Dermal Blood Flow (DBF) response (mean ± 95 % CI, number of subjects = 11, number of observations after capsaicin/placebo per arm = 22) during intra-arterial infusion of L-NMMA (0.2 $mg.min^{-1}.dL^{-1}$ forearm) in combination with SNP (0.2 $\mu g.min^{-1}.dL^{-1}$ forearm). DBF increase was similar in the L-NMMA infused arm (IA) and the non-infused arm (NIA) after application of 1000 µg capsaicin. Resting DBF (i.e. after placebo application) was slightly lower in the L-NMMA infused arm (IA) compared to the non-infused arm (NIA) (p=0.01, paired Student's t-test comparing AUC_{0-30})

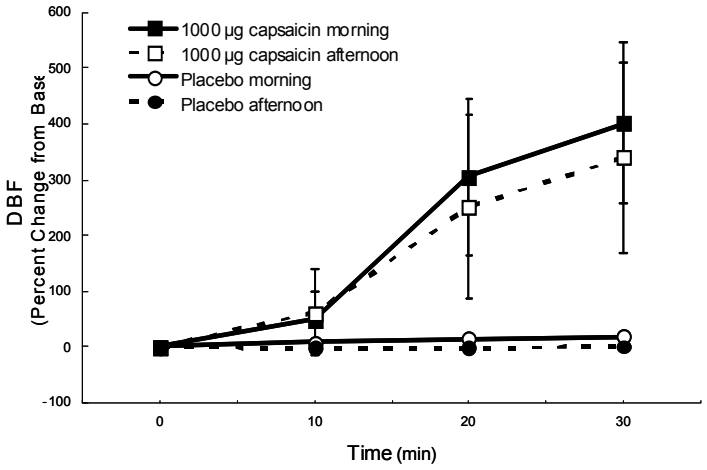
Study 2

Figure 3A: Dermal Blood Flow (DBF) response (mean ± 95 % CI, number of subjects = 11, number of observations after capsaicin/placebo per arm = 22) during morning and afternoon. DBF increase was similar in the left arm in the morning and the right arm in the afternoon after application of 1000 µg capsaicin. Resting DBF (i.e. after placebo application) was also similar during the morning and afternoon session.

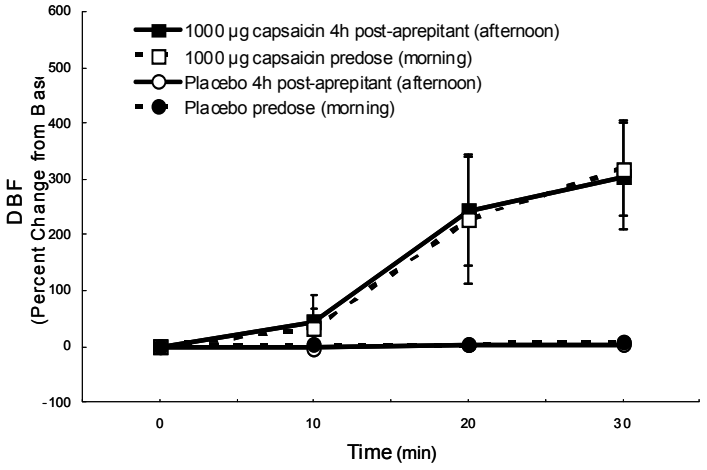
Figure 3B Dermal Blood Flow (DBF) response (mean ± 95 % CI, number of subjects = 11, number of observations after capsaicin/placebo per arm = 22) after aprepitant (375mg) administration. DBF increase was similar in the left arm before aprepitant administration and the right arm 4 hours after the intake of aprepitant. Resting DBF (i.e. after placebo application) was unaffected by aprepitant.

Figure 3

A
Diurnal Influence on DBF Response



B
Effect of aprepitant on DBF response



Study 2: Effect of oral administration of aprepitant on capsaicin-induced dermal vasodilation

Mean ± SD (range) for age, weight and height of the 11 included subjects was 26 ± 5 (19-41) years, 72 ± 12 (55-94) kg and 180 ± 7 (165-192) cm, respectively. No changes in systolic and diastolic blood pressure or heart rate were observed during the experiments.

In the control period, before capsaicin application, baseline DBF was similar in morning and afternoon at 0.39 (0.37, 0.42) AU (i.e. left arm) and 0.42 (0.38, 046) (i.e. right arm), respectively. The response to capsaicin showed no diurnal variation. In the morning, DBF response expressed as t_{30} averaged 402 (258, 546) % versus 340 (170, 510) % in the afternoon. This corresponds to an AUC_{0-30} of 5,551 (3,084; 8,017) %.min in the morning and 4,829 (2,051; 7,608) %.min in the afternoon (Figure 3A).

In the treatment period, baseline DBF averaged 0.41 (0.37, 0.45) AU in the morning (i.e. before aprepitant administration). Four hours post-aprepitant, DBF before capsaicin administration was slightly higher at 0.49 (0.43, 0.51) AU ($P < 0.05$). Aprepitant did not affect capsaicin-induced increase in DBF (Figure 3B): expressed as t_{30} the increase in DBF averaged 319 (233, 405) % pre-aprepitant versus 305 (210, 400) % 4 hours post-dose; AUC_{0-30} averaged 4,201 (2,465; 5937) %.min pre- and 4,403 (2,812; 5995) %.min post-aprepitant.

Discussion

This study demonstrates in healthy male volunteers *in vivo* that DBF response following capsaicin application to the skin is to a large extend antagonized by a CGRP receptor antagonist, whereas inhibition of SP, NO and prostaglandins had no substantial effect.

The central role of CGRP in the DBF response to capsaicin was largely expected based on both animal and human studies (23, 24). The observation that the inhibition of DBF increase by $CGRP_{8-37}$ is incomplete, does not discard CGRP as an important mediator of the vasodilatory response to capsaicin, but is probably due to the limited potency of $CGRP_{8-37.}$ $CGRP_{8-37}$ is known to have a 2000 time lower affinity to the CGRP-receptor of human arteries than the non-peptide CGRP receptor antagonist BIBN4096BS (25). When the dose of $CGRP_{8-37}$ used in the present study, was intra-arterially co-infused with a 120-fold lower dose of CGRP into the brachial artery of the human forearm, the degree of inhibition of the CGRP-induced vasodilation was comparable with the inhibition of the capsaicin-induced DBF response observed in the present study (i.e. around 50%) (14). Furthermore, when

$CGRP_{8-37}$ and CGRP are simultaneously injected into the volar forearm of healthy volunteers, the ability of CGRP to increase DBF is again only inhibited by about 50% (26). Recently, the potent oral CGRP antagonist telcagepant was shown to inhibit the dermal vasodilation following capsaicin application by approximately 80%, which further substantiates CGRP as an important mediator of capsaicin-induced vasodilation in the skin (27). We do realize however that the present study set-up differs, which makes it hard to compare results across studies. For example, with telcagepant, inhibition of CGRP receptors within the central nervous system could account for a more robust inhibition of capsaicin-induced DBF responses, whereas brachial infusion of $CGRP_{8-37}$ only inhibits CGRP released from peripheral nociceptive nerves in the skin of the forearm. On the whole, our findings indicate that CGRP is an important mediator of capsaicin-induced vasodilation in the skin, validating the use of the proposed pharmacodynamic model in the early clinical development of CGRP receptor antagonists. It is also interesting to note that $CGRP_{8-37}$ infusion had no effect on resting DBF, which is in agreement with our previous observation that $CGRP_{8-37}$ does not affect resting forearm blood flow (14). These data suggest that CGRP is not an important mediator of resting blood flow and that CGRP receptor antagonists are unlikely not affect tissue perfusion under resting conditions.

The absence of a substantial contribution of the other mediators (i.e. vasodilating prostaglandins, NO and SP) to capsaicin-induced DBF increase may seem rather surprising, especially for SP. Studies on neurogenic inflammation in animal skin have pointed to SP as a major inflammatory mediator (28, 29). In mice, capsaicin-induced vasodilation increases when neurokinin-1 receptors are functionally blocked. In contrast, also in mice, $CGRP_{8-37}$ is only able to inhibit capsaicin-induced vasodilation in combination with a SP antagonist (29). Thus an interaction between functional neurokinin-1 and CGRP receptors is suggested. Our results demonstrate that in humans no such interaction seems to exist as aprepitant does neither increase nor inhibit capsaicin-induced vasodilation, whereas $CGRP_{8-37}$ clearly decreases the response to capsaicin. This confirms findings by Petersen et al., who could not detect SP nor histamine release of the skin following intradermal capsaicin administration to humans (30). We therefore suggest that, in humans, SP has no major role in capsaicin-induced vasodilation.

Although, it can not be excluded that in case of blockade of CGRP receptors, SP may in part take over to compensate for inhibition of the CGRP-dependent vasodilation, this hypothesis was not tested because of the recent observation that over 80% of capsaicin-induced dermal vasodilation was inhibited by telcagepant (27). Taking in account the reproducibility of capsaicin-induced vasodilation, it would require an unfeasibly large number of subjects to detect any additional inhibition by aprepitant (10). In view of Petersens and our findings, we conclude that, under physiological conditions, SP has no major role in capsaicin-induced vasodilation in the human skin.

As aprepitant could not be locally administered by arterial infusion, it was impossible to compare the DBF response simultaneously between the infused (treated) arm and the non-infused (control) arm. As the DBF response to capsaicin does not demonstrate a circadian rhythm, this non-simultaneous assessment is valid.

The inability of indomethacin to inhibit the increase of capsaicin-induced DBF indicates that potent cyclo-oxygenase inhibition does not affect this response. We propose therefore, that vasodilating prostaglandins are not responsible for the dermal vasodilation component of neurogenic inflammation in humans. The role of prostaglandins in neurogenic vasodilation has been debated by conflicting findings in different research settings. On the one hand, cyclo-oxygenase inhibitors have been shown to inhibit CGRP release at the spinal level in rats (31, 32). Furthermore, treating human keratinocytes with capsaicin results in a dose-dependent expression of cyclo-oxygenase-2 (33). On the other hand Herbert et al. demonstrated that neither acetylsalicylic acid nor indomethacin affected capsaicin-induced increase in DBF in human skin (34), though they did find a significantly lower increase of DBF with indomethacin pre-treatment in the area directly underlying the application site of capsaicin, but not in the area surrounding the application site. The latter confirms the contention that in their study set-up the inflamed area underlying capsaicin-soaked plasters should be distinguished from proper neurogenic inflammation.

We demonstrated that the arterial infusion of a non-selective NO synthase inhibitor L-NMMA does not affect the dermal vasodilation following capsaicin application (16). This finding is particularly interesting as there is much debate surrounding the role of NO in the vasodilator mechanism of CGRP. If one considers endogenous CGRP release from capsaicin-sensitive nociceptors in the skin pivotal for the capsaicin-induced DBF response and one takes into account that the vasodilator effect of CGRP in humans is partly mediated by the release of NO (15), one would expect an inhibitory effect of L-NMMA. Hughes et al. demonstrated that in the rabbit cutaneous microvasculature, NO synthase inhibition had no effect on the CGRP-induced vasodilation, whereas it did reduce capsaicin-induced vasodilation (35). This would suggest that in rabbit skin the vasodilator mechanism of CGRP is independent of NO, whereas NO may play an important role in the release of CGRP from capsaicin-sensitive nerves. In contrast, Goldsmith et al. were able to inhibit CGRP-induced DBF increase by injecting L-NMMA into skin of the human forearm (36). Our findings indicate that, in the human skin, NO is neither involved in the release of CGRP nor in its vasodilator mechanism. As in our model we compare the capsaicin-induced dermal vasodilation between infused and non-infused arm, it could be argued that “spillover” of L-NMMA from the infused arm to the non-infused arm would prevent us from detecting an inhibitory effect of L-NMMA. However, spillover of L-NMMA to the non-infused arm

seems very unlikely. First, L-NMMA has been shown to quickly disappear from plasma (37). Secondly, the capsaicin-induced increase in DBF in the non-infused arm did not differ from the DBF responses in the non-infused arm during the other study periods including the screening period. Finally, the slight increase in diastolic blood pressure after L-NMMA infusion is most likely procedure related as it was also seen in the other treatment periods and reported by other authors using intra-arterial brachial infusion even when infusing vasodilators (38). Therefore, we are confident to conclude that there is no substantial contribution of de novo synthesized NO to capsaicin-induced neurogenic inflammation. It should be noted that L-NMMA slightly decreased resting DBF in the infused arm when compared to the non-infused arm. This reflects the limitations of the "NO clamp" technique in which the co-infusion of nitroprusside as NO donor to compensate for the inhibition of basal endothelial NO release in incomplete.

Our findings validate the pharmacodynamic model using capsaicin-induced vasodilation to assess CGRP antagonists in vivo in humans (10). We thus successfully translated the pharmacodynamic assay of Hershey et al. into the human species (24). This is valuable, as our study also clearly illustrates the variability of mediators involved in neurogenic inflammation between species.

In summary as capsaicin-induced vasodilation in the human skin is to a large extend antagonized by $CGRP_{8-37}$, but not by L-NMMA, aprepitant or indomethacin, CGRP seems to be a major mediator involved in this response. Therefore, the approach presented in this study is proposed as a human in vivo pharmacodynamic model which can be used in the early clinical development of CGRP receptor antagonists. Moreover, we demonstrated that the capsaicin-induced vasodilation does not display a circadian rhythm.

Acknowledgements

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Chapter 5

The potent CGRP receptor antagonist, telcagepant, does not affect nitroglycerin-induced vasodilation in healthy men

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Submitted

Abstract

Objective: To assess the effect of the calcitonin gene-related peptide (CGRP) receptor antagonist, telcagepant, on the hemodynamic response to sublingual nitroglycerine (NTG).

Methods: Twenty two healthy male volunteers participated in a randomized, placebo-controlled, double-blind, 2-period, cross-over study. Subjects received 500 mg telcagepant or placebo followed, 1.5 hours later, by 0.4 mg NTG. To assess the hemodynamic response the following vascular parameters were measured: blood pressure, aortic augmentation index (AIx) and brachial artery diameter (BAD). Data are presented as mean (95% confidence interval, CI).

Results: The aortic AIx following NTG decreased by -18.50 (-21.02, -15.98) % after telcagepant versus -17.28 (-19.80, -14.76) % after placebo. The BAD fold increase following NTG was 1.14 (1.12, 1.17) after telcagepant versus 1.13 (1.10, 1.15) after placebo. For both AIx and BAD, the hypothesis that telcagepant does not significantly affect the changes induced by NTG is supported ($P < 0.0001$).
In addition, no vasoconstrictor effect of telcagepant could be demonstrated.

Conclusion: Telcagepant did not affect NTG-induced hemodynamic changes. These data suggest that NTG-induced vasodilation is not CGRP dependent.

Introduction

The introduction of the so-called “triptans” (i.e. 5-$HT_{1B/1D}$ receptor agonists), with sumatriptan being the first compound of this class, improved migraine therapy considerably over the past decade (1, 2). However, despite their well-established efficacy and the demonstrable need to reduce migraine-associated disability, triptans remain underused in clinical practice (3). Several factors, such as cost and availability, contribute to the relatively restricted use of triptans. In addition, concern among patients and doctors about their vasoconstrictor properties and the fact that triptans are contraindicated in patients with cardiovascular disease or at high cardiovascular risk, limits their more widespread use (4).

At the same time, the development rationale for acute anti-migraine drugs radically changed due to conceptual changes in the understanding of the pathophysiology of migraine (2, 5). Whereas triptans were developed as cranial vasoconstrictors, the focus later shifted towards compounds that counteract inflammation resulting from the release of neuropeptides from the pain-producing trigeminal neurons innervating the cranial circulation and dura mater (6). In the following years, several compounds that were able to inhibit neurogenic inflammation in animal models failed to show efficacy in human migraine trials (7). Numerous observations linked migraine headache with the release of one neuropeptide in particular, namely calcitonin-gene related peptide (CGRP) (8-10). Interestingly, experimental data also demonstrated an important role for CGRP receptors in nociceptive trigeminovascular processing in animal models (11). The hypothesis that CGRP receptor antagonism would relief pain in migraine was first confirmed by the intravenous administration of olcegepant (BIBN4096, Boehringer-Ingelheim) (12). More recently, telgacepant (MK-0974, Merck & Co., Inc.), an oral CGRP antagonist, was also shown to be effective in treating moderate to severe migraine with an efficacy comparable to the triptans while a more favourable side effect profile was observed (13).

The exact site of action of CGRP receptor antagonists remains controversial as CGRP is present in both the central and peripheral nervous system and in blood vessels (7). But, at this time, CGRP receptor antagonists seem devoid of direct vasoconstrictor activity and may thus present an alternative and safe treatment for migraineurs with cardiovascular risk/disease (14-16). Nevertheless, it has been hypothesized that CGRP antagonism could result in inhibition of CGRP-dependent vasodilation and might thus have cardiovascular consequences (17, 18). Furthermore, data suggest that administration of nitroglycerin (NTG), which is widely used in the treatment of cardiac ischemic pain (i.e. angina pectoris), causes release of CGRP in the cardiovascular system resulting in coronary artery vasodilatation (19). The degree to which the vasodilatory properties of NTG depend on the release of CGRP is not known and the observations concerning the effect of CGRP receptor antagonism on response to NTG lack clinical study (20, 21).

This study was aimed at evaluating the effect of a single 500 mg dose of the CGRP receptor antagonist telcagepant on the vasodilatory response to a therapeutic dose of 0.4 mg NTG. To that end, two techniques were applied: pulse wave analysis and high resolution ultra-sound. Pulse wave analysis allows for derivation of the aortic pressure waveform (PWF) from the peripheral PWF obtained by applanation tonometry at the radial artery using a validated transfer function (22). From the aortic PWF aortic diastolic and systolic blood pressure (ADBP, ASBP) values and augmentation

index (aortic AIx) can be calculated. The aortic AIx is the proportion of central pulse pressure that results from arterial wave reflection and is a commonly used measure of arterial stiffness. This PWF information has become a powerful tool to investigate the cardiovascular response *in vivo* in humans to disease states and drugs. It was notably shown more reliable in detecting arterial triptan and nitrate-induced vascular effects than the classically used brachial artery diastolic (DBP) and systolic blood pressure (SBP) (23-26). High resolution ultra-sound allows the real time measurement of changes in the brachial artery diameter (BAD) induced by vasoactive drugs, notably triptans and nitrates (23, 27). In addition to exploring the effects of telcagepant on NTG response, the present study allowed us to investigate the vascular effects of a single 500 mg dose of telcagepant itself using the same highly sensitive techniques.

Methods

Subjects

After approval by the ethics committee of the University Hospital of Leuven, 43 healthy, non-smoking, male subjects were screened from the general population. Written informed consent was obtained during a screening visit from all subjects. Major exclusion criteria were: age <20 and >50 years; a history of major gastrointestinal, genitourinary, renal, hepatic, pulmonary, haematological, immunological, endocrine or metabolic, neurological or cerebrovascular abnormalities or disease and use of any kind of drugs. Subjects with a history of previous myocardial infarction or any clinically relevant cardiovascular disease were also excluded from participation in the study. On the basis of medical history, physical examination, laboratory tests and electrocardiography underlying disease was excluded. During the screening session, the subject's pressure waveform was assessed for reliability as per Sphygmocor system specifications. Only subjects with a positive aortic AIx during the screening visit were enrolled. The study was conducted in accordance with the guidelines on GCP and ethical standards for human experimentation established by the declaration of Helsinki (28).

Study design

All predefined criteria were fulfilled by 22 screened subjects. These 22 eligible subjects participated in a randomized, placebo-controlled, double-blind, two-way, cross-over study in which the treatments were 500 mg telcagepant or placebo. In each period, each subject received a single oral dose of either 500 mg telcagepant or matching placebo followed 1.5 hours later, by a dose of 0.4 mg sublingual NTG (flow chart in Fig. 1). There was at least a 5-day washout interval between both study periods. For each individual subject, treatment was always administered at the same time of the day during each study period. Placebo and telcagepant doses were given as 3 capsules and taken under the supervision of a study nurse with 240 mL of water.

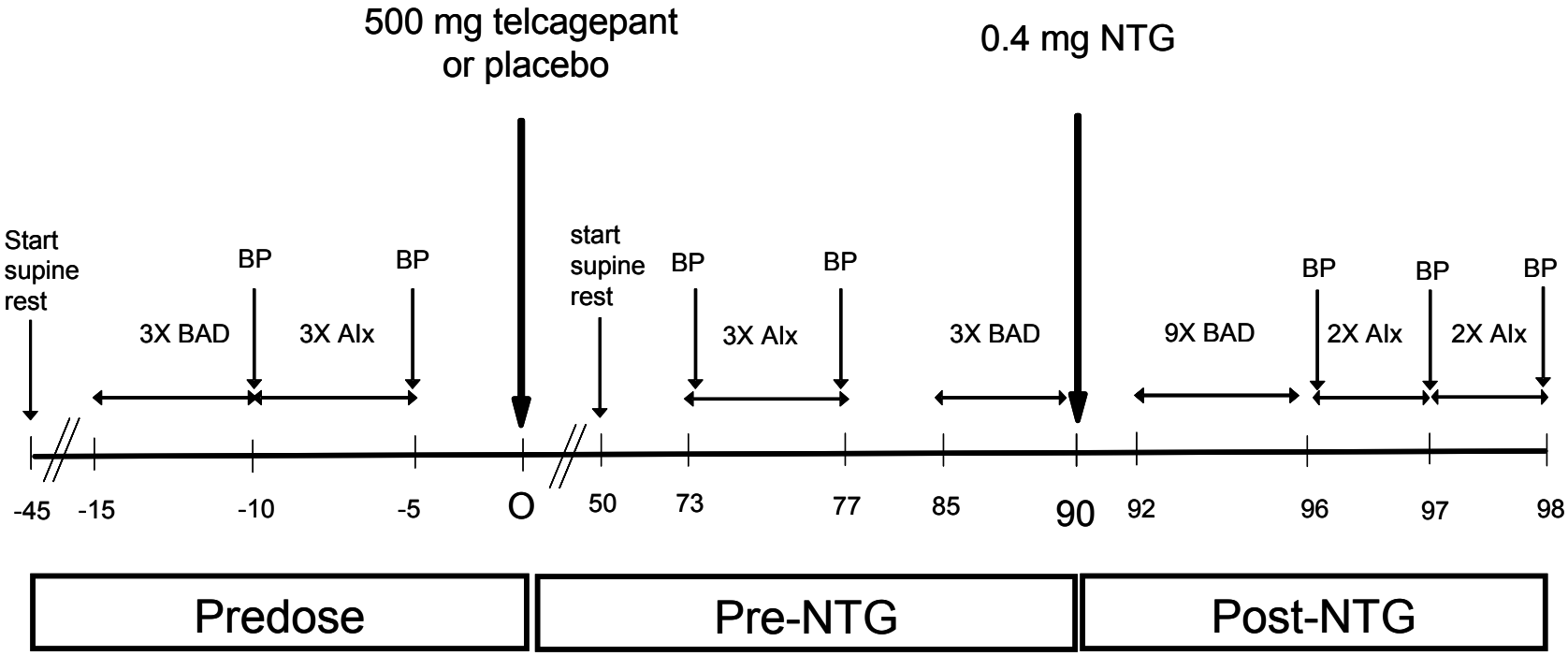
Figure 1

Study flowchart

BP: brachial blood pressure

BAD: brachial artery diameter

AIx, pressure waveform measurements with resulting aortic and radial augmentation index.


500 mg telcagepant
or placebo
0.4 mg NTG
Start
supine
rest
BP
BP
start
supine
rest
BP
BP
BP
BP
BP
3X BAD
3X AIx
3X AIx
3X BAD
9X BAD
2X AIx
2X AIx
-45
-15
-10
-5
O
50
73
77
85
90
92
96
97
98
Predose
Pre-NTG
Post-NTG

Measurements

All measurements were performed in a quiet, temperature-controlled (24°C ± 1°C) room by two blinded observers. Subjects abstained from any drug for at least 3 days and from alcohol- and caffeine-containing beverages for at least 12 hours before drug administration. Subjects fasted at least 8 hours before drug administration and remained in a fasting state during all measurements. Before performing baseline measurements, subjects rested for 30 minutes in supine position on a comfortable bed for acclimatisation (flow chart in Fig. 1). Fifteen minutes prior to drug administration, BAD followed by PWF measurements were performed in threefold. Seventy three minutes after the administration of telcagepant or placebo, three PWF measurements were repeated. Thereafter and 5 minutes prior to NTG administration (i.e. 85 minutes postdose), three BAD recordings were performed. The same image of the brachial artery was maintained by use of a probe-holder and arm supports throughout the sublingual administration of 0.4 mg NTG at exactly 90 minutes after drug intake. The time-point of NTG administration was chosen at the expected time of maximal plasma concentration of telcagepant (29). Between 2 and 6 minutes following NTG administration (i.e. between 92 and 96 minutes postdose), a maximum of 9 BAD measurements was performed, followed by 4 PWF recordings. All brachial oscillometric SBP and DBP values were obtained before and after PWF recordings and used for calibration of the PWFs. The study flow chart is given in Figure 1.

Brachial blood pressure and heart rate

Supine SBP, DBP and heart rate (HR) were recorded at the left upper arm by use of a validated, non-invasive semi-automated oscillometric device (OMRON 705IT; Omron Healtcare, Hamburg, Germany) (30).

Applanation tonometry

PWFs were recorded with a high-fidelity tonometer (SPT-301; Millar instruments). Each recording lasted 8 seconds, and the mean of at least 2 recordings at each time point was used for analysis. After calibration by use of brachial oscillometric SBP and DBP values, these PWFs were automatically transformed by computer software (Sphygmocor; Atcor Medical, West Ryde, New South Wales, Australia) into the corresponding aortic PWF by use of a generalized transfer function (31). The following parameters were calculated by the software: ADBP, ASBP and both radial and aortic AIx. The Sphygmocor system has been validated and demonstrated adequate test-retest reproducibility in healthy volunteers (32, 33).

Ultrasound

The end-diastolic BAD was measured at the antecubital crease of the right arm by use of an echo-tracking system (Wall track System; Pie Medical Imaging, Maastricht, The Netherlands). The Wall Track system consists of an ultrasound device (Esaote AU5; Esaote Biomedica, Genoa, Italy) equipped with a 7.5- to 10 MHz linear-array transducer connected to a data acquisition and processing unit (34). This system has previously been validated and has demonstrated acceptable reproducibility in healthy subjects (35). The brachial artery was scanned in longitudinal sections to obtain satisfactory ultrasonographic images. At each time point repeated measures were made to obtain at least 15 cardiac cycles for analysis.

Data analysis

Power calculation

Prior to the study, power calculations were performed for aortic AIx and BAD. For aortic AIx, in house data that were collected previously by our group were available. The power calculation assumed a within-subject variance and between subject variance of 17.79 and 101.14, respectively for aortic AIx change from baseline. It also assumed a true effect of -12 % when NTG is administered with placebo. With n = 21 subjects, α = 0.05 (one-sided) and n - 2 degrees of freedom for error, there is 99 % power to conclude that the true aortic AIx ratio (effect of NTG with telcagepant/effect of NTG with placebo) is > 0.5, if the true ratio is 1.0.

For BAD, the power calculation was based on data from the literature (36). The power calculation assumed a true within-subject variance for ln-brachial artery diameter change from baseline of 0.0322. With n = 21 subject, α = 0.05 (one-sided), and n – 2 degrees of freedom for error, there is > 0.99 probability that the 90 % confidence interval lower boundary for the true mean fold treatment difference (effect of NTG with telcagepant/effect of NTG with placebo) ≥ 0.50, if the true ratio is 1.0.

Primary analyses to evaluate the effect of telcagepant on NTG-induced vascular changes

The study was powered to detect changes in the maximal vasodilatory NTG response due to telcagepant for the parameters aortic AIx and BAD. Therefore, in the primary analyses, only aortic AIx and BAD were used. As NTG causes a decrease in aortic AIx and the baseline aortic AIx was positive (cf. inclusion criteria), the change in aortic AIx is a negative value. Therefore, the maximum effect on aortic AIx for a subject in a given treatment period was defined as the largest negative difference between the post and pre-NTG measurements over the 2 predefined post-NTG time-points. The maximum increase in BAD induced by NTG was defined as the maximum fold-change obtained over the 4 predefined post-NTG time-points. Individual maximal effect values were analyzed in a mixed effects analysis of variance (ANOVA) with fixed effects for treatment, period and treatment sequence and a random effect for subject nested within treatment sequence. For BAD, values were ln-transformed, whereas for AIx the data were analyzed on the original scale. Results for BAD were back-transformed to the original scale to obtain a geometric mean and corresponding 95% confidence interval (95% CI).

For aortic AIx, the hypothesis was tested that at least 50% of the maximum NTG effect is maintained following administration of telcagepant as compared to placebo, where 50% was predefined as the threshold for clinical significance. The p-value is for the test that the fold treatment difference (effect of NTG with telcagepant/effect of NTG with placebo) is less than 0.50 (null hypothesis) versus at least 0.50 (alternative hypothesis). P<0.05 signifies that at least 50% of the NTG effect is maintained when administered with telcagepant.

In addition, the hypothesis was tested that at least 50% of the maximum NTG effect on BAD is maintained following administration of telcagepant as compared to placebo. Therefore, a two-sided 90% CI (equivalent to a one-sided lower 95% CI) for the difference (effect of NTG with telcagepant – effect of NTG with placebo) in ln-BAD maximum effect was calculated using the mean square error from the ANOVA and referencing a t-distribution. These confidence limits were exponentiated to obtain a 90% CI for the fold treatment difference (effect of NTG with telcagepant/effect of NTG with

placebo). It was predefined that if the lower bound of the 90% CI was ≥ 0.50, the hypothesis that telcagepant has no effect on NTG-induced vascular changes would be supported.

Secondary analyses to evaluate the effect of telcagepant on NTG-induced vascular changes

In secondary analyses, the effect of telcagepant on the vasodilatory response to NTG was assessed for all vascular parameters. To this end, the change for each individual vascular parameter was calculated as the mean difference and corresponding 95% confidence interval (95% CIs) between measurements recorded post-NTG minus those obtained pre-NTG. The mean NTG effect was then compared between both treatment periods using a mixed effects analysis of variance model (ANOVA) with fixed effects for treatment, period, treatment sequence, time and treatment by time interaction and a random effect for nested within treatment sequence.

Secondary analysis to evaluate the effect of telcagepant

In secondary analyses, the mean and corresponding 95% CI for each vascular parameter at each individual time-point (i.e. predose, pre-NTG and post-NTG) was calculated. Further, each vascular parameter was evaluated in the mixed effects analysis of variance model (ANOVA) with fixed effects for treatment, period, treatment sequence, time and treatment by time interaction and a random effect for nested within treatment sequence. The treatment main effect was assessed by comparing each vascular parameter between the treatment and the placebo period over the 3 time-points and at each individual time-point using the ANOVA.

Results

All 22 subjects completed the study. One subject, with a positive aortic AIx at screening, had a negative aortic AIx at baseline during both study periods. Moreover, in this subject NTG had no effect on aortic AIx and thus the subject was considered to be a non-responder to NTG and his data were excluded from further analyses. The remaining 21 subjects were aged 38 ± 10 years and had a body mass index of 24.4 ± 2.8 kg/m^2 (mean ± SD).

Primary analyses to evaluate the effect of telcagepant on NTG-induced vascular changes

Aortic AIx

The maximum change in aortic AIx following sublingual NTG averaged -18.50 (-21.02, -15.98) % after telcagepant versus -17.28 (-19.80, -14.76) % after placebo. The hypothesis that telcagepant does not significantly affect the mean change from baseline in aortic AIx induced by NTG is supported (P < 0.0001).

Brachial artery diameter

The maximum fold change in BAD following sublingual NTG averaged 1.14 (1.12, 1.17) after telcagepant versus 1.13 (1.10, 1.15) after placebo. The geometric mean ratio (effect of NTG with telcagepant/effect of NTG with placebo) was 1.02 with a corresponding 90% CI of (1.00, 1.03). Since the confidence interval lies completely above 0.50, the hypothesis that telcagepant does not significantly affect the mean fold change from baseline in BAD induced by NTG is supported.

Secondary analyses to evaluate the effect of telcagepant on NTG-induced vascular changes

Using the previously described ANOVA, no difference could be detected for any of the vascular parameters in mean response to NTG following telcagepant compared to placebo(Table1,Figure2&3)

Table 1: Vascular parameters.

	Period	Predose	Pre-NTG	Post-NTG	NTG-induced Change
HR beats/min	Placebo	53 (50, 57)	53 (49, 56)	56 (52, 61)	4 (2, 6)
	Telcagepant	54 (50, 57)	53 (49,57)	58 (53, 62)	5 (3, 6)
SBP mmHg	Placebo	119 (115, 123)	119 (116, 123)	118 (114, 121)	-2 (-4, 0)
	Telcagepant	119 (115, 122)	121 (117,124)	121 (117,125)	0 (-2, 2)
DBP mmHg	Placebo	72 (69, 76)	75 (71, 78)	67 (64, 70)	-8 (-9, -6)
	Telcagepant	73 (70, 76)	75 (71, 78)	69 (65, 72)	-6 (-7, -5)
ASBP mmHg	Placebo	105 (101, 110)	107 (103, 111)	99 (95, 102)	-8 (-10, -7)
	Telcagepant	104 (101, 108)	107 (104, 111)	101 (97, 104)	-7 (-8, -5)
ADBP mmHg	Placebo	73 (70, 76)	75 (72, 79)	67 (64, 71)	-8 (-10, -6)
	Telcagepant	74 (71, 76)	75 (72, 79)	69 (66, 73)	-6 (-8, -5)
Radial AIx %	Placebo	65 (59, 71)	67 (61, 70)	44 (39, 48)	-23 (-26, -20)
	Telcagepant	62 (56, 67)	66 (61, 70)	42 (38, 47)	-23 (-26, -20)
Aortic AIx %	Placebo	13 (9, 18)	15 (11, 19)	-2 (-5, 2)	-16 (-19, -13)
	Telcagepant	11 (7, 15)	15 (11, 18)	-3 (-6, 0)	-17 (-19, -15)
BAD µm	Placebo	6430 (6089, 6770)	6445 (6134, 6756)	7048 (6705, 7391)	603 (464, 741)
	Telcagepant	6441 (6112, 6770)	6410 (6048, 6771)	7069 (6676, 7461)	659 (533, 785)

HR, SBP, DBP, ADBP, ASBP, radial and aortic AIx and BAD during both treatment periods (telcagepant and placebo) at each time-point and the change induced by 0.4 mg NTG.

Data (mean ± 95% CI); n= 21; HR, heart rate; SBP, brachial systolic blood pressure; DBP, brachial diastolic blood pressure; ASBP, aortic systolic blood pressure; ADBP, aortic diastolic blood pressure; radial AIx, augmentation index from radial pressure waveform; aortic AIx, augmentation index from aortic pressure waveform; BAD, brachial artery diameter.

Figure 2

Heart rate (A), brachial and aortic systolic blood pressure (B), diastolic blood pressure (C) and radial and aortic augmentation index (D) after oral administration of 500 mg telcagepant (full line, open circle) or placebo (dotted line, full circle) in combination with 0.4 mg of sublingual NTG.

Number of subjects = 21

Data presented as mean ± SEM

P values refer to the previously described ANOVA comparing telcagepant with placebo period averaged over all timepoints.

SBP, Systolic blood pressure; DBP, Diastolic blood pressure; AIx, Augmentation Index

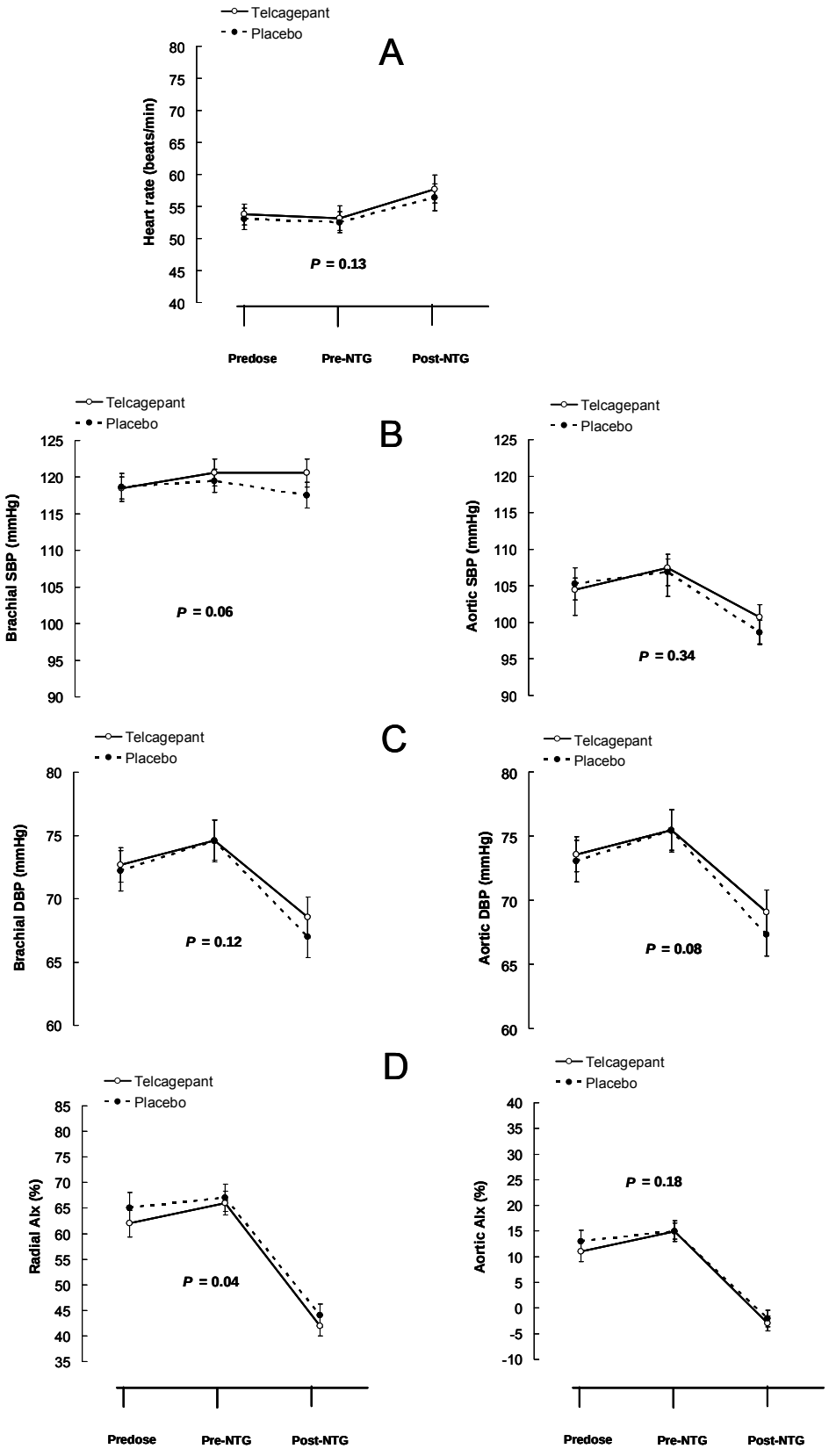

Telcagepant
Placebo
A
Heart rate (beats/min)
80
75
70
65
60
55
50
45
40
P = 0.13
Predose
Pre-NTG
Post-NTG
B
Brachial SBP (mmHg)
125
120
115
110
105
100
95
90
P = 0.06
Aortic SBP (mmHg)
P = 0.34
C
Brachial DBP (mmHg)
80
75
70
65
60
P = 0.12
Aortic DBP (mmHg)
P = 0.08
D
Radial AIx (%)
85
80
75
70
65
60
55
50
45
40
35
P = 0.04
Aortic AIx (%)
40
35
30
25
20
15
10
5
0
-5
-10
P = 0.18

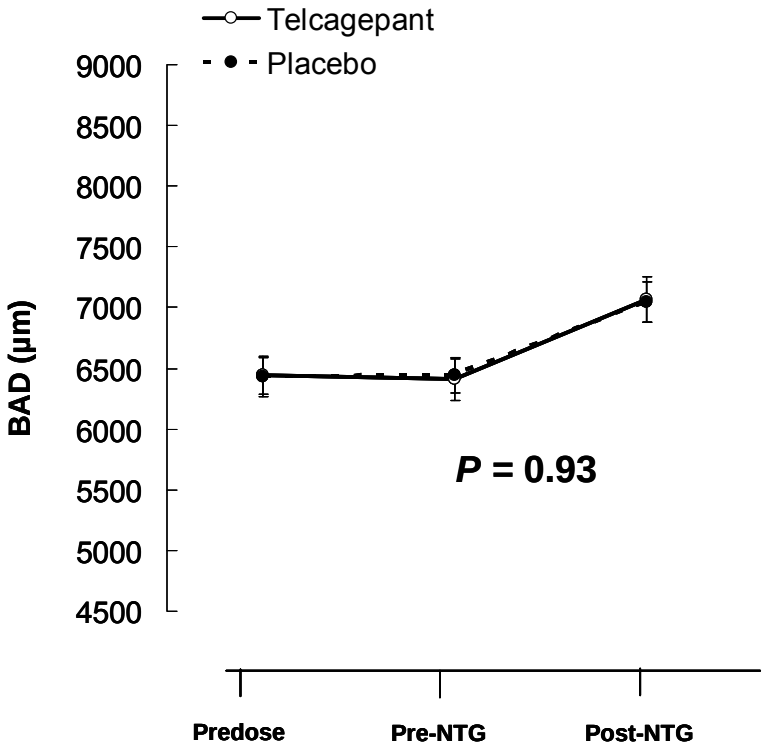
Figure 3

Brachial artery diameter (BAD) after oral administration of telcagepant (full line, open circle) or placebo (dotted line, full circle) in combination with 0.4 mg of sublingual NTG

Number of subjects = 21

Data presented as mean ± SEM

P values refer to the previously described ANOVA comparing telcagepant with placebo period averaged over all timepoints.


Telcagepant
Placebo
9000
8500
8000
7500
7000
6500
6000
5500
5000
4500
BAD (µm)
P = 0.93
Predose
Pre-NTG
Post-NTG

Secondary analyses to evaluate the effect of telcagepant

Averaged over the 3 time-points, vascular parameters did not differ between treatment periods except for the radial AIx, which was lower during treatment period with telcagepant as compared to the placebo period (*P* = 0.040, previously described ANOVA) (Table 1, Figure 2). This difference is largely explained by a lower value at the predose time-point during the telcagepant period (*P* = 0.027, previously described ANOVA). Though small, the unexplained differences in the values obtained at baseline might contaminate the data.

At the pre-NTG time-point, which falls at the time of the expected maximal plasma concentration of telcagepant, none of the vascular parameters differed between both treatment periods (previously described ANOVA).

Discussion

We found that the orally administered CGRP antagonist, telcagepant, had no effect on the hemodynamic response of healthy volunteers to therapeutic doses of sublingual NTG. In addition, no vasoconstrictor effect was seen after a single 500 mg dose of telcagepant compared to placebo. These findings support a more favourable cardiovascular safety and tolerability profile of CGRP antagonists over the triptans (13, 16, 37).

Animal research suggesting a role for CGRP in the vasodilatory effect of NTG led us to investigate whether telcagepant attenuated NTG-induced vascular changes in healthy subjects (20). NTG-induced vasodilation is associated with an endogenous CGRP release in both feline cerebral arterioles and rat aortas (38, 39). These findings in animals suggest that CGRP antagonism might impede on the vasodilation induced by exogenous NO donors such as NTG. In humans, however, there is still much debate surrounding the interaction between NO and CGRP. In the resistance vessels of the human forearm, $CGRP_{8-37}$, a CGRP receptor antagonist, has no effect on the vasodilation induced by the NO donor sodium nitroprusside (14). The vasodilator mechanism of CGRP itself on the other hand seems to be partly mediated by the release of NO in the forearm vascular territory but not in the human skin (15, 40). The interaction between NO and CGRP is thus different between vascular territories and between species. This clearly illustrates the need for sensitive techniques by which one can assess in vivo in humans the drug-induced vascular effects of new compounds at an early stage of clinical development

In this respect, pulse wave analyses and brachial diameter measurements present very suitable techniques; they provide a non-invasive and more sensitive assessment of drug-induced hemodynamic changes than those obtained by more conventional oscillometric blood pressure measurements (23, 24). In addition, the effect of NTG on these parameters is well known. Oliver et al. conducted a study in which they established the dose-relationship of sublingual NTG to changes in BAD and radial and aortic AIx in healthy men (26). The change in heart rate, brachial diastolic blood pressure, aortic and radial AIx and BAD following 0.4 mg of NTG in our study are largely in agreement with their data.

Given the results, we conclude that telcagepant does not have a clinical significant effect on the decrease in aortic AIx following a single therapeutic dose of NTG in healthy men. Importantly, these data support the conclusion that NTG remains an effective treatment for ischemia on the background of telcagepant therapy. This is crucial as one of the most important mechanisms of actions of NTG to relieve angina is to reduce the afterload of the heart, which is adequately reflected by the decrease in the aortic AIx (41, 42). More generally, this finding indicates that treatment with a potent CGRP-receptor antagonist does not interfere with the pharmacological effect of NTG, thus discarding a role for CGRP in NTG-induced vasodilation.

The analysis of the BAD permits an online assessment of the vasoactive effects of a drug on the brachial artery (23). Whether the effect of a drug on the BAD reflects coronary artery behaviour has never been determined and would require simultaneous acquisition of both peripheral arterial ultrasound measurements and invasive coronary angiography. It is nevertheless of interest that previous studies have linked impairment of brachial artery response to NTG with coronary artery disease, suggesting similarities between both vascular territories (43, 44). The results obtained in the present study indicate that telcagepant has no clinically significant effect on brachial vasodilation induced by a single dose of NTG in healthy men.

It was recently demonstrated, both in a dose finding phase II study and in a confirmatory phase III study, that a 300 mg dose of telcagepant has an efficacy comparable to that of the triptans for the treatment of acute migraine headache (13, 45). The anticipated clinical dose of telcagepant (relative to the formulation used in this study) is a single 300 mg dose, with an optional second 300 mg dose 2 hours later, if needed. A single 500 mg dose, as used in this study, achieves pharmacokinetic exposures similar to 2 X 300 mg doses, administered 2 hours apart. Despite the use of a high clinical dose in the present study (i.e. 500 mg), telcagepant alone (prior to NTG treatment) did not exert any unwanted cardiovascular effects neither on the peripheral nor on the central circulation of healthy men. The radial AIx was marginally lower when telcagepant was administered compared to placebo (P = 0.040), which results mostly from an unexplained difference in the predose values and therefore merely reflects normal variability of the measurement. Our observations are in agreement with the findings of Petersen et al. who reported the absence of effect of olcegepant (a distinct CGRP receptor antagonist) on cerebral blood flow, temporal and radial artery diameter and oscillometric blood pressure measurements (16). These data suggest that although CGRP circulates under measurable concentrations in the blood in basal conditions, it does not exert a homeostatic vasodilator activity in humans under resting conditions. As a consequence, a CGRP receptor antagonist, in contrast with the triptans, seems devoid of vasoconstrictor effects under resting conditions.

A number of limitations of the present study need to be taken into account. First, the vascular effects of telcagepant were investigated in healthy men. Though CGRP has been demonstrated to play a role in ischemic preconditioning in rats and is believed to mediate the cardioprotective effects of NTG-induced preconditioning in rabbits, there was no evidence of a major role of CGRP in regulating ischemic blood flow in dogs (46-48). Because of these differences between species and the lack of human studies, additional studies may be useful in patients with known cardiovascular disease (20). In a first study in patients with stable cardiovascular disease, telcagepant did not appear to exacerbate

spontaneous ischemia (49). Nevertheless, this study was performed under standardized resting conditions, whereas the effect of CGRP antagonism in cardiovascular patients might be rather different during normal daily activities or exercise. Second, central pressure effects were not measured invasively but were estimated by use of a generalized transfer function. This transfer function has received some criticism and has not been convincingly validated in young healthy subjects (41). In addition calibrating the peripheral pressure wave form by use of brachial SBP and DBP might further flaw the prediction of aortic blood pressures (31). However, in this study the use of radial AIx and aortic AIx yield to the same conclusions, confirming that the untransformed radial pressure wave form provides similar information on pressure wave augmentation as the aortic pressure wave form does. Third, the present study was not performed in migraine patients, the future patient population of telcagepant. This might be relevant as migraine patients are known to have alterations in arterial function when compared to healthy volunteers (50).

In summary, this study shows that telcagepant, at a high clinical dose for the treatment of migraine headache, had no clinically relevant effect on NTG-induced hemodynamic changes in healthy male volunteers. Nor was there a measurable vasoconstrictor effect of telcagepant as such in both the central and peripheral vascular bed. These results support the favourable cardiovascular safety profile of CGRP-receptor antagonists and indicate that CGRP is not involved in NTG-induced vasodilation in humans.

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Part II

Nitric oxide as a target for migraine treatment:

Chapter 6:

No evidence of increased nitric Oxide synthase activity in migraine: Is there still a role for NO?

Chapter 7

Does the unfavourable pharmacokinetic and pharmacodynamic profile of the iNOS inhibitor GW273629 lead to inefficacy in acute migraine?

Chapter 6

No evidence of increased nitric oxide synthase activity in migraine: Is there still a role for NO?

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Submission due

Abstract

Objectives

To assess whether migraine patients display a chronic nitric oxide synthase (NOS) hyperactivity by comparing the nitric oxide (NO) production before and following a loading dose of L-arginine between migraine patients (interictally) and matched healthy control subjects. In addition, we evaluated whether a loading dose of L-arginine triggers an acute migraine headache in migraineurs.

Methods

Twenty healthy subjects and 20 migraine patients participated in a 2-period, randomized, double-blind, placebo-controlled study. Each subject received a 30-min infusion, by peripheral vein, of 30 g L-arginine hydrochloride or placebo (i.e. an equal volume of 0.9% saline solution). Meanwhile, biomarkers associated with the L-arginine-NO pathway (i.e. exhaled/nasal NO (eNO/nNO), plasma citrulline and urinary excretion of nitrite/nitrate and cGMP) were assessed before and for 6 hours following the start of the infusion.

Results

At baseline, eNO and nNO were higher in migraineurs compared to healthy subjects (mean ± 95% confidence interval): 15.9 (8.8, 23.0) ppb versus 10.8 (7.0, 14.5) ppb for eNO (P = 0.04) and 76.3 (61.2, 91.4) versus 61.6 (51.2, 72.0) ppb for nNO (P =0.03), respectively. The AUC_{0-6} in ppb for eNO and nNO following L-arginine or saline infusion did not differ between both groups. The increase in L-citrulline, following L-arginine infusion, was smaller in migraine patients (15 (13,18) µmol/L) compared to healthy volunteers (19 (16,23) µmol/L) (P = 0.046). In healthy subjects, both nitrate and cGMP excretion were higher following L-arginine compared to placebo infusion: 132.63 (100.24, 165.02) versus 92.07 (66.33, 117.82) µmol $mmol^{-1}$ creatinine for nitrate (P = 0.014) and 50.53 (42.19, 58.87) versus 39.64 (33.94, 45.34) nmol $mmol^{-1}$ creatinine for cGMP (P = 0.0003), respectively. In migraineurs, excretion of these biomarkers was comparable following L-arginine or saline infusion.

Conclusion

The results of the present study do not support the idea of a generalized increase in NO synthase activity in migraine patients outside of a migraine attack. The smaller increase in plasma L-citrulline, urinary nitrate and cGMP excretion following L-arginine infusion in migraine patients might indicate dysfunction of endothelial NO synthase.

Introduction

Olesen et al. proposed that nitric oxide (NO) plays a crucial role in the pathophysiology of migraine headache (1). They observed that both administration of exogenous NO donors and stimulation of endogenous NO production by histamine induce delayed headaches in migraineurs which resemble the characteristics of spontaneous migraine attacks (2-4). In addition, they reported that the non-specific NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) provides pain relief to patients experiencing a spontaneous migraine headache (5). Numerous studies suggested an increased NO production in migraineurs, both ictally and interactally, but did not permit a conclusive and final answer regarding the presumed hyperactivity of the L-arginine-NO pathway (6-9). Recently, our group observed plasma levels of 3-nitrotyrosine, a relevant biomarker of NO-dependent oxidative stress, which were over 4-fold higher during a migraine headache compared to in between attacks (10, 11). In addition, exhaled NO (eNO) and nasal NO (nNO) also tended to be higher during a migraine attack compared to outside of an attack (10). All these observations have strengthened the belief that NO is a key molecule in the pathogenesis of migraine. Caution however is warranted, as one of the major difficulties encountered in all studies is the elusive nature of the NO molecule (12). The search for stable end-products of NO furnished inconsistent results. In addition, inhibition of NO synthesis, long time seen as a promising target for the treatment of migraine headache, has recently fallen short of expectations (10, 13, 14).

Three isoforms of NO synthase (NOS) are distinguished: inducible NOS (i.e. iNOS or NOS II) and two constitutively expressed NOS isoforms: neuronal NOS (i.e. nNOS or NOS I) and endothelial NOS (i.e. eNOS or NOS III), respectively. As inhibition of eNOS is undesirable due to its widespread physiological role in healthy vascular tissue, research has focused on the development of potent and specific iNOS inhibitors (15, 16). Potent iNOS inhibitors unfortunately failed to show any beneficial effect in both the acute and prophylactic treatment of migraine (10, 13, 14). Despite these recent findings, evidence to support NOS as a potential target for migraine treatment remains strong. In particular for nNOS, which is expressed throughout the trigeminal system and plays an important role in the sensitization of spinal second order nociceptors (17, 18). Neuronal NOS inhibition was shown to have antagonist actions within the trigeminovascular system, thereby reducing neurogenic dural vasodilation (19). It has been suggested that the delayed headache induced by the administration of an NO donor is due to nNOS activation leading to the production of more NO and the subsequent activation of the trigeminovascular system. Therefore, nNOS might be an interesting target for the treatment of migraine and the question whether NO plays a role in the pathophysiology of migraine thus remains unanswered.

In the present study, the objective was to assess whether migraine patients display a chronic, interictal, NOS hyperactivity. Endogenous NO is synthesised by NOS from the amino-acid L-arginine, yielding L-citrulline as a by-product. The administration of L-arginine in humans results in an increase of eNO and nNO, a slight decrease in mean arterial pressure (MAP), an increased plasma citrulline concentration and an increase in urinary nitrate and cyclic guanosine monophosphate (cGMP) (20, 21). If the L-arginine/NO conversion in migraine patients is increased due to NOS hyperactivity, this should be reflected by the associated biomarkers characteristic for NO production (eNO, nNO, plasma L-

citrulline and L-arginine and urinary nitrite/nitrate and cGMP). In addition, the administration of L-arginine would result in a larger increase of NO production in migraine patients compared to healthy volunteers. Furthermore, the increase in NO might provoke a delayed migraine headache in migraineurs as seen following the administration of exogenous NO donors.

To that end, this study compares the NO production before and following a loading dose of intravenously administered L-arginine between a group of migraine patients outside of a migraine headache period and a matched group of healthy control subjects. In addition, we evaluated whether a loading dose of L-arginine triggers an acute migraine headache in migraineurs.

Methods

Subjects

After approval by the ethics committee of the University Hospital of Leuven, 30 migraine patients with or without aura, as defined by the International Headache Society diagnostic criteria, were recruited (22). In addition, 32 healthy volunteers matched for age, gender and body mass index (BMI) were screened from the general population. Major exclusion criteria were: age < 18 and ≥ 50 years, history suggestive of asthma, rhinitis or atopy, history of severe herpes infection (i.e. contra-indication for L-arginine administration), diabetes, history of cardiovascular disease, smoking, hypertension (systolic blood pressure > 140 mm Hg or diastolic blood pressure > 90 mm Hg), pregnancy or lactation and use of chronic drugs (except hormonal contraceptives). Migraine patients with a history of migraine < 6 months, having > 15 days of headache per month at any given time since the onset of migraine attacks, using anti-migraine drugs > 10 days /month or using prophylactic medication, were excluded. Control subjects experiencing more than 1 headache per month, with a history of moderate or severe headaches or migraine headache or with a first degree relative with migraine were excluded. Female subjects had to use oral, injected or implanted hormonal methods of contraception from at least the commencement of their last normal menstrual period prior to the first administration of the challenge agent.

The diagnosis of migraine was made by a neurologist or general physician in accordance with the International Headache Society diagnostic criteria (22). Migraine patients fulfilling criteria for both migraine with and without aura were considered as migraine patients with aura. The Migraine Disability Assessment Score (MIDAS) was used to assess headache-related disability (23).

Based on the medical history, physical examination and routine laboratory tests, 9 patients and 11 healthy volunteers were excluded. From the remaining 21 migraine patients, one was excluded because of severe hypotension following L-arginine administration. One of the healthy volunteers withdrew from the study because of the difficult venous puncture. The 20 remaining migraine patients and 20 healthy volunteers completed the study successfully.

The study was conducted in accordance with the Declaration of Helsinki. All subjects provided written informed consent prior to participation.

Study design

All eligible subjects participated in a 2-period, randomized, double-blind, placebo-controlled study. Each subject received a 30-min infusion, by peripheral vein, of 30 g L-arginine hydrochloride (R-Gene®, 100 ml of 10% Arginine hydrochloride injection for intravenous use, Pfizer Inc, New York, NY, USA) or an equal volume of 0.9% saline solution (B Braun, Melsungen, Germany) (flow chart in Figure 1). There was at least a 7-day washout interval between both study periods. The infusion was always administered in the morning in order to avoid potential diurnal variations. Placebo and L-arginine doses were given under the supervision of a research physician.

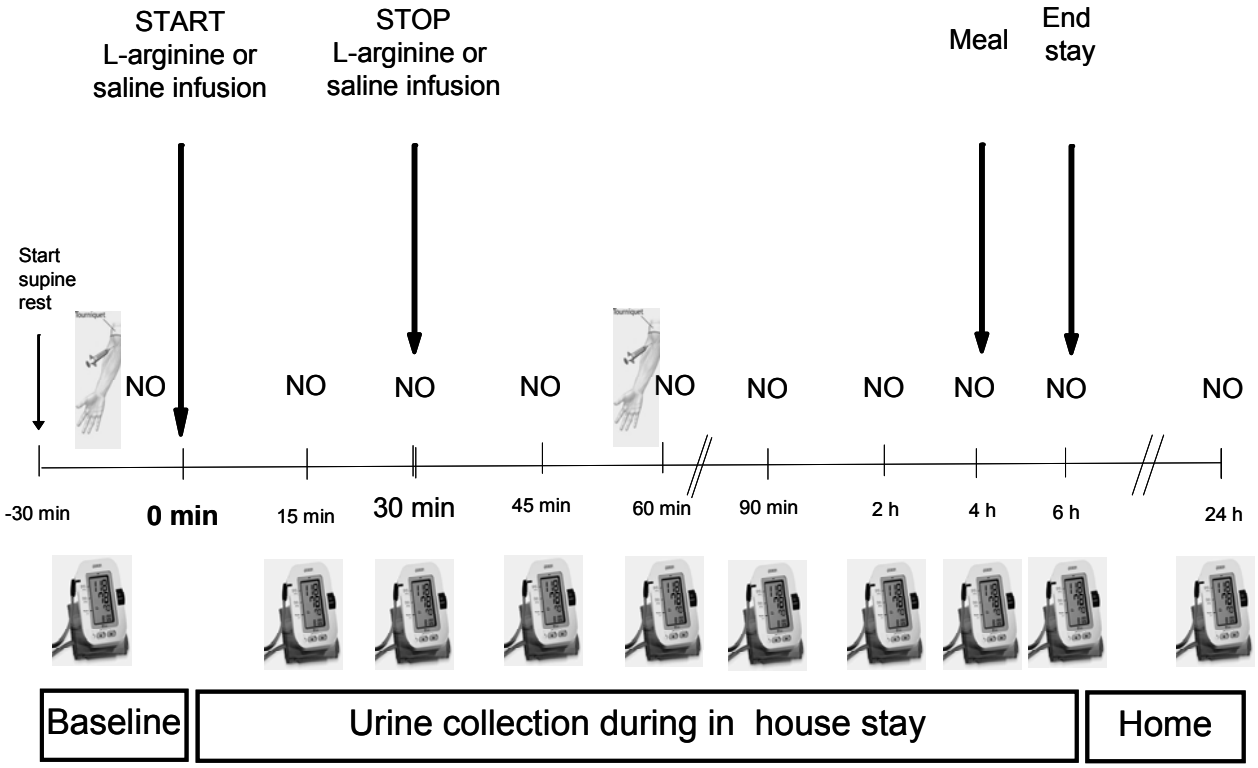


Figure 1

Flow chart. NO, eNO and nNO measurements in threefold; , blood sampling; , vital signs in twofold.

Measurements

Measurements were performed in a quiet, temperature controlled (23 ± 1°C) room. Female subjects were studied outside the "pill free" period. Each subject received a list of dietary sources of nitrates/nitrites (e.g. red meat or cured meat) and was instructed to refrain from such foods for at least 48 hours before the start of the experiments. Subjects abstained from any drugs (except oral contraceptives) for at least 3 days. Migraine patients had to be headache free for at least 3 days. Subjects fasted at least 8 hours before L-arginine or placebo administration and no food was allowed until 4 hours and 15 minutes after the infusion. Six hours and 15 minutes after the infusion, subjects were allowed to go home but were still expected to refrain from dietary sources of nitrates/nitrites up to 24 hours after L-arginine infusion as was required in the 48 hour period prior to the experimental session.

In each study period, during the 60 minutes period following the start of the infusion, eNO, nNO, blood pressure and heart rate were measured at 15-minutes intervals. Measurements were repeated at 1.5, 2, 4, 6 and 24 hours after the start of the infusion.

Blood samples for L-citrulline and L-arginine were sampled at baseline and 1 hour after the start of the infusion. Urine was collected for cGMP and nitrite/nitrate assays. The collection was performed during the 6 hour period following the start of the L-arginine/placebo infusion. The headache response was evaluated according to the criteria of the International Headache Society (IHS, 2004) in the 24 hour period following the administration of the L-arginine/placebo loading dose. Headache intensity was scored on a visual scale that measures from 0 to 10. The study flow chart is given in Figure 1.

Brachial blood pressure and heart rate

Supine systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were recorded at the left upper arm by use of a validated, non-invasive semi-automated oscillometric device (OMRON 705IT; Omron Healtcare, Hamburg, Germany) (24). At each time-point, measurements were performed twice.

Exhaled and nasal NO

During each study period, eNO and nNO were measured using the CLD 88sp NO analyze supplied by Eco Medics, UK. At each time-point, eNO and nNO were measured 3 times and the mean of these 3 readings was used for analysis. eNO and nNO measurements were performed according to the guidelines of the American Thoracic Society (ATS) (25).

Plasma L-arginine and L-citrulline

Plasma L-arginine and L-citrulline concentrations were determined by liquid chromatography (LC)-tandem mass spectrometry (MS) analysis as described by Chace et al. (26).

A 50-µL aliquot of plasma was spiked with stable isotope-labelled L-arginine and L-citrulline, which served as internal standards. Protein was precipitated with 100 µL of methanol, filtrated through a 0.22-µm hydrophilic membrane (Multiscreen HTS^{TM}; Millipor, Molsheim, France), derivatized with

butanolic HCl (1N, 65°C, 17 min) and analysed by LC-tandem MS. Quantification was performed by selected reaction monitoring of the respective daughter ions of analytes and internal standards.

Urinary nitrite/nitrate concentrations

Urinary nitrite/nitrate levels were determined using nitrate reductase and the colorimetric Griess reaction (27). A 1:4 dilution in PBS of the urinary samples (200 µl) was ultra filtrated (6,000 *g*, 45 minutes, 4°C) through 10,000-kDa molecular weight filters (Ultrafree-MC Biomax; Millipore). Fifty microliters of the filtrates were analyzed in duplicate. A standard curve was prepared, ranging from 0 µM up to 50 µM and was included in duplicate in each assay. Nitrate in the standards and samples was converted to nitrite by addition of 50 µL PBS with 600 U/L nitrate reductase from Aspergillus (Roche Diagnostics Corp.) and 100 µM NADH (Sigma-Aldrich). Complete conversion was achieved with an incubation time of 4 hours at room temperature, as evaluated by comparison of a converted nitrate standard curve with a nitrite standard curve. Nitrite was then measured after addition of 50 µL PBS, 50 µL 1% sulfanilamide in 5% phosphoric acid and 50 µL 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride. The absorbance was read at 540 nM and corrected for absorbance at 650 nM. Urinary excretion rates of nitrite/nitrate were corrected for creatinine excretion.

Urinary cyclic guanosine monophosphate concentrations (cGMP)

Urinary cGMP levels were determined by a cGMP enzyme immunoassay (R&D systems Europe, Ltd.). This assay is commercially available and based on the competitive binding technique in which cGMP, present in the sample, competes with a fixed amount of horseradish peroxidase (HRP)-labeled cGMP for sites on a rabbit polyclonal antibody. During the incubation, the polyclonal antibody becomes bound to a goat anti-rabbit antibody coated onto a microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The color development is stopped and the absorbance is read at 450 nm. The intensity of the color is inversely proportional to the concentration of cGMP in the sample.

Data analysis

Exhaled and nasal NO values are expressed in parts per billion (ppb). The change in eNO and nNO following L-arginine or placebo administration is expressed as the absolute change from baseline in ppb. At each time-point, the mean of observations and standard error of the mean are given.

As the data for eNO and nNO are not normally distributed, they were ln-transformed prior to statistical analysis. The baseline values were then compared between migraine patients and healthy volunteers using a two sample Student's *t* test. Of the remaining time-points, only those from the six hours of in house stay were included in the analyses, as the measurements of the 24 h time-point proved to be unreliable due to poor compliance, notably with the dietary requirements. The areas under the curve from baseline up to six hours following infusion (i.e. AUC_{0-6}) were calculated as summary measures for the absolute values and for the change from baseline for eNO and nNO. Subsequently, they were compared between migraine patients and healthy volunteers using a two sample Student's *t* test on ln-transformed data. The AUC_{0-6} for the absolute values and for the change from baseline were also

compared between the L-arginine and the placebo period using a paired Student's *t* test after ln-transformation.

Plasma L-citrulline and L-arginine data are expressed in µmol/L. Both the baseline values and the values 1 hour after the start of the infusion with L-arginine/placebo were compared between migraine patients and healthy volunteers using a two sample Student's *t* test. In addition, the L-arginine and the placebo period were compared using a paired Student's *t* test.

Urinary nitrite/nitrate and cGMP concentrations were simultaneously quantified to allow differentiation between impaired NO synthesis (in which case nitrite/nitrate levels and cGMP levels are expected to be low) and oxidative inactivation (in which case cGMP levels are expected to be low, but nitrite/nitrate levels should be normal) (28, 29). Urinary nitrite/nitrate and cGMP concentrations were corrected by urinary creatinine concentrations to depict the urinary excretion rates of these metabolites and eliminate variability due to differences in renal excretory function (30). Both the values following L-arginine and saline infusion were compared between migraine patients and healthy volunteers using a two sample Student's *t* test on ln-transformed data. In addition, the L-arginine and the placebo period were compared using a paired Student's *t* test on ln-transformed data.

To analyze brachial blood pressure changes, the MAP was calculated. MAP is defined as the sum of the level of DBP plus one third of the difference between SBP and DBP. The baseline MAP values were compared between migraine patients and healthy volunteers using a two sample Student's *t* test. In addition, the maximal decrease in MAP following L-arginine infusion was compared between migraineurs and healthy volunteers using a two sample Student's *t* test. Finally, the AUC_{0-6} for the MAP was compared between migraineurs and control subjects after placebo and L-arginine infusion by a two sample Student's *t* test.

Based on the within-subject standard deviation, observed in a reproducibility study with L-arginine infusions in 12 healthy volunteers, and given a type I error probability (α) of 0.05, sample size calculations were performed to provide 80% power for detecting a given difference between the group of patients and the healthy volunteers for the biomarkers eNO, nNO and plasma L-citrulline. Thirteen subjects had to be included in each group in order to detect a 25% difference in the baseline values of nNO. For a 25% difference in the AUC_{0-6} of nNO after L-arginine administration, a sample size of only 11 subjects was needed. For eNO, the sample sizes needed to detect a 25% difference were 55 and 43 subjects for the difference in baseline and AUC_{0-6}, respectively. To detect a 25% difference in the increase of L-citrulline following L-arginine infusion, a sample size of 6 subjects was sufficient.

Results

Mean ± SD for age and BMI was 28 ± 8 years and 24 ± 3 kg/m^2 for migraine patients and 27 ± 8 years and 24 ± 3 kg/m^2 for healthy volunteers, respectively. Both groups consisted of 4 men and 16 women. Migraine patients had an average MIDAS (range) of 19 (2-46) and used no prophylactic medication. All patients and healthy volunteers completed the study. Except for one female healthy volunteer who developed severe genital herpes in the week following L-arginine administration, no adverse events of note were reported. Her data were included in the analysis as L-arginine was administered during the last study period.

At baseline, both eNO and nNO were significantly higher in migraineurs compared to healthy volunteers: 15.9 (8.8, 23.0) ppb compared to 10.8 (7.0, 14.5) ppb for eNO (mean (95% CI), P = 0.04, two sample Student's *t* test) and 76.3 (61.2, 91.4) ppb compared to 61.6 (51.2, 72.0) ppb for nNO(mean (95% CI), P = 0.03, two sample Student's *t* test). Figure 2 shows eNO and nNO following the infusion of L-arginine or saline. In table 1, the AUC_{0-6} for the absolute value in ppb is given for both migraine patients and healthy volunteers following L-arginine or saline infusion. In addition the AUC_{0-6} for the change from baseline is given for the period in which L-arginine was infused.

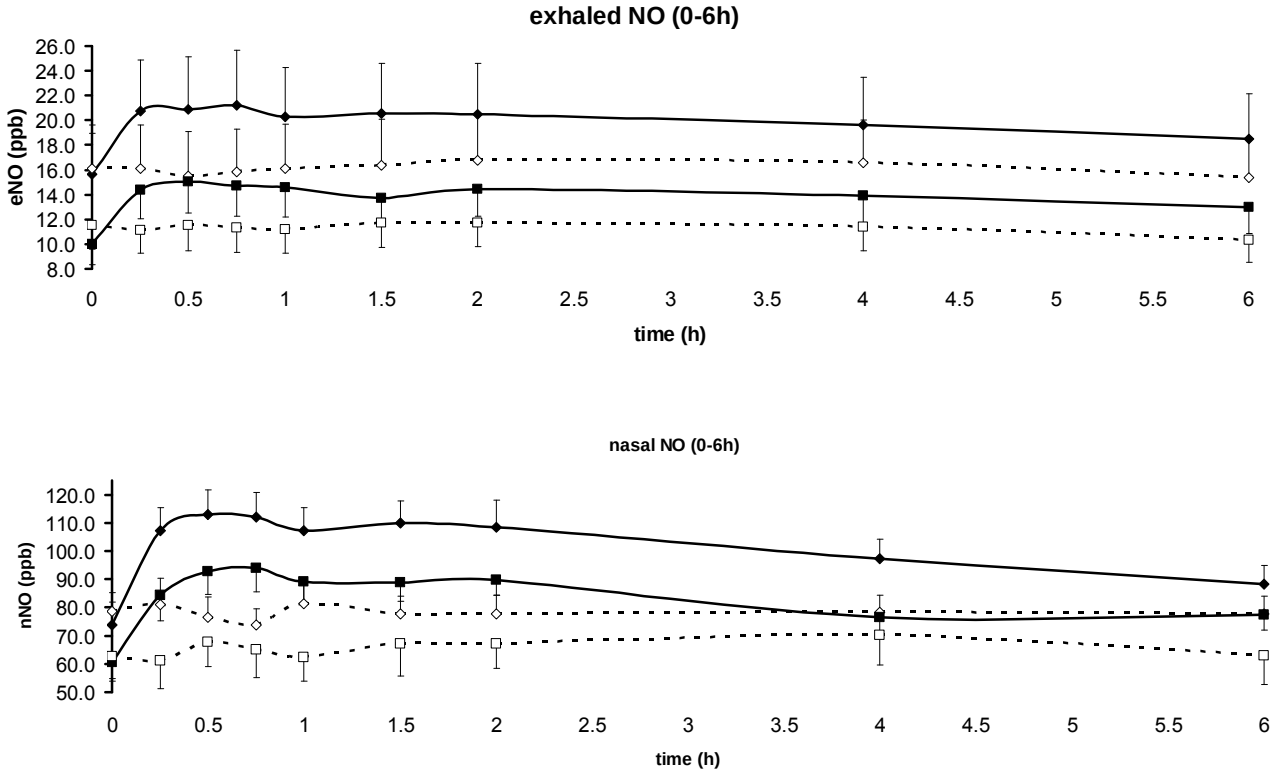


Figure 2

Exhaled/nasal NO (mean ± SEM); number of migraine patients = 20; number of healthy volunteers = 20. Diamonds are migraine patients, squares are healthy volunteers. Full line and symbols correspond to the period in which L-arginine was infused; dotted lines and open symbols correspond to the placebo period.

Table 1

AUC_{0-6} for the absolute value of exhaled NO (eNO) and nasal NO (nNO) and for the change from baseline during the L-arginine period.

	Parameter	Infusion	Migraine patients (MP)	Healthy volunteers (HV)	P-value MP-HV	$^{\$}$P-value A-P
AUC_{0-6} Absolute value (ppb)	eNO	L-arginine (A)	118.8 (69.3, 168.3)	83.4 (55.5, 111.4)	**0.21**	**< 0.0001**
		Saline (P)	97.6 (53.9, 141.4)	67.7 (43.8, 91.6)	**0.15**	
	nNO	L-arginine (A)	605.7 (512.2, 699.1)	496.0 (424.2, 567.8)	**0.06**	**< 0.0001**
		Saline (P)	468.7 (392.7, 544.7)	401.2 (232.9, 455.5)	**0.14**	
AUC_{0-6} Change from baseline (ppb)	eNO	L-arginine (A)	25.0 (13.4, 36.5)	23.3 (10.8, 35.8)	0.73	Non applicable
	nNO		162.2 (116.1, 208.2)	133.3 (94.5, 172.2)	0.65	

Data are presented as the mean ± 95% confidence interval; number of migraine patients = 20; number of healthy volunteers = 20

P-value assessed by a two sample Student's *t* test on ln-transformed data; $^{\$}$P-value assessed by a paired Student's *t* test on ln-transformed data

Plasma L-citrulline levels at baseline did not differ averaging 26 (23, 29) and 29 (26, 32) µmol/L for migraine patients and healthy volunteers, respectively (mean (95% CI), P = 0.32, two sample Student's *t* test). The increase in L-citrulline following L-arginine infusion was smaller in migraine patients (mean (95% CI), 15 (13, 18) µmol/L) compared to healthy volunteers (mean (95% CI), 19 (16,23) µmol/L; P = 0.046, two sample Student's *t* test) (Figure 3A). Plasma levels of L-arginine were similar at baseline at 65 (56,75) and 68 (60, 76) µmol/L for migraine patients and healthy volunteers, respectively (mean (95% CI), P = 0.66, two sample Student's *t* test). Plasma L-arginine levels increased after L-arginine infusion by 2,602 (2,267, 2,937) and 2,651 (2,391, 2,910) µmol/L in migraine patients and healthy volunteers, respectively (mean (95% CI), P = 0.81, two sample Student's *t* test; Figure 3B).

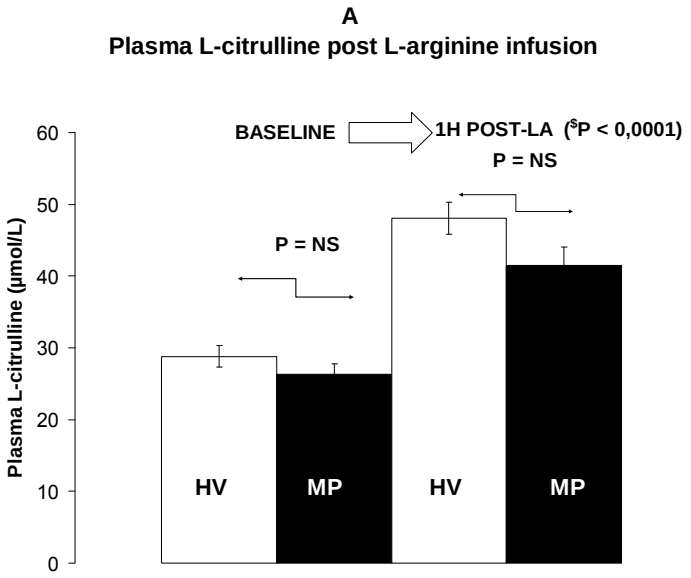


Figure 3A: Plasma L-citrulline (mean ± SEM); number of migraine patients = 20; number of healthy volunteers = 20. HV; healthy volunteers. MP; Migraine patients. P: Two sample Student's *t* test comparing migraine patients to healthy volunteers. $P: Paired Student's *t* test comparing baseline to 1h post-L-arginine.

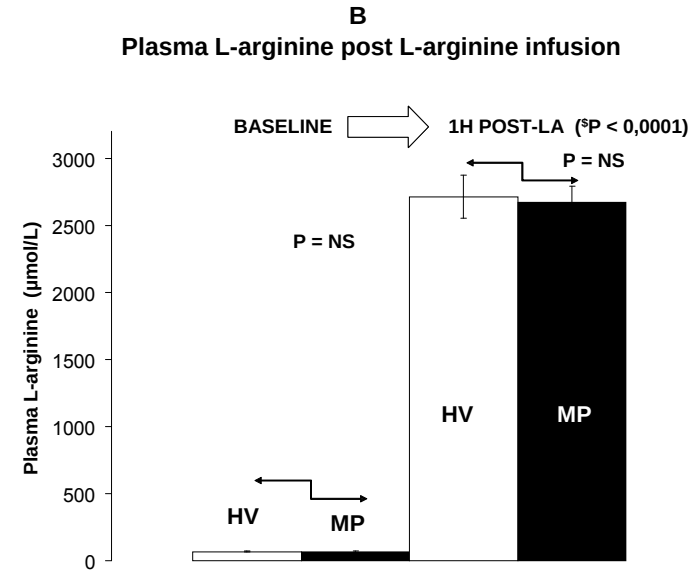


Figure 3B: Plasma L-arginine (mean ± SEM); number of migraine patients = 20; number of healthy volunteers = 20. HV; healthy volunteers. MP; Migraine patients. P: Two sample Student's *t* test comparing migraine patients to healthy volunteers. $P: Paired Student's *t* test comparing baseline to 1h post-L-arginine.

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Urinary nitrite/nitrate and cGMP excretion did not differ between migraine patients and healthy volunteers during the placebo period (Table 2). Interestingly, whereas no significant increase in urinary nitrite/nitrate and cGMP excretion could be detected in migraine patients between the L-arginine and the placebo period, there was a significant increase of both biomarkers in healthy volunteers following L-arginine infusion (Table 2).

MAP was similar at baseline in migraine patients and healthy volunteers. The maximal decrease in MAP following L-arginine infusion did not differ between migraine patients and healthy volunteers: -11 (-14, -8) mmHg in migraine patients versus -10 (-13, -8) mmHg in healthy volunteers (mean (95 % CI); P = 0.72, two sample Student's *t* test). In addition, no difference in AUC_{0-6} was detected between migraine patients and healthy volunteers following L-arginine or placebo infusion (Figure 4).

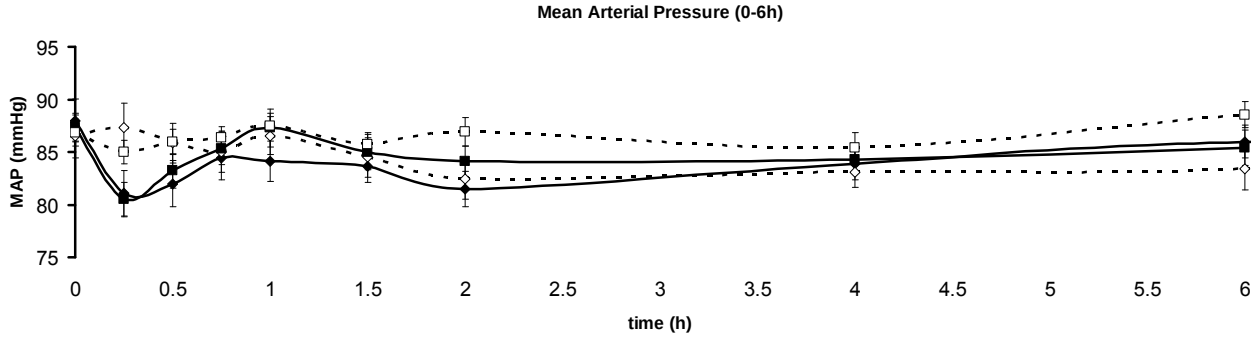


Figure 4: Mean arterial pressure (mean ± SEM); number of migraine patients = 20; number of healthy volunteers = 20. Diamonds are migraine patients, squares are healthy volunteers. Full line and symbols correspond to the period in which L-arginine was infused; dotted lines and open symbols correspond to the placebo period.

Seven migraine patients developed a migraine headache in the 72 hour interval following L-arginine infusion. Three of those also developed a migraine headache after saline infusion. In total, 7 patients developed migraine following placebo administration. None of the biomarkers was significantly different between those patients who developed a migraine headache and those who did not.

Table 2

Urinary nitrite/nitrate and cGMP excretion from 0 till 6 hours after infusion

Biomarker	Infusion	Migraine patients	Healthy volunteers	$P-value comparing migraine patients to healthy volunteers
Nitrite/Nitrate (μmol $mmol^{-1}$ creatinine)	L-arginine	105.04 (80.51, 129.57)	132.63 (100.24, 165.02)	0.07
	Saline	90.38 (74.65, 106.11)	92.07 (66.33, 117.82)	0.70
cGMP (nmol $mmol^{-1}$ creatinine)	L-arginine	37.16 (31.11, 43.22)	50.53 (42.19, 58.87)	**0.009**
	Saline	37.31 (31.52, 43.11)	39.64 (33.94, 45.34)	0.46
P-value comparing nitrite/nitrate excretion between saline and L-arginine period		0.21	**0.014**	Non applicable
P-value comparing cGMP excretion between saline and L-arginine period		0.99	**0.0003**	

Data are presented as the mean ± 95% confidence interval; number of migraine patients = 20; number of healthy volunteers = 20

$P-value assessed by a two sample Student's *t* test on ln-transformed data; P-value assessed by a paired Student's *t* test on ln-transformed data

Discussion

The results of the present study do not support the idea of a generalized increase in NO synthase activity in migraine patients outside of a migraine attack. When comparing migraine patients to healthy subjects, challenge with L-arginine does not result in a larger increase in biomarkers associated with NO production (i.e. eNO, nNO, plasma L-citrulline and urinary excretion of nitrite/nitrate and cGMP). On the contrary, following L-arginine infusion, the increase in plasma L-citrulline levels and urinary excretion of nitrite/nitrate and cGMP is smaller in migraine patients compared to healthy volunteers.

L-arginine infusion results in a systemic hypotensive response due to an increase in endogenous NO production as indicated by both the increased plasma L-citrulline and the expired NO. The magnitude of the response to L-arginine infusion in our study is comparable to previous reports in healthy volunteers and confirms the concept that changes in expired NO may be a useful reflection of changes in systemic NO production (20).

An interesting finding in the present study is that the increase in L-citrulline following L-arginine infusion is slightly smaller in migraine patients. In addition, no significant increase in urinary nitrite/nitrate and cGMP excretion can be detected in migraine patients when the L-arginine period is compared to the placebo period. This is in sharp contrast with the healthy volunteers in whom a robust and significant increase in both urinary excretion of nitrate and cGMP is observed (Table 2). If anything, this would rather suggest a *decreased* NOS activity in migraine patients compared to healthy volunteers. The plasma L-citrulline level and the urinary excretion of nitrite/nitrate and cGMP largely depend on vascular eNOS activity (29-32). Therefore, the smaller increase in both plasma L-citrulline and urinary nitrite/nitrate and cGMP excretion following L-arginine challenge might indicate dysfunction of eNOS in migraine patients. This adds to the ongoing discussion about migraine being an endotheliopathy leading to decreased eNOS activity (33). Indeed, several studies suggest that endothelial function is abnormal in migraine patients (34, 35). Notably, our group conducted a study in which brachial artery flow mediated dilation (FMD) was measured to compare endothelial vasomotor function between migraine patients and healthy volunteers (36). FMD of the brachial artery was significantly decreased in migraine patients, suggesting that endothelial dysfunction is present in migraine patients and impedes on endothelium-dependent vasodilation. Our findings suggest that the decrease in endothelium-dependent vasodilation in migraineurs might be due to hypoactivity of eNOS. Nevertheless, reserve is warranted as large discrepancies exist between reports concerning endothelial function in migraine patients (37, 38).

A second interesting finding is the slightly higher values at baseline for eNO and nNO in migraine patients *outside* of an attack compared to healthy volunteers at baseline. Our group previously reported a tendency towards higher eNO and nNO values in migraine patients *during* an acute attack (10). This was based on pre-dose measurements of eNO and nNO in the context of an open label, non-randomised, two-period study in which the potent iNOS inhibitor GW273629 was administered once while patients suffered from a moderate to severe migraine headache and once during a migraine free period. Though it is tempting to hypothesize that the increase in expired NO reflects an increased NO production as part of the pathophysiology of migraine, such claims should, in view of our current results, be made cautiously. First, the differences observed between both groups in the

present study are small: expired NO is barely 5 and 15 ppb higher in migraine patients compared to healthy volunteers for eNO and nNO, respectively. Taking into account that the 5^{th} and 95^{th} percentiles for eNO in healthy subjects are considered to be 4.5 and 20.6 ppb for males while 3.6 and 18.2 ppb for females, one can't but wonder what a 5 ppb difference in eNO between two groups clinically implies (39). For nNO, the amplitude to ensure that a difference is genuine and not ascribable to the noise of measurement is even larger, which implies that interpretation of the difference in nNO between migraine patients and healthy volunteers might be over-interpretation (40). Second, it is generally accepted that expired NO is mainly of airway epithelial origin (41, 42). Nevertheless, some studies have suggested that a systemic increase of NO production can translate into an increase in expired NO (20, 43, 44). In this study however, none of the other biomarkers support differences in systemic NO production between migraine patients and healthy volunteers at baseline. Third and last, studies in humans demonstrate that iNOS is the most important contributor to the formation of NO in human breath (10, 41). The potent iNOS inhibitor GW273629 was able to decrease both eNO and nNO by approximately 80% (10). If excess NO would be present in the expired air of migraine patients, it would thus most likely be of iNOS origin. Whilst, until recently iNOS was considered a promising target for the treatment of migraine, recent clinical trials have failed to show any efficacy of potent iNOS inhibitors, both in the acute and the prophylactic treatment of migraine headache (10, 13, 14). Consequently, possible differences in iNOS activity between migraine patients and healthy volunteers are very unlikely to be involved in the pathophysiology of migraine.

In those patients who developed a migraine headache following L-arginine or placebo infusion, no significant early increases in biomarkers associated with NO production are observed. This further discredits NO as a main player in the pathophysiology of migraine. Though, caution is warranted while interpreting the data concerning the latter, as these were retrieved only from the 14 patients who developed a migraine headache during one of the study periods.

The observed higher interictal eNO and nNO values in migraine patients remain puzzling. In this respect, one might evoke the fact that an association between migraine and asthma, rhinitis and atopy has been reported by some authors (45, 46). Nevertheless, other reports have denied such associations (47, 48). Moreover, in this study, patients with a history or complaints suggestive of asthma, rhinitis or atopy were excluded, making it very unlikely that a potential association might have affected the data. Interpreting the higher eNO and nNO values in migraineurs as a proof for the involvement of NO in the pathophysiology of migraine, would be thoughtless. Nonetheless, it would also be wrong to discard any role for NO in the pathophysiology of migraine solely based on the results of this study which has several limitations.

First, the biomarkers associated with the L-arginine-NO pathway are sampled peripherally and might thus not adequately reflect variations in NO production within the trigeminovascular system itself. Changes in nNOS activity in non-adrenergic, non-cholinergic (NANC) fibres supplying cerebral blood vessels might have been obscured. Indeed, several studies suggest a relation between NO and the phenomenon of neurogenic inflammation in the trigeminovascular system, which appears to be a key step in the development of neurovascular headaches (49). Neurogenic inflammation is characterized by the release of various vasoactive peptides, including calcitonin gene-related peptide (CGRP), from

trigeminal perivascular nerve endings and results in vasodilation of the cranial blood vessels and sensitization of perivascular sensory afferents. The latter leads to increased transmission of nociceptive information to the brainstem. While the link between the production of NO and the release of CGRP is controversial, CGRP and NOS do seem to coexist in trigeminal ganglion cells (50, 51). In addition, nNOS appears to coordinate NO production to provoke CGRP release and vasodilation at the level of the trigeminovascular system (19). As nNOS involvement might have been missed by the peripheral sampling, it should still be considered as a potential therapeutic target in the treatment of migraine. Second, the L-arginine-NO pathway is assessed during a headache free period. In the 14 patients who developed a migraine headache, no data are available concerning the biomarkers associated with NO production as the headache occurred only 6 to 24 hours after L-arginine or placebo infusion. Third, the search for end-products of NO furnishes inconsistent results, mainly because of a broad range of inter-individual variation. This is partly due to dietary habits and therefore a nitrite/nitrate-restricted diet was imposed to all participants. Nevertheless, differences in physical activity between migraine patients and healthy volunteers might also have introduced bias (52, 53). Last, the evidence for the involvement of NO in the pathophysiology of migraine remains strong (1, 54). This however does not imply that there should be an overproduction of NO in migraineurs, as the relation between migraine and NO might be due to hypersensitivity to NO.

In summary, the results of the present study do not support the idea of a generalized increase in NOS activity in migraine patients outside of a migraine attack. In combination with the results of recently conducted clinical trials with potent iNOS inhibitors, which turned out to be ineffective, this study favours the involvement of nNOS in the pathogenesis of migraine above iNOS and eNOS. Therefore, nNOS remains a potentially valid target in the treatment of migraine The smaller increase in plasma L-citrulline, urinary nitrite/nitrate and cGMP excretion following L-arginine infusion in migraine patients might indicate dysfunction of eNOS in migraine patients.

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Chapter 7

Does the unfavourable pharmacokinetic and pharmacodynamic profile of the iNOS inhibitor GW273629 lead to inefficacy in acute migraine?

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Abstract

Objectives: To investigate the pharmacodynamics and pharmacokinetics of a single dose of GW273629, a selective iNOS inhibitor, given during and outside a migraine attack.

Methods: GW273629 1500mg was administered to 15 migraine patients both ictally and interictally. Nasal and exhaled NO, plasma 3-nitrotyrosine and nitrates were measured to assess systemic NO production. In addition PK and treatment response were assessed. Data are mean (95% CI).

Results: Plasma 3-nitrotyrosine was higher ictally: 11.96(8.22, 15.71) versus 2.74(2.24, 3.24)ng/10mg, respectively ($p<0.0001$). Exhaled and nasal NO showed a similar trend: 12.5(6.5,18.6) and 62.2(41.5, 82.8)ppb ictally versus 9.9(6.3, 13.4)ppb and 52.5(38.5, 66.0)ppb interictally, respectively. Early absorption of GW273629 (AUC_{0-2}(90% CI)) was reduced by 41(22, 55)% during an attack. There was no improvement of headache or on associated symptoms.

Conclusions:

Migraine headache is associated with reduced early absorption of GW273629 and excess NO production. In this open label study, GW273629 was ineffective in the treatment of acute migraine.

GW273629 (3-[[2-[(1-iminoethyl)amino]ethyl]sylphonyl]-L-alanine) is a novel, potent inhibitor of human inducible nitric oxide synthase (i.e. iNOS or NOS II) with a half maximal inhibitory concentration (IC_{50}) of 9.03 µM ± 0.85 (mean ± SEM) for recombinant human iNOS in vitro (1). The compound was in clinical development for the acute treatment of migraine headache. The rationale for the development of NOS inhibitors for the treatment of migraine finds it's origin in the so-called nitric oxide (NO) hypothesis put forward by Olesen et al. They observed that both administration of exogenous NO and stimulation of endogenous NO production by histamine, induce genuine migraine attacks in migraineurs (2, 3). Plasma levels of nitrites, a stable product of NO degradation, are known to be higher in migraine patients outside an attack and have been shown to further increase when a headache occurs (4, 5). The candidacy of NO as a pivotal molecule in the initiation and maintenance of spontaneous migraine attacks was further supported when the non-specific NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) was shown to provide pain relief to patients experiencing a spontaneous migraine headache (6).

NOS and NO have widespread physiological roles in healthy vascular and neuronal tissues (7). Consequently, the potential side-effects of non-specific NOS inhibition could by far outweigh any possible treatment benefit. Therefore inhibition of the constitutively expressed neuronal and endothelial isoforms of NOS (nNOS or NOS I and eNOS or NOS III, respectively) is undesirable. If in migraine iNOS is implicated, an inhibitor of iNOS with a high degree of selectivity over nNOS and eNOS, could have great therapeutic potential with few expected side-effects (8). In vitro, GW273629 is >150-fold and >365-fold more selective for iNOS than eNOS and nNOS, respectively (1). In animal studies, this high selectivity for iNOS was confirmed *in vivo*. The lack of effect on blood pressure and heart rate, when GW273629 was administered to healthy volunteers at doses up to 1800 mg, further indicates the absence of eNOS inhibition *in vivo* in humans.

Though selective iNOS inhibition is a new and controversial target in migraine therapy, it is supported by evidence from studies both in animals and in humans (8, 9). To our knowledge, GW273629 is the first selective iNOS inhibitor to be evaluated in migraine patients. Nevertheless, the efficacy of this orally administered compound does not only depend on the validity of iNOS as a target, but also on the its pharmacokinetic profile. In healthy subjects, GW273629 was rapidly absorbed following oral administration, with the maximum plasma concentration (C_{max}) occurring at 2-3.5 hours post-dose. However, a migraine attack is known to be associated with gastric stasis, which may lead to impairment and delay in drug absorption (10). Moreover, even outside of an acute attack, migraine patients manifest autonomic dysfunctions, which may account for altered pharmacokinetics of orally administered drugs in these patients (11). Therefore, it is advised to assess the pharmacokinetic properties of novel anti-migraine drugs in migraine patients both during and outside of an acute migraine headache in early clinical development (12).

The present study provided this opportunity and also enabled us to investigate whether a migraine attack is indeed associated with an increased NO production. In addition, the effect of an iNOS inhibitor upon NO production was assessed by measuring characteristic biomarkers associated with the L-arginine/NO pathway. To that end exhaled and nasal NO, plasma nitrate and plasma 3-

nitrotyrosine (protein bound and free) were selected because of evidence, that these parameters might accurately reflect systemic NO production (13-15).

The primary objective of this study was to investigate the pharmacokinetics of GW273629 in migraine patients both during and outside of an acute attack. In addition, the effects of migraine and of selective iNOS inhibition upon systemic NO production were assessed. A secondary objective was to obtain preliminary evidence concerning the efficacy of GW273629 for treating acute migraine headache.

Methods

Subjects

After approval by the ethics committee of the University Hospital of Leuven, 35 patients suffering from migraine with or without aura, according to the diagnostic criteria of the International Headache Society (International Committee on the Classification of Headache Disorders, 2004) were recruited through advertisements. The study was conducted in accordance with the Declaration of Helsinki. Diagnosis was initially made by a general physician or neurologist and was confirmed using a validated questionnaire and structured interview (16). Written informed consent was obtained during a screening visit from all patients. Major exclusion criteria were: age <18 and ≥65 years; women of child bearing potential not using an appropriate method of contraception; history of cardiovascular or cerebrovascular disease; smoking; hypertension; diabetes; hypercholesterolemia; pregnancy or lactation and daily use of drugs (except hormonal contraceptives). The use of any acute non-specific or specific antimigraine drug was allowed as a first-line treatment. Patients with a history of migraine of < 6 months, using prophylactic therapy or taking medication for acute headache on more than 10 days per month were excluded. Based on medical history, physical examination and routine laboratory tests, 5 patients were excluded. All other migraine patients were otherwise in good health and eligible for inclusion into the study, except for two patients that withdrew consent. Of the 28 remaining patients, 15 were dosed both during and outside of a migraine headache.

Study design

This was an open label, non-randomised, two-period study. During the first study period, a single 1500mg dose of GW273629 was administered while the patients suffered from a moderate to severe migraine headache. In the second study period, the same dose of GW273629 was given during a migraine free period. Both study periods were separated by a wash-out period of at least one week and a minimum of 72 hours had elapsed since the end of the previous migraine attack. The date and time of the last meal and caffeinated drink were recorded. Both dosing sessions were conducted in the Center for Clinical Pharmacology and subjects remained in-house until at least 12 hours post-dose. During the first study period, patients were asked to participate only if the ongoing migraine headache, if not treated, would remain moderate to severe for at least another two hours. In addition, they agreed to refrain from rescue medication for the migraine headache until two hours after the intake of GW273629.

Pharmacokinetics

During each study period, blood samples for PK analysis were collected just before dosing and at the following time-points after GW273629 administration: 5, 15, 30, 45 minutes and 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12 and 24 hours. Plasma samples were analysed for GW273629 using a validated analytical method based on solid phase extraction, followed by high pressure liquid chromatography/mass spectroscopy/mass spectroscopy analysis. In brief, plasma was assayed for parent compound by fluorescence derivatisation with a quaternary ammonium reagent followed by HPLC with mass spectrometric detection. The method has been fully described elsewhere (1, 17). The lower limit of quantification (LLQ) for GW273629 was 0.05 μg/mL using a 50 μL aliquot of human plasma with a higher limit of quantification (HLQ) of 10 μg/mL.

Pharmacodynamics

Exhaled and nasal NO

During each study period, eNO and nNO were measured prior to dosing and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24 hours after GW273629 administration. The expired NO was measured using a chemiluminescence analyzer (CLD 88sp, Eco Medics, Switzerland) which lower and upper limit of detection was 0.002 ppb and 5 ppm, respectively. The instrument was calibrated at 0 and 400 ppb as recommended by the manufacturer. Exhaled NO and nNO measurements were performed according to the guidelines of the American Thoracic Society (ATS) (18). The subjects were asked to exhale at a constant flow, which they could readily see displayed on the computer screen in the form of a bar that remained red until target flow was obtained, and then turned green. If the flow dropped below or increased above the desired range, the green bar changed back to red. Three measurements varying by <10% were made at each post-dose time-point (with a maximum of 6 measurements) and the average value was recorded. Prior to dosing, the procedure was done in twofold (i.e. 6 measurements for eNO and nNO each). The influence of ambient NO levels was excluded by placing an NO-scrubbing filter in the inspiratory limb of the collection apparatus.

Plasma nitrite/nitrate

Plasma samples were collected pre-dose and at 2, 6 and 24 hours post-dose. Plasma samples were diluted 2.5-fold with 14 mM sodium phosphate pH 7.4 (assay buffer). Separate series of nitrate and nitrite standards with concentrations from 200 μM with six serial two-fold dilutions from this were prepared in assay buffer. A blank containing only assay buffer was also included. Duplicate 50 μl samples of diluted plasma, standards or blank were added to separate wells of a 96-well plate. To one of these samples, a further 40 μl assay buffer was added. The second sample was treated with 40 μl of assay buffer containing glucose-6-phosphate (2.5 mM), NADPH-dependent nitrate reductase (200 U l^{-1}) and glucose-6-phosphate dehydrogenase (400 U l^{-1}). A further 10 μl assay buffer containing NADPH (10 μM) was added to initiate the conversion of nitrate to nitrite. The conversion was allowed to run to completion over 1 h at room temperature. Griess reagent was prepared just before use by mixing equal volumes of *N*-(1-naphthyl) ethylenediamine (0.1% w v^{-1} in MilliQ water) and sulphanilamide (1% w /v^{-1} in 5% v v^{-1} concentrated orthophosphoric acid). Griess reagent was added as a 100 μl aliquot to each sample following its 1 h incubation. After a further 15 min incubation, the

microplate was read in an absorbance reader with a 550 nm filter and a 630 nm reference filter. Standard curves for conversion of nitrate to nitrite were linear between 1.5 and 100 μM.
The method is fully described elsewhere (1, 19, 20).

Plasma 3-nitrotyrosine, tyrosine

Plasma samples were collected pre-dose and at 2, 6 and 24 hours post-dose for tyrosine and 3-nitrtyrosine. The bioanalytical method (based upon a liquid chromatography – mass spectrometry (LC-MS/MS) procedure involved the extraction of tyrosine and nitrotyrosine and internal standard ($^{13}C_9$-nitrotyrosine) from aliquots of human plasma by adding methanol to the plasma which was then centrifuged. The supernatant was then drawn off leaving a precipitated protein slurry which was freeze dried under vacuum. Protein (1 or 5mg) was weighed and transferred to a heat resistant tube. Sodium hydroxide (5 mL; 4M) was added, along with internal standard and analyte if required. Samples were then incubated at 120°C for *ca*. 16 hours. The hydrolysate (1 mL) was taken and citrate buffer was added to adjust the sample to pH 5. The whole sample was then loaded onto a LC-18 SPE cartridge (500 mg/3 mL). The cartridge was washed with water and the compound eluted with methanol/water. An aliquot (200μL) was removed for tyrosine analysis and the remainder of the sample was evaporated to near dryness by centrifugal evaporation for nitrotyrosine analysis. The residues were reconstituted in acetonitrile : water (10 : 90) and an aliquot chromatographed by injecting onto a 150 mm x 2.1 mm id x 5 μm C18 analytical column with a gradient mobile phase of acetonitrile : 0.1% formic acid. Nitrotyrosine and $^{13}C_9$-nitrotyrosine were detected by MS/MS by Turboionspray in the positive ionisation mode, monitoring ions at m/z 227 to m/z 181 for nitrotyrosine and m/z 236 to m/z 189 for ^{13}C-nitrotyrosine. Tyrosine and internal standard were detected by GC-MS in the negative chemical ionisation mode, monitoring ions at m/z 407 for tyrosine and m/z 411 for deuterated tyrosine.
Quality control samples were prepared for use during method re-establishment at endogenous and enhanced levels. The high enhanced quality control samples were prepared by the addition of nitrotyrosine standard to control plasma protein. The analysis is fully described by Frost et al. (15). As can be deducted from the method used, tyrosine and 3-nitrotyrosine are expressed per precipitated protein weight (ng/10mg for 3-nitrotyrosine).

Vital signs

Supine systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were measured in the dominant arm with a validated semi-automated oscillometric device (Omron HEM-705CP; Omron Healthcare, Hamburg, Germany). In addition, mean arterial pressure (MAP) was approximated using the formula MAP= DBP + 1/3 (SBP-DBP).

Preliminary efficacy assessment

The efficacy of GW273629 was assessed during a migraine attack by recording the following information:

1. Headache severity assessment on a four-point scale (0=none, 1=mild, 2=moderate, 3=severe) at baseline (pre-dose) and 0.5, 1, 1.5 and 2 hours after dosing;

2. Nausea, vomiting, photophobia and phonophobia assessment on a two-point scale (0=absent, 1=present) at baseline (pre-dose) and 0.5, 1, 1.5 and 2 hours after dosing;
3. Recurrence of moderate to severe headache (from mild or none) for the time period from 2 to 48 hours post-dose;
4. Use of rescue medication up to 48 hours post-dose.

Data analysis and statistics

Pharmacokinetics

Using non-compartmental analysis, AUC_{0-2}, AUC_{0-4} and time AUC_{0-t} were calculated. In addition, t_{lag}, C_{max}, t_{max} and $t_{1/2}$ were assessed. On the ln-transformed data AUC_{0-2}, AUC_{0-4}, AUC_{0-t}, and C_{max} were statistically analysed by ANOVA. A point estimate and the corresponding 90% CIs was constructed using the residual variance from the ANOVA for the difference "period 1 (migraine)-period 2 (no migraine)". By back-transforming the result, the point estimates and 90% CIs for the ratio period 1/period 2 were obtained.

Pharmacodynamics

Descriptive summary statistics (mean, 95% CI) were calculated for all pharmacodynamic endpoints.

For eNO and nNO, which are the parameters that allow direct assessment of the effect of GW272629 on iNOS activity, data were ln-transformed. Subsequently, baseline values were compared between both study periods using a paired Student's *t* test. In addition, the Spearman rank correlation coefficients for the eNO and nNO values during against outside of a migraine attack were calculated to assess the effect of a migraine attack on the inhibitory time-profile of GW273629. The data were also analyzed using analysis of variance (ANOVA). Pre-dose values (from period 1 (migraine) and period 2 (no migraine)) were investigated as a covariate. On the ln-transformed data, a point estimate and the corresponding 95% CI was constructed using the residual variance from the ANOVA for the difference "period 1 (migraine)-period 2 (no migraine)". By back-transforming the result, the point estimates and 95% CIs for the ratio period 1/period 2 were obtained.

Preliminary efficacy assessment

The number and proportion of subjects with a headache response, defined as a decrease in headache intensity from moderate or severe to mild or none, were summarized by time point up to 2 hours post-dose.

Results

The demographic and migraine characteristics of all 15 patients who completed the study are summarized in table 1.

Table 1: Demographic and migraine characteristics (n=15)

Sex, number (percentage)	
Male	4 (27%)
Female	11 (73%)
Age (years)	31 ± 10.6
Height (cm)	168 ± 8
Weight (kg)	64.4 + 13.1
Body Mass Index (kg/m^2)	22.7 ± 3.3
Migraine, number (percentage)	
With aura	8 (53%)
Without aura	7 (47%)
Age of onset of migraine (years)	17.4 (11-28)
Average number of migraine attacks/month	2.4 (1-5)
Average duration of migraine attacks (hours)	16.6 (1-72)

Unless indicated, data are presented as mean ± SD or mean (range)

Plasma concentrations and pharmacokinetics

Plasma GW273629 concentrations showed considerable between subject variability both during and outside of a migraine attack.

Figure 1
Mean plasma GW273629 concentration-time plot

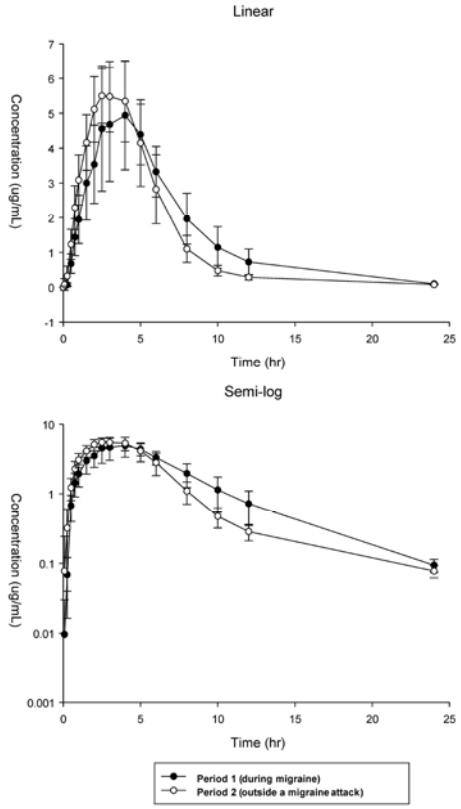


Figure 1: Mean GW273629 concentration-time plot on a linear (top) and semi logarithmic scale (bottom).
Number of subjects = 15
Date presented as mean ± 95% confidence interval.

Arithmetic means of pharmacokinetic parameters are given in table 2. In addition, point estimates and 90% confidence intervals (CI) for the comparison between dosing of GW273629 during a migraine attack and outside of a migraine attack are shown. Early absorption of GW273629 was reduced during a migraine attack as reflected by an average decrease of the area under the plasma concentration-time curve from time 0 to 2 hours (AUC_{0-2}) and from time 0 to 4 hours (AUC_{0-4}) by 41% and 35%, respectively. Both the delay between the time of dosing and the time of appearance of plasma concentration (t_{lag}) and the time of the highest observed plasma concentration (t_{max} and C_{max}) increased significantly while C_{max} decreased, on average, by 21% during a migraine attack compared to migraine-free conditions. Neither the overall extent of absorption (area under the plasma concentration-time curve from time 0 to last quantified concentration (AUC_{0-t})) nor the terminal phase elimination half life ($t_{1/2}$) of GW273629 were altered during a migraine attack.

Table 2

Pharmacokinetic parameters both during (Period 1) and outside (Period 2) a migraine attack and the corresponding ratio (Period 1/Period 2)

PK Parameter	Period 1 (n=15)	Period 2 (n=15)	Ratio Period1/Period2 (n=15)
AUC_{0-2} (µg.h/mL)	3.66 (2.40, 4.91)	5.47 (4.39, 6.55)	[a]0.59 (0.45, 0.78)
AUC_{0-4} (µg.h/mL)	12.79 (8.45, 17.12)	16.25 (14.20, 18.30)	[b]0.65 (0.48, 0.88)
AUC_{0-t} (µg.h/mL)	34.35 (26.93, 41.77)	31.76 (27.04, 36.48)	1.05 (0.94, 1.18)
$t_{1/2}$ H, mean (95% CI)	4.05 (2.94, 5.16)	4.82 (3.68, 5.95)	Not applicable
C_{max} (µg/mL)	5.73 (4.33, 7.13)	7.02 (6.13, 7.92)	[c]0.79 (0.70, 0.88)
t_{max} (h)	3.97 (3.16, 4.79)	3.23 (2.56, 3.91)	Not applicable
t_{lag} (h)	0.17 (0.11, 0.22)	0.10 (0.06, 0.13)	Not applicable

Pharmacokinetic parameters are given as arithmetic mean (95% Confidence Interval) and the ratios as geometric mean (90% Confidence Interval)

P = [a]0.0046, [b]0.0257 and [c]0.0020 (ANOVA following ln-transformation comparing period 1 with 2)

Pharmacodynamics

Vital signs

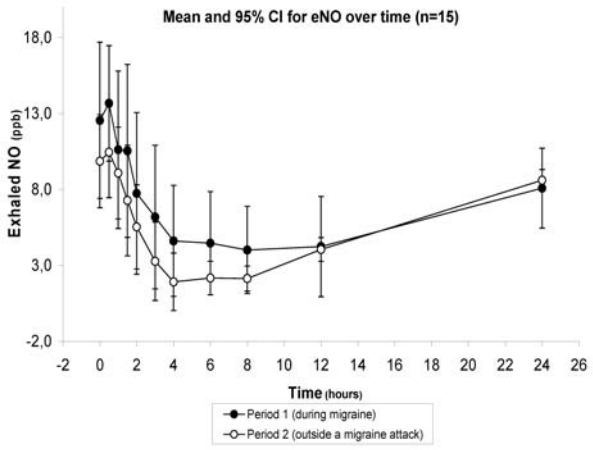
The pre-dose vital signs (blood pressure and heart rate) were similar during and outside of a migraine attack. They were not affected by the administration of GW273629.

Exhaled NO and nasal NO

Predose, both eNO and nNO tended to be higher during a migraine attack. Before dosing eNO averaged (mean (95% CI)) 12.5 (6.5, 18.6) ppb during versus 9.9 (6.3, 13.4) ppb outside of an acute migraine attack (P = 0.23, paired Student's *t* test). Nasal NO averaged 62.2 (41.5, 82.8) ppb ictally versus 52.5 (38.5, 66.0) ppb interictally (P = 0.16, paired Student's *t* test). Values of eNO and nNO over time are presented below.

Figure 2

A



B

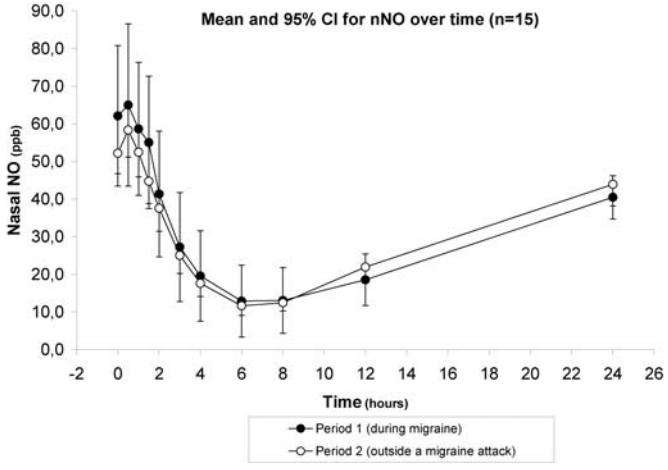


Figure 2: Mean exhaled NO (eNO) (**Figure 2A)**/nasal NO (nNO) (**Figure 2B)** both during and outside of a migraine attack.

Number of subjects = 15. Data presented as mean ± 95% confidence interval

Table 3

Pooled vital signs over periods 1 and 2 (n=15)

Parameter	Baseline	1 hour	2 hour	4 hour	8 hour	24 hour
SBP (mm Hg)	**116 (110,122)**	**117 (111,123)**	**118 (111,124)**	**119 (113, 125)**	**118 (111, 125)**	**114 (108,120)**
DBP (mm Hg)	**70 (66,74)**	**71 (67,76)**	**72 (67,76)**	**71 (66,76)**	**71 (65, 76)**	**70 (66,74)**
MAP (mm Hg)	**86 (81,90)**	**87 (82,91)**	**87 (82,91)**	**87 (82,91)**	**87 (81,92)**	**85 (81,89)**
HR (bpm)	**65 (61,70)**	**62 (58,67)**	**62 (57,67)**	**64 (59,69)**	**68 (63,72)**	**71 (66,76)**

All data presented as arithmetic mean (95% Confidence Interval)

SBP=systolic blood pressure; DBP=diastolic blood pressure; MAP=mean arterial blood pressure; HR= heart rate

There was a lag in inhibitory effect of GW273629, with the maximal decrease in eNO and nNO only being achieved 4 to 6 hours post-dose, whilst the t_{max} was reached at approximately 4 hours post-dose. The Spearman rank correlation coefficient for expired NO during versus outside of a migraine attack was 0.71 and 0.81 for eNO and nNO, respectively. Overall, the eNO profiles decreased from baseline until 4 hours post-dose and increased from 8 hours post-dose (figure 2A). The nNO values decreased until 6 hours post-dose, where after they increased (figure 2B). At two hours post-dose expired NO was decreased by 30 to 40 %, with a maximum decrease of approximately 80 % 4 to 6 hours after dosing GW273629

Plasma 3-nitrotyrosine and tyrosine

Pre-dose, plasma protein bound 3-nitrotyrosine was higher ($p < 0.0001$) during compared to outside of a migraine attack with a mean (95% CI) of 11.96 ng/10mg (8.22, 15.71) and 2.74 ng/10mg (2.24, 3.24), respectively. The ratio of protein bound 3-nitrotyrosine/protein bound tyrosine, as a measure for the production of reactive nitrogen species, is plotted in figure 3A. This graph shows that GW273629 did not have any effect on the plasma 3-nitrotyrosine levels.

Plasma nitrite/nitrate

Pre-dose, plasma nitrate levels did not differ averaging (95% CI) 35.740 (23.377, 48.103)µM during compared to 39.747 (26.277, 53.217)µM outside of an acute migraine headache. In contrast, at the 6 hours post-dose time-point, plasma nitrate was higher outside of an acute migraine attack (55.320 (44.924, 65.716) µM interictally compared to 34.820 (27.541, 42.099) µM ictally) (Figure 3B).

Preliminary Efficacy

The number of subjects with a headache response, defined as a decrease in headache intensity from moderate or severe to mild or none within 2 hours following GW273629 administration, was 2 out of 15 (13.3%) (table 4). No subjects reported being pain-free during the first 2 hours post-dose and there was no appreciable effect on symptoms associated with the migraine attack. Consequently, 12 subjects out of 15 (80.0%) received rescue medication at the earliest time-point allowed (i.e. 2 hours post-dose). Rescue medication used was acetaminophen, acetaminophen + codeine, sumatriptan and diclophenac by 5, 3, 3 and 1 patient, respectively

Figure 3

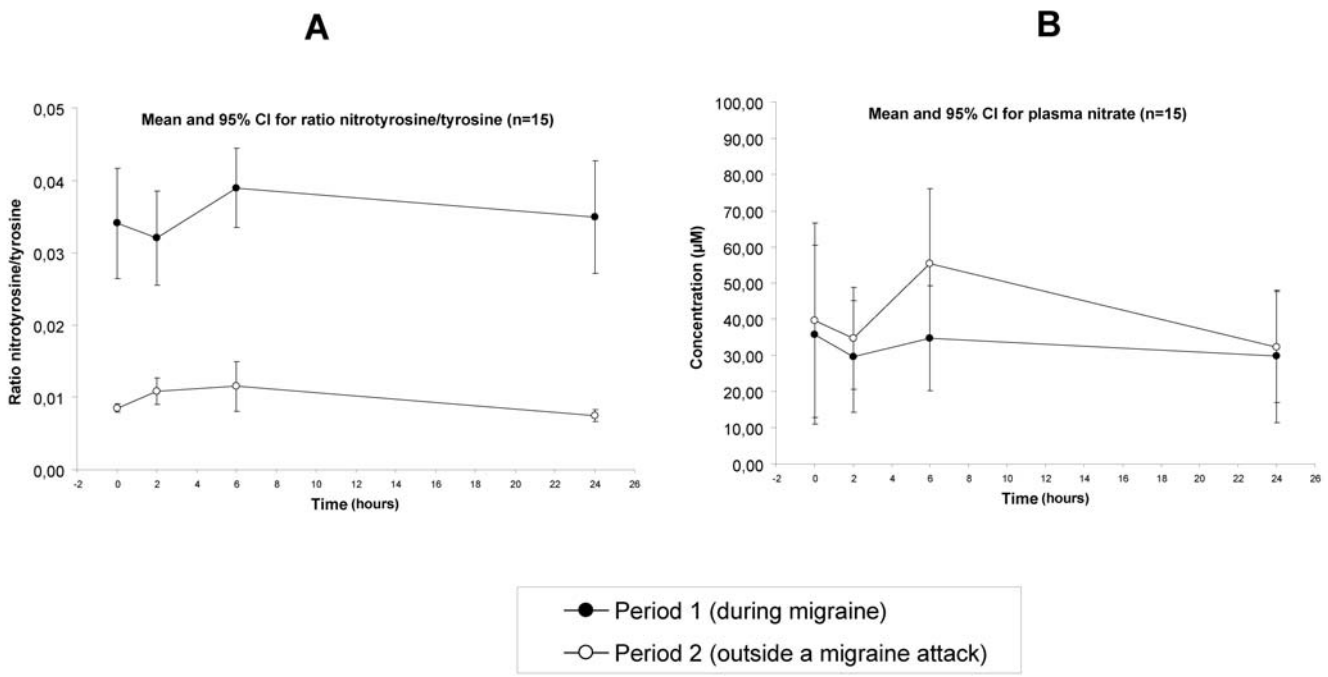


Figure 3A: Mean ratio nitrotyrosine/tyrosine both during and outside of a migraine attack
Number of subjects = 15
Data presented as mean ± 95% confidence interval

Figure 3B:: Mean nitrate plasma concentration both during and outside of a migraine attack
Number of subjects = 15
Data presented as mean ± 95% confidence interval

Table 4

Clinical assessment during migraine attack (Period 1; n=15)

	30 minutes	1 hour	1.5 hours	2 hours
Decreased intensity	0	0	0	0
Pain Free	0	0	0	2
Photophobia	15	15	15	15
Phonophobia	13	12	12	12
Nausea	0	0	1	2

Number of the 15 patients showing headache response

or number of the 15 patients presenting symptoms associated with the migraine attack

Discussion

This is the first study performed with a highly selective iNOS inhibitor, GW273629, for the treatment of acute migraine headache in migraine patients.

First, the pharmacokinetic properties of GW273629 were assessed both during and outside of an acute migraine headache. The present study clearly demonstrates that the speed of absorption of orally administered GW273629 is abated during a migraine attack compared to the migraine-free period. The delay in early absorption is best illustrated by the decreased $AUC_{0\text{-}2}$ and C_{max} combined with an increased t_{max}. During a migraine attack, none of our patients achieved the maximal plasma concentration within the first two hours after dosing. The absorption of GW273629 was previously shown not to be affected by food and thus it is unlikely that this effect is due to a different duration since the last meal between the two study periods. The overall absorption was not affected by the migraine headache. As is the case with most currently available acute anti-migraine drugs, this delay in early absorption is probably due to the delayed gastric emptying which is characteristic for the migraine syndrome (10).

Second, the pharmacodynamics of GW273629 were assessed. Despite the delayed early absorption during a migraine attack, the result of the Spearman rank correlation coefficient indicates that this delay had hardly any effect on the time-course of iNOS inhibition. The Spearman rank correlation coefficient being close to 1 for both eNO and nNO, we can conclude that the time-course of the expired NO decrease following GW273629 administration is nearly identical during and outside of a migraine attack.

Pre-dose eNO and nNO tended to be higher during a migraine attack. In addition, plasma levels of 3-nitrotyrosine were more than 4-fold higher when patients presented during a migraine headache compared to in between attacks. Our results concerning eNO and nNO suggest that expired NO can indeed measure NO produced outside the respiratory tract. As migraine is not known to be strongly associated with lung inflammation, it would be very unlikely that this trend towards increased expired NO during a migraine attack is due to NO production within the respiratory tract itself. Therefore, one might hypothesize that the increases in eNO and nNO reflect an increased NO production as part of the pathophysiology of migraine. The anatomical origin of NO in expired air remains a matter of debate. It is generally accepted that expired NO is mainly of airway epithelial origin (13). The biological fate of NO produced elsewhere in the body is still poorly understood. NO is a highly labile species with a half-live of only a few seconds (7). Therefore, one might be surprised that numerous studies have demonstrated the presence of NO from outside the respiratory tract in expired air. Studies in rabbits and lambs have shown that the intravenous administration of nitrate vasodilators cause increased concentrations of exhaled NO (21, 22). In humans, a dose-dependent increase in exhaled NO was found after administration of both nitroglycerin and sodium nitroprusside (23). Another study in healthy volunteers demonstrated an increase in exhaled NO when L-arginine, a precursor of NO, was given intravenously. This increase correlated with the magnitude of the systemic hypotensive response to L-arginine (24). All these observations suggest that a systemic increase of NO translates into an increase in expired NO. We hypothesize that plasma proteins can serve as a carrier for NO and allow it to travel significant distances (25). When NO reaches the vasculature of the airways, its charge

neutrality and relatively hydrophobic behaviour allow it to diffuse freely across cell membranes and to reach the air which is emitted through the airways. This has led to the believe that expired NO could prove to be useful in the identification and monitoring of patients with inflammatory processes, though, said to say, the debate on this issue continues (26).

The higher 3-nitrotyrosine levels in migraineurs suggest that migraine is associated with an excess in NO production and thus seem to confirm the earlier NO-hypothesis by Olesen and colleagues (6, 8, 27). Plasma nitrate concentrations failed to confirm an increased NO production being similar during and in between migraine attacks at the pre-dose time-point. At the 6 hours post-dose time-point, plasma nitrate levels were even higher interictally than ictally and, despite potent iNOS inhibition, increased when compared to baseline in the interictal study period. These observations are in keeping with the contention that circulating nitrate does not reflect systemic NO production (14). Plasma nitrate concentration is thought to reflect constitutive eNOS activity and thus the finding that the plasma nitrate concentration remains stable or even increases after administering GW273629 further confirms its selectivity for iNOS over eNOS and nNOS. The increase of plasma nitrate levels at the 6 hours post-dose time-point was only observed in the interictal period and is most likely due to the meal given at 4 hours post-dose, which was refused by all patients during an attack. Outside of an attack, all subjects ate at the 4 hours time-point.

The present study does not allow evaluation of whether a putative excess in NO production during a migraine attack is generated by iNOS, nNOS or eNOS. Inducible NOS is a good candidate because, once activated, it is able to synthesize large amounts of NO for extensive periods of time. The local increase in NO during a migraine attack must be substantial as indicated by the quadrupling of the 3-nitrotyrosine plasma levels. Only iNOS induction would be capable to sustain such a massive NO production (28). Furthermore, if the excess NO was generated by eNOS, this would inevitably have had hemodynamic consequences which was not the case. The baseline vital signs were equal during and outside of a migraine attack, which discards any role for eNOS. As said before, the absence of effect of GW273629 on plasma nitrate levels further confirms this. For nNOS, no such indicators are present and it is thus impossible to exclude a role for nNOS. Considering GW273629s maximal plasma concentration (7.02 µg/mL) and its IC_{50} in vitro for nNOS (170 µg/mL), eNOS (>237 µg/mL) and iNOS (2 µg/mL), inhibition of nNOS and eNOS is presumably negligible compared to iNOS inhibition (1). In no way should the observation that 3-nitrotyrosine levels do not drop in the 24 hours following iNOS inhibition be interpreted as an indication that iNOS is not responsible for the higher NO production with higher 3-nitrotyrosine levels. It merely reflects the long half life of 3-nitrotyrisine in vivo, which is known to amount up to 6 days (29, 30).

Preliminary efficacy of GW273629 on acute migraine was also assessed in this study. Whilst the study was open label and not placebo controlled, the low percentage of subjects reporting headache relief and the frequent use of rescue medication are indicative of poor efficacy. The headache relief rate following GW273629 administration is even below the usual placebo response seen in other trials for the treatment of acute migraine (31). The inefficacy of GW273629 is inkeeping with the unfavourable kinetic profile of GW273629 with a t_{max} of nearly 4 hours during an attack which is an obstacle for its efficacy for treating acute migraine. Furthermore, there is a lag in the inhibitory effect of GW273629 as

assessed by eNO and nNO meaning that maximal inhibition of iNOS is only being achieved 4 to 6 hours post-dose. Our findings regarding eNO, nNO and 3-nitrotyrosine suggest that there might be an excess NO production in the run-up to an acute migraine attack. Nevertheless, the preliminary efficacy results of this study do not favour iNOS inhibition as a potential candidate for the treatment of migraine. Indeed, in spite of 30 to 40 % inhibition of expired NO at the two hours post-dose time-point, no favourable effect on headache was observed whatsoever. That being said, for the treatment of acute migraine, the unfavourable pharmacokinetic and pharmacodynamic of the compound might be in itself an impediment for its efficacy.

Efficacy of NOS inhibition for the treatment of migraine has only been shown with the non-selective NOS inhibitor L-NMMA which is also a potent vasoconstrictor. Therefore, the question remains whether its efficacy was driven by iNOS, nNOS or eNOS inhibition (6). Akerman et al. tried to determine which NOS was the better potential therapeutic target using an animal model. Their results favoured the involvement of nNOS in the pathogenesis of migraine above iNOS (32). Our current results do support increased NO production in acute migraine, but strong iNOS inhibition by GW273629 had no beneficial effect probably partially because its efficacy was impeded by the unfavourable pharmacokinetic and pharmacodynamic profile of GW273629. However, an other potent selective iNOS inhibitor, GW274150, with a more favourable pharmacokinetic profile than GW273629, also proofed ineffective in both prophylactic and acute treatment of migraine. This discredits iNOS as a key player in the pathophysiology of migraine (33, 34)

In summary, this study shows that the early absorption of orally administered GW273629 is delayed during an acute migraine attack. The biomarkers eNO, nNO and 3-nitrotyrosine, measured to assess the L-arginine/NO pathway after the administration of a selective iNOS inhibitor, suggest that acute migraine headache is associated with an excess NO production and allow for the assessment of systemically generated NO. The absence of any effect on vital signs supports the cardiovascular safety of GW273629 in general and absence of eNOS inhibition in particular. We conclude that eNO, nNO and plasma 3-nitrotyrosine are useful biomarkers in the early clinical development of NOS inhibitors. These results do not support the continued development of GW273629 for acute migraine and confirm recent studies in which iNOS inhibition for both prophylactic and acute treatment of migraine fell short of expectations.

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Part III

The endocannabinoid system as a target for migraine treatment:

Chapter 8

Increased cerebral type 1 cannabinoid receptor availability in migraine patients

Chapter 8

Increased Cerebral Type 1 Cannabinoid Receptor Availability in Migraine Patients

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Abstract

It has been suggested that the endocannabinoid system may be involved in the pathophysiology of migraine.. We studied type 1 cannabinoid receptor (CB1R) availability in female migraine patients compared with age matched healthy women using [^{18}F]MK-9470 PET imaging. CB1R availability was globally increased in migraine patients but was not correlated with severity or frequency of migraine attacks. Increases in CB1R availability were most pronounced in the cingulo-frontal cortex and limbic system regions of the brain known to be involved in pain modulation., Our findings support further study of the role of endocannabinoids and CB1R in migraine patients.

Introduction

Although cannabinoids have been used for the treatment of migraine headache for many centuries, proof of their efficacy is obscured by the anecdotal nature of reporting (1). Since the genetic and pharmacological characterization of the endocannabinoid system, its role in the modulation of pain has been the focus of renewed interest. Although little is known about its role in primary headache disorders, recent findings suggest that a failure of the endogenous cannabinoid system might play a role in the pathophysiology of migraine and its tendency to progress to chronic migraine (2-4). In particular, platelets of female migraineurs exhibit an increased activity of anandamide hydrolase and the presumed anandamide membrane transporter, pointing towards an imbalance of endocannabinoid degradation possibly sensitizing female migraine patients to pain (2, 5). The relationship between the endocannabinoid system and migraine is further supported by experimental observations showing strong inhibition of trigeminovascular neuron activation and resulting vasodilation by the endocannabinoid anandamide (3, 6).

In vivo, investigation of the type 1 cannabinoid receptor (CB1R) has become possible with the development of second generation positron emission tomography (PET) radioligands with good imaging characteristics (7, 8). We compared CB1R availability between a group of female migraineurs outside the acute phase of a migraine attack and an matched healthy female control group using [^{18}F]MK-9470. Within the migraine group, we related changes in CB1R availability to pre-specified migraine characteristics and usual treatment regimen for acute headache.

Methods

Subjects

Twenty female migraine patients, as defined by the International Headache Society diagnostic criteria (9), and 18 healthy women were recruited. Major exclusion criteria for all participants were: age < 18 or > 50, pregnancy or lactation, history of psychiatric disease or first- or second-degree relative with hospitalization for psychiatric disease, hypertension (systolic blood pressure > 140 mmHg or diastolic blood pressure > 90 mmHg), history of substance abuse and use of psychoactive medication in the past 3 months. Migraine patients with a history of migraine < 1 year or having > 15 days of headache per month at any given time since the onset of migraine attacks were excluded. The healthy control women were matched to the migraine patients for age and body mass index (BMI). Controls experiencing more than 1 headache per month, with a history of moderate or severe headaches or migraine headache or a first degree relative with migraine were excluded.

The diagnosis of migraine was made by a neurologist or general physician in accordance with the International Headache Society diagnostic criteria (9). Patients with or without aura were included. Migraine patients fulfilling criteria for migraine both with and without aura were considered as migraine patients with aura. The following migraine characteristics were also evaluated : time since onset of migraine; median attack frequency; median duration of migraine attacks if untreated or treated unsuccessfully; usual lateralization of the headache and usual drug regimen in case of an attack. The Migraine Disability Assessment Score (MIDAS) was used to assess headache-related disability (10).

The study was approved by the local university Ethics Committee and conducted in accordance with the Declaration of Helsinki. All subjects gave written informed consent.

Study design and imaging procedure

The study was a cross-sectional observational study. During the screening visit, all subjects underwent thorough physical examination including neurological examination, blood and urine testing (including toxicology for all major known addictive drugs), as well as magnetic resonance (MR) imaging of the brain using a 1.5 Tesla Vision Scanner (Siemens, Germany) to exclude structural brain abnormalities. Volumetric T1 MPRAGE was used (sequence: TR=0, TE=4ms, flip angle=12°, inversion time 300 ms, matrix 256X256, 160 sagittal contiguous slices of 1 mm). In addition, routine transaxial T2 imaging was performed.

PET acquisition was performed after an overnight fast and between 11 AM and 7 PM, on a HR+ camera (Siemens, Ehrlangen, Germany). Study participants were instructed to abstain from any drugs (including acetaminophen and aspirine) during three days and from chocolate-, alcohol- and caffeine-containing beverages and food during 48 hours preceding the scan. For migraine patients, a minimum of three days had to be elapsed since the end of the last migraine headache episode.

Radiotracer synthesis was performed as previously described (8). Subjects received on average 300.5±45.3 MBq of [^{18}F]MK-9470 in slow intravenous bolus injection under standardized injection circumstances (supine, low ambient noise, dimly lit room). The subject's head was placed in a vacuum head holder to avoid head movement. PET acquisition was started 90 minutes after injection, for a

total duration of 30 minutes. Images were reconstructed using a three-dimensional filtered back-projection algorithm including scatter and measured attenuation correction.

Image processing and data analysis

Correction for motion between frames was performed using the realignment module in SPM2 (Statistical Parametric Mapping, Wellcome Department of Imaging Neuroscience, London, UK). Parametric images of [^{18}F]MK-9470 binding were calculated at each voxel from the area under the time-activity curve obtained in the 30 minute scanning period, and expressed as standardized uptake values (SUV) using PMOD v.2.7 (PMOD Inc, Zurich, Switzerland). Activity concentration at each voxel was therefore divided by the amount of tracer injected and normalized to the subject's body weight: SUV = Activity (kBq/cc)/(injected dose (MBq)/(weight (kg)) (8).

Each [^{18}F]MK-9470 parametric image was co-registered to the subject's MR image and spatially normalized on a CB1R binding template in standard MNI (Montreal Neurological Institute) space. As previously described (11), a volume-of-interest (VOI) map was loaded on all SUV images, representing cortical Brodmann areas and subcortical nuclei. [^{18}F]MK-9470 SUV values of the cortical Brodmann areas were grouped after weighing for volume into larger anatomical regions (Table 2). Additionally, we used statistical parametric mapping (SPM) to detect differences between the migraine and control group on a voxel level. Images were 10 mm-smoothed and only significant clusters ($P_{cluster}$ < 0.05, corrected for multiple comparisons) were retained, combined with sufficient localizing power (P_{height} < 0.005, uncorrected for multiple comparisons). The cluster extent threshold was set at 50 voxels (0.4 cm^3). SPM correlation analyses were performed within the group of migraine patients for MIDAS, time since onset of migraine, median attack frequency, median duration of migraine attacks and for time elapsed between the last migraine headache and PET acquisition using the ***Spearman's*** rank ***correlation*** test. Additionally, the group of migraine patients with aura was compared to the group having migraine without aura (two-sample Student's *t* test). For comparison between usual treatment regimens, migraine patients were divided into three groups according to whether they usually treated an attack by acetaminophen, non-steroidal anti-inflammatory drugs or triptans. Subsequently, these three groups were compared by one-way analysis of variance (ANOVA). Conventional statistics were carried out using Statistica v 7.1 (Statsoft Inc, Tulsa, OK, USA).

Power analysis based upon test-retest and intersubject variability showed that a difference in CB1R availability of 5-10% between the two groups would be detectable (age, sex and BMI matched).

Results

Mean ± SD for age and BMI was 24 ± 9 years and 23 ± 4 kg/m^2 for healthy women and 28 ± 8 years and 23 ± 3 kg/m^2 for migraine patients, respectively. Migraine patients had an average MIDAS (range) of 20 (3-75) and used no prophylactic medication. Ten patients suffered from migraine with aura, 10 from migraine without aura. An attack was usually treated by acetaminophen (7 patients), non-steroidal anti-inflammatory drugs (6 patients) or triptans (7 patients). All included subjects completed the study.

Globally, cerebral [^{18}F]MK-9470 binding was significantly increased in migraine patients compared to healthy controls (average grey matter difference +16%; P = 0.009, two-sample Student's *t* test)

Figure 1

Global increase in CB1R availability

in migraine patients compared to healthy women

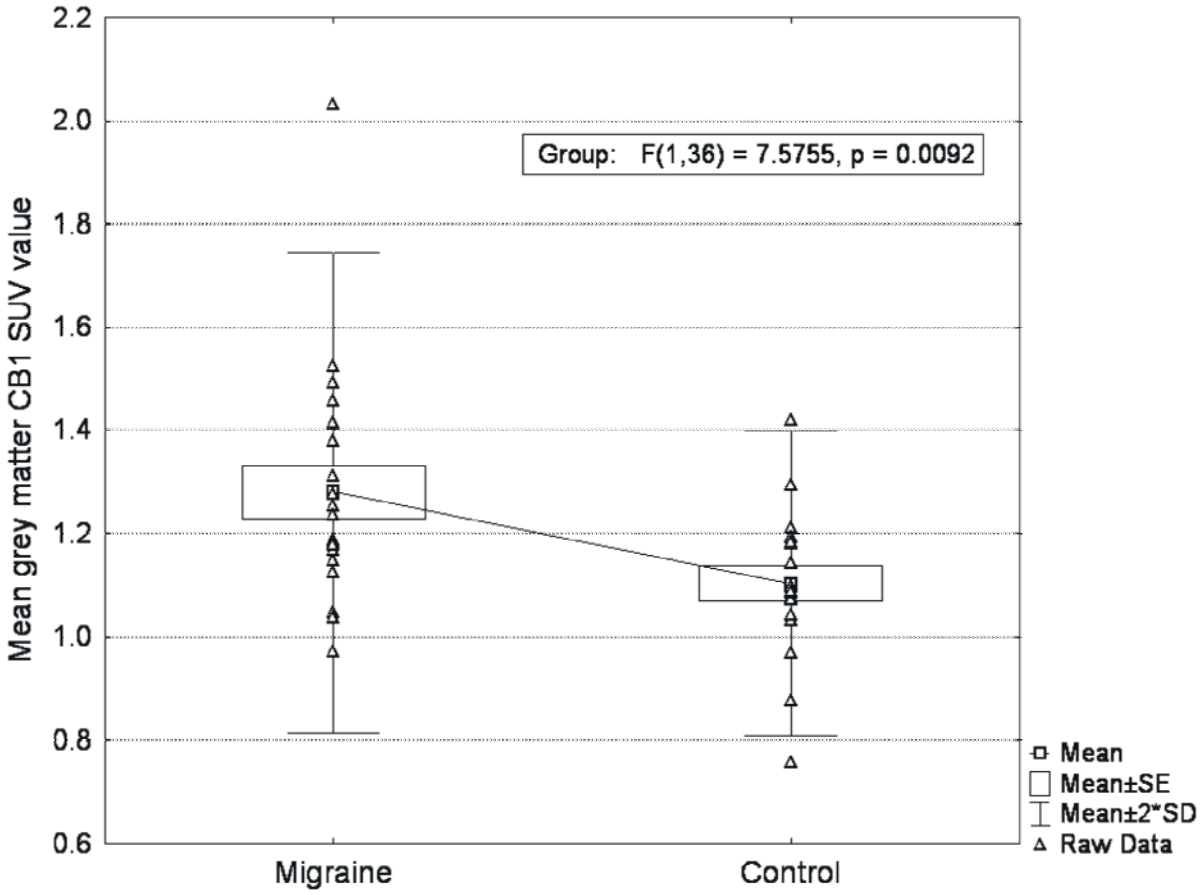


Figure 1: Boxplot of [^{18}F]MK-9470 CB1R binding in the migraine patients compared to healthy women (global grey matter value) with individual data points.

Number of migraine patients = 20; number of healthy women = 18

Volume-of-interest based SUV values for different brain regions, differences in percent between both groups and the corresponding P-values are given in table 1. SPM located the most significant increases in migraineurs the anterior cingulate, mesial temporal, prefrontal and superior frontal cortices ($P_{height} < 0.005$ and $P_{cluster} = 0.0002$; two-sample Student's *t* test) (Figure 2).

Table 1

Regional [^{18}F]MK-9470 CB1R binding.

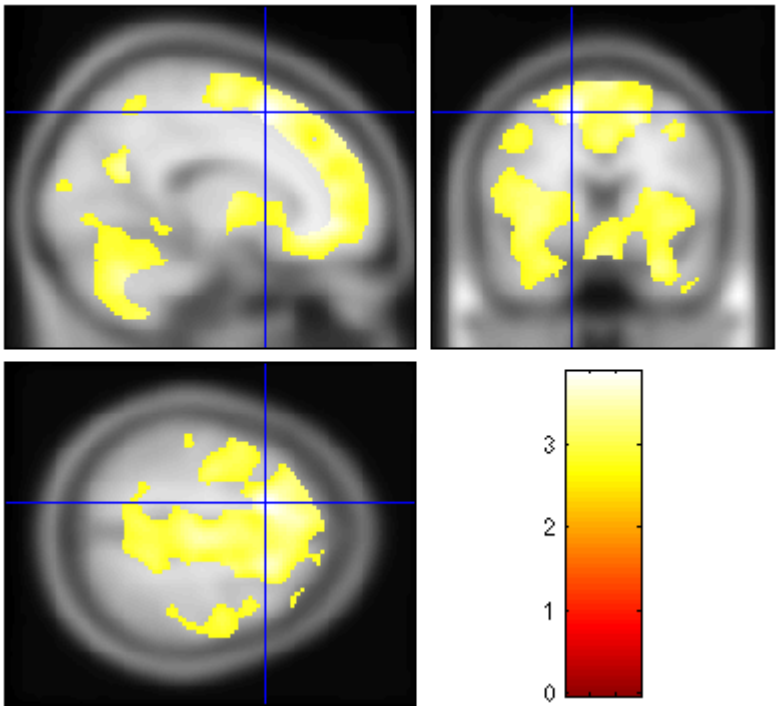
Region	Migraine patients Mean SUV ± SD	Healthy volunteers Mean SUV ± SD	Difference %	p-value
Frontal	1.31 ± 0.25	1.13 ± 0.15	16.65%	0.008
Temporal	1.30 ± 0.24	1.12 ± 0.15	15.77%	0.011
Mesial temporal	1.08 ± 0.18	0.93 ± 0.12	17.00%	0.004
Parietal	1.32 ± 0.23	1.15 ± 0.15	14.36%	0.016
Occipital	1.32 ± 0.24	1.15 ± 0.15	14.42%	0.016
Central sensorimotor	1.23 ± 0.24	1.06 ± 0.14	15.81%	0.012
Insula	1.37 ± 0.25	1.17 ± 0.15	17,61%	0.005
Cingulate	1.49 ± 0.27	1.27 ± 0.16	17.33%	0.005
Striatum	1.48 ± 0.27	1,28 ± 0.18	15.11%	0.015
Thalamus	1.05 ± 0.19	0.91 ± 0.12	15.22%	0.013
Pons	0.88 ± 0.16	0.77 ± 0.09	14.57%	0.013
Cerebellum	1.17 ± 0.22	1.00 ± 0.13	16.95%	0.007

Data are presented as mean ± SD, P-value from two-sample Student's *t* test

number of migraine patients = 20, number of healthy women = 18

Figure 2

Voxel-based maps of regional increase in CB1R availability in migraine patients compared to healthy controls



Legend to figure 2: Increases in [^{18}F]MK-9470 CB1R binding in migraine patients compared to healthy women. SPM T-map is overlaid on a T1 MRI template at $p_{height} < 0.005$ (uncorrected) and T-values are indicated by the color bar. Cluster extent threshold is 50 voxels. Number of migraine patients = 20; number of healthy women = 18

There was a positive correlation between global cortical CB1R availability and the time elapsed since the last migraine headache (Pearson r = 0.64, p = 0.002). However, the significance of this correlation was lost when the single data point from a subject with highest CB1R availability and longest time since the last migraine attack (35 days) was treated as outlier and omitted from the analysis (r = 0.32, p = 0.2)

We found no correlation between CB1R availability and the migraine disability score, attack frequency, or age of migraine onset, nor was there a difference in CB1R availability between migraine patients suffering from migraine with aura compared to without aura (Pearson correlation analysis and two-sample Student's *t* test, respectively). The usual treatment of the patients during a migraine attack did not affect CB1R availability (one-way ANOVA).

Discussion

This is the first study to characterize the *in vivo* cerebral distribution of the type 1 cannabinoid receptor (CB1R) in female migraine patients in comparison to healthy women. Migraine patients presented an increased CB1R availability, especially in the cingulo-frontal cortex and the limbic system that are. known to be involved in pain modulation. Our findings add further support to the recent observations of anomalies in the endocannabinoid system in female migraineurs (2, 12, 13). It could be hypothesized that lower levels of circulating endocannabinoids, that have been suggested in migraineurs, might theoretically lead to a global CB1R receptor up-regulation in the brain in an attempt to maintain endogenous analgesic tone (2). Thus, mismatches between the levels of the endogenous ligand and receptor availability may predispose to migraine. Indeed it has been suggested that cannabinoid agonists may provide migraine headache relief (1, 14, 15). However, in this study, no simultaneous measurement of endocannabinoid levels (i.e. anandamide) were performed and this is an important hypothesis that could be explored in future studies..

The increase of CB1R availability in migraine patients was present in all brain regions, indicating a global effect. The exact meaning of this generalized effect remains uncertain as there was no correlation to attack frequency or severity. However this correlation is dependent on the patient based reports of their migraine attacks and a degree of subjectivity could be responsible for variability and thus obscure putative correlations. The reliability of the migraine history could be improved in future studies by using physician based assessments along with proven clinical instruments such a migraine diaries that chronicle attacks over prolonged periods of time. A global increase in CB1R availability between migraine patients and healthy volunteers may reflect the regular use of analgesic medications. Interestingly however, no differences in CB1R availability were observed between patient groups who apparently used different classes of drugs to treat their migraine attacks although the exclusivity of class of drug used is uncertain since many use multiple classes of drugs in their attempts to gain headache pain relief.

A positive correlation between CB1R availability and the time elapsed since the previous migraine headache depended on an outlier and thus needs cautious interpretation (increased availability may be protective against migraine attack), but certainly warrants further study. Future studies could therefore investigate the relationship between CB1R availability and the onset of a migraine attack although this may be complicated by the permanent constitutive cycling of CB1R between plasma membranes and endosomes. It is currently unknown whether [^{18}F]MK-9470 stays bound during the cycling process or can access binding sites on these intracellular receptors (16). Finally, this study was conducted solely in female patients and volunteers and migraine is known to be more prevalent in women than men and often associated with the menstrual cycle. Previous studies have shown that there are gender differences in CB1R availability with significantly lower values in women compared to men (11) raising the possibility that our observations reflect differences in hormonal status between the female migraineurs and normal healthy volunteers groups (11).

In conclusion the results of the present study suggest that there are changes in cannabinoid signaling in the brain of migraine patients as manifest by an increase in CB1R availability. Future studies are therefore warranted into the role of this important endogenous pain modulatory system in predisposition and the pathophysiology of migraine that could lead to pharmacological advances in its treatment.

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Chapter 9

Summary and General Conclusions

Summary

Migraine is a common neurovascular disorder which ranks among the world's most disabling medical conditions and has been estimated to be the most costly neurological disease in the European Community. Although knowledge of the main molecular players is still incomplete, intensive research during the last decade has led to conceptual changes in our understanding of migraine headache (**Chapter 1**). Current acute and prophylactic treatment options are far from optimal and consequently improved treatments based on the new insights in the pathophysiology of migraine are desperately needed.

In the present thesis, a number of clinical pharmacological experimental methods are used to evaluate emerging new targets for the treatment of migraine *in vivo* in humans (**Chapter 2**). The main aim is to link findings from basic science to their clinical applications quickly, exemplifying the relevance of clinical pharmacology and its contribution to translational medicine and the explorative phase of clinical drug development. Our research focuses on three promising new treatment strategies: (1) calcitonin gene-related peptide (CGRP) receptor antagonism **(Part I)**, (2) nitric oxide synthase (NOS) inhibition **(Part II)** and (3) intervention in the endocannabinoid system **(Part III)**.

In **Part I** of the thesis, we translate a non-invasive pharmacodynamic model used in animals to allow rapid evaluation of CGRP receptor antagonists into an *in vivo* human model. As the selectivity of CGRP receptor antagonists is known to differ between species this translational step is crucial for investigating proof-of-mechanism of CGRP antagonists in humans. The model uses topical application of capsaicin to the skin of healthy volunteers to provoke CGRP release leading to an increase in dermal blood flow (DBF) which can be measured by Laser Doppler perfusion imaging. In a first exploratory study in 12 healthy men, we identify the dose of capsaicin needed to produce a robust and reproducible dermal blood flow (DBF) response measured by Laser Doppler perfusion imaging after topical application to the human forearm (**Chapter 3**). Simultaneously, the influence of the forearm location on the capsaicin response is assessed. In the proximal forearm of healthy volunteers a 1000 µg capsaicin dose elicits a reproducible response in both proximal sites at the 30-minutes time point. Subsequently, a reproducibility study is performed in 21 healthy men to assess the within-subject arm-to-arm and period-to-period reproducibility of the forearm DBF response to capsaicin using the 1000 µg capsaicin dose and the proximal forearm locations. In this second study, the data of 11 subjects with an increase in forearm blood flow from baseline ≥ 100 % (i.e. responders) during both study visits are included in a reproducibility analysis. The arm-to-arm reproducibility of the DBF after capsaicin application is almost perfect and based on the within-subject standard deviation the sample-size needed to detect differences in capsaicin-induced DBF increase between arms are calculated.

Subsequently, we characterize and indentify the mediators involved in the dermal vasodilation induced by capsaicin application to the human skin (**Chapter 4**). To assess the involvement of CGRP, the CGRP receptor antagonist $CGRP_{8-37}$ is infused into the brachial artery of one arm. In this way, the non-infused arm is not treated and serves as the control arm. The involvement of nitric oxide (NO) and prostaglandins in capsaicin-induced DBF increase are evaluated in a similar way by infusing the non-selective NO synthase inhibitor, N^G-monomethyl-L-arginine monoacetate (L-NMMA), and the cyclo-

oxygenase inhibitor indomethacin, respectively. To assess to involvement of substance P (SP) the potent SP antagonist aprepitant is given orally, and capsaicin-induced vasodilation is measured predose on the left arm and postdose (T_{max}) on the right arm. The main finding from this study is that capsaicin-induced vasodilation in the human skin is to a large extent antagonized by $CGRP_{8-37}$, but not by L-NMMA, aprepitant or indomethacin. Therefore CGRP seems to be the major mediator involved in this vasodilatory response to capsaicin. In addition, $CGRP_{8-37}$ does not affect resting DBF, indicating that CGRP does not play a role in maintaining basal DBF. The proposed model allows the assessment of the pharmacodynamic efficacy of CGRP receptor antagonists in a small group of healthy subjects and may guide the finding of a clinical effective dose.

In **Part I** of the thesis, we also use tools from clinical pharmacology to assess the vascular effects and safety of CGRP receptor antagonists. Data in animals suggest that administration of nitroglycerin (NTG), which is widely used in the treatment of cardiac ischemic pain (i.e. angina pectoris), causes release of CGRP in the cardiovascular system resulting in coronary artery vasodilatation. We therefore want to investigate the effect of CGRP receptor antagonism on response to NTG *in vivo* in humans (**Chapter 5**). We conduct a study in 22 healthy men and assessed vascular changes using non-invasive techniques, including ultrasound measurement of the brachial artery diameter and systolic pulse contour analysis. By administering NTG following a high clinical dose of the potent CGRP receptor antagonist, telcagepant, we demonstrate that CGRP antagonism has no clinically relevant effect on NTG-induced hemodynamic changes in healthy men. Nor is there a measurable vasoconstrictor effect of telcagepant as such in both the central and peripheral vascular beds. These results support the favourable cardiovascular safety profile of CGRP receptor antagonists, especially when compared to triptans.

In **Part II** of the thesis, the involvement of NO synthase activity in the pathogenesis of migraine is investigated by measuring associated biomarkers of systemic NO production. In clinical pharmacology, the measurement of biomarkers is used to identify potential treatment targets. Later, the same biomarkers can often be used as “surrogates” to evaluate preliminary clinical efficacy of newly developed compounds. There is abundant evidence for the involvement of NO in the pathogenesis of migraine. NO induces delayed headaches in migraineurs which resemble the characteristics of spontaneous migraine attacks and the non-selective NO synthase inhibitor L-NMMA provides pain relief to patients experiencing a spontaneous migraine headache.

In a first study, we assess whether migraine patients display a chronic NOS hyperactivity (**Chapter 6**). Based on the results of a reproducibility study in 12 healthy volunteers, we set up a study to compare NO production before and following a loading dose of L-arginine between 20 migraine patients, outside of a migraine attack, and 20 healthy volunteers by measuring biomarkers associated with the L-arginine/NO conversion (i.e. nasal and exhaled NO, plasma L-citrulline and L-ariginine and urinary excretion of nitrates and cyclic guanosine monophosphate (cGMP)). The results of this study do not support the idea of a generalized increase in NOS activity in migraine patients outside of a migraine attack. On the contrary, following L-arginine infusion, the increase in plasma L-citrulline levels and urinary excretion of nitrite/nitrate and cGMP is smaller in migraine patients compared to healthy volunteers. The latter might indicate dysfunction of endothelial NOS in migraine patients and adds to

the ongoing discussion about migraine being an endotheliopathy leading to decreased endothelial NOS.

The second study in **Part II** of the thesis investigates the pharmacokinetic and pharmacodynamic properties of GW273629, a potent inducible NOS (iNOS) inhibitor, in 15 migraine patients both during and outside of an acute migraine attack (**Chapter 7**). As in the first study, systemic NO production is evaluated by measuring biomarkers associated with the L-arginine/NO pathway (i.e. nasal and exhaled NO, plasma nitrate and plasma 3-nitrotyrosine). This allows to assess the effect of migraine headache and selective inducible NO synthase inhibition upon systemic NO production. Although the early absorption of orally administered GW273629 is delayed during a migraine attack, the time-course of iNOS inhibition, as assessed by nasal and exhaled NO, is similar during and outside of a migraine attack. So while migraine headache changes the pharmacokinetic properties of GW273629, its pharmacodynamic profile remains unaffected . Results from this study suggest that migraine headache might be associated with excess systemic NO production, but strong iNOS inhibition by GW273629 has no beneficial effect in this small open label study. Recent large clinical trials have also failed to show any efficacy of potent iNOS inhibitors, both in the acute and the prophylactic treatment of a migraine headache.

In **Part III** of the thesis, we use recent advances in neuroimaging to evaluate putative changes in the endocannabinoid system associated with migraine. In vivo investigation of the type 1 cannabinoid receptor (CB1R) has become possible with the development of positron emission tomography (PET) radioligands. We compare cerebral CB1R availability between 20 female migraineurs outside the acute phase of a migraine attack and 18 healthy women. Migraine patients present an increase in CB1R availability in all brain regions, especially in the cingulo-frontal cortex and the limbic system which presumably exert top-down influences to modulate pain. This is in accordance with recent findings suggesting that a failure of the endogenous endocannabinoid system might play a role in the pathophysiology of migraine and its tendency to progress to chronic migraine. This study is another illustration of how clinical pharmacology uses a large variety of tools to identify potential treatment targets. Our finding that there exists a dysregulation in the endocannabinoid system in migraine patients should encourage further investigation into cannabinoid signalling and might ultimately lead to pharmacological advances in the treatment of migraine.

General conclusion

This thesis presents a number of tools from clinical pharmacology which allow to both identify and facilitate clinical development of new pharmacological migraine treatments. Whereas preclinical research remains essential in the quest for more efficient and safer migraine therapy, our results clearly underline the need for human models as there seems to be large variability with regard to the mediators involved in the pathophysiological processes associated with the development of a migraine headache. In addition, the mere nature of migraine renders the development of animal research models difficult and therefore their validity remains uncertain.

Whereas CGRP receptor antagonists for migraine treatment will soon appear on the market, the future of NOS inhibition and modulation of the endocannabinoid system remains uncertain. Non-selective

NOS inhibition is effective in the treatment of migraine but unattractive because of cardiovascular safety concerns. Our results favour the involvement of nNOS in the pathogenesis of migraine above iNOS and eNOS but clinical data are lacking at this moment. Finally, therapeutic intervention in the endocannabinoid system does seem attractive, but the recent withdrawal from the market of cannabinoid receptor antagonists clearly illustrates that caution is warranted with regard to central nervous system side effects.

Samenvatting en algemene conclusie

Samenvatting

Migraine is een veel voorkomende neurovasculaire, aanvalsgewijs optredende vorm van hoofdpijn en één van 's werelds meest invaliderende aandoeningen. In de Europese Gemeenschap plaatsen schattingen migraine bovenaan de lijst van neurologische aandoeningen qua kostprijs. Dit heeft in het afgelopen decennium geleid tot intensief onderzoek waardoor onze visie op de ontstaanswijze van migraine sterk veranderd is (**Hoofdstuk 1**). Hoewel onze kennis over de moleculaire mechanisme die migraine veroorzaken ongetwijfeld nog onvolledig is, bieden deze gewijzigde inzichten de mogelijkheid om nieuwe medicamenteuze behandelingen te ontwikkelen. Daar de huidige acute en profylactische medicamenteuze behandelingen verre van optimaal zijn, is er een grote vraag naar nieuwe, efficiëntere en veiligere antimigraine geneesmiddelen.

De belangrijkste doelstelling van dit proefschrift is klinisch farmacologische experimentele methoden te ontwikkelen om nieuwe behandelingstrategieën *in vivo* te toetsen bij de mens (**Hoofdstuk 2)**. Op die manier probeert men snel bevindingen uit de fundamentele wetenschap te vertalen naar hun potentiële klinische toepassingen. Dit proefschrift is als dusdanig een mooie illustratie van de belangrijke plaats die klinische farmacologie inneemt in translationele geneeskunde en de exploratieve fase van het geneesmiddelenonderzoek.

Wij bestuderen drie veelbelovende nieuwe behandelingstrategieën voor migraine: (1) het blokkeren van de calcitonine gene-related peptide (CGRP) receptor (**Deel I**), (2) het remmen van stikstofmonoxide synthase (NOS) enzymen (**Deel II**) en (3) het induceren van veranderingen in het endocannabinoïd systeem (**Deel III**).

In **Deel I** van het proefschrift, vertalen we een niet-invasief farmacodynamisch diermodel naar de mens. Daar de selectiviteit van CGRP receptor antagonisten erg verschilt tussen verschillende diersoorten en de mens, is deze translationele stap van onschatbare waarde voor het onderzoek naar het werkingsmechanisme van CGRP receptor antagonisten bij de mens. In het door ons ontwikkelde model wordt capsaïcine lokaal aangebracht op de huid van beide onderarmen van gezonde vrijwilligers. Dit leidt tot CGRP vrijzetting en een toename in huiddoorbloeding die kan gemeten worden door gebruik te maken van Laser Doppler perfusie beeldvorming. In een eerste studie bij twaalf gezonde mannen wordt de adequate capsaïcine dosis gezocht die leidt tot een sterke en reproduceerbare toename van de huiddoorbloeding (**Hoofdstuk 3**). Bovendien bestuderen we of de plaats waarop capsaïcine wordt aangebracht op de onderarm invloed heeft op de toename in huiddoorbloeding. Na dertig minuten resulteert 1000 µg capsaïcine in een reproduceerbare toename van de huiddoorbloeding in de proximale onderarm bij gezonde vrijwilligers.

In een daaropvolgende studie wordt de reproduceerbaarheid van de toename in huiddoorbloeding geëvalueerd door 1000 µg capsaïcine aan te brengen op beide proximale onderarmen van 21 gezonde mannen. De reproduceerbaarheid van de capsaïcine respons wordt hierbij per vrijwilliger vergeleken tussen beide onderarmen (inter-arm reproduceerbaarheid) en tussen twee opeenvolgende studieperioden (inter-periode reproduceerbaarheid). Bij de reproduceerbaarheidanalyse werd enkel rekening gehouden met de gegevens van de 11 vrijwilligers waarvan de huiddoorbloeding met ≥ 100%

toenam na het aanbrengen van capsaïcine (i.e. responders). Uit deze analyse blijkt dat de inter-arm reproduceerbaarheid van de toename in huiddoorbloeding na het aanbrengen van capsaïcine op de onderarm quasi perfect is. Bovendien kunnen we op basis van deze reproduceerbaarheidgegevens berekenen hoeveel proefpersonen nodig zijn om een verschil in de toename van de huiddoorbloeding na het aanbrengen van capsaïcine te kunnen detecteren tussen beide onderarmen.

In een daaropvolgende studie, bestuderen we welke moleculaire spelers bij de mens verantwoordelijk zijn voor de toename in huiddoorbloeding na het aanbrengen van capsaïcine (**Hoofdstuk 4**). Om de betrokkenheid van CGRP na te kijken dienen we de CGRP receptor antagonist $CGRP_{8-37}$ toe via de arteria brachialis van één arm. Op deze manier wordt de door capsaïcine veroorzaakte toename in huiddoorbloeding in de andere, niet-behandelde, arm gebruikt als controle en kan het effect van een CGRP receptor antagonist op de capsaïcine respons worden geëvalueerd. Op een vergelijkbare manier wordt ook de betrokkenheid van stikstofmonoxide (NO) en prostaglandines bij de capsaïcine respons onderzocht. Hiertoe worden respectievelijk de niet-selectieve NO synthase remmer, N^G-monomethyl-L-arginine monoacetate (L-NMMA), en de cyclo-oxygenase remmer, indomethacine, intra-arterieel toegediend in één van beide armen. De rol van substance P (SP) wordt bestudeerd door per orale toediening van de krachtige SP antagonist, aprepitant. De capsaïcine-geïnduceerde vaatverwijding wordt hiertoe vóór toediening van aprepitant gemeten op de linker arm en 4h na toediening (T_{max}) op de rechterarm. De belangrijkste conclusie van deze studie is dat capsaïcine-geïnduceerde vasodilatie in de huid van de mens grotendeels wordt geremd door $CGRP_{8-37}$, maar niet door L-NMMA, aprepitant of indomethacine. Hieruit leiden we logischerwijze af dat CGRP een belangrijke rol speelt in de toename van de huiddoorbloeding na het aanbrengen van capsaïcine. We stellen tevens vast dat $CGRP_{8-37}$ geen invloed heeft op de huiddoorbloeding na het aanbrengen van placebo. Dit geeft aan dat CGRP geen rol speelt bij de handhaving van de normale huiddoorbloeding in rust. Het hier voorgestelde model laat toe om de werkzaamheid van CGRP receptor antagonisten te evalueren bij een kleine groep gezonde proefpersonen en kan zo helpen bij het onderzoek naar een klinische werkzame dosis.

In **Deel I** van het proefschrift hebben we tevens de vasculaire effecten en veiligheid van CGRP receptor antagonisten geëvalueerd door gebruik te maken van niet-invasieve technieken waarmee in de klinische farmacologie structurele en functionele arteriële eigenschappen worden gemeten. Uit dierexperimenteel onderzoek blijkt immers dat de toediening van nitroglycerine (NTG), dat op grote schaal gebruikt wordt bij de behandeling van cardiale ischemie (bv. angina pectoris), leidt tot het vrijzetten van CGRP in het cardiovasculaire systeem wat onder andere resulteert in vaatverwijding ter hoogte van de kransslagaders. Daarom wensen wij *in vivo* bij de mens te onderzoeken welk effect CGRP receptor antagonisten hebben op de door NTG veroorzaakte vaatverwijding (**Hoofdstuk 5**). Hiertoe, voeren we een studie uit bij 22 gezonde mannen en beoordelen de vasculaire veranderingen met behulp van niet-invasieve technieken zoals ultrageluidonderzoek en applanatie tonometrie. Wanneer NTG wordt toegediend na een hoge klinische dosis van de krachtige CGRP receptor antagonist, telcagepant, is er geen klinisch relevant effect op de door NTG geïnduceerde hemodynamische veranderingen bij gezonde mannelijke vrijwilligers. Er is tevens geen meetbaar vaatvernauwend effect van telcagepant als dusdanig. Deze bevindingen ondersteunen het gunstige

cardiovasculair veiligheidsprofiel van CGRP receptor antagonisten, vooral wanneer men de vasculaire effecten vergelijkt met deze van de bestaande triptanen.

In **Deel II** van dit proefschrift, bestuderen wij of de activiteit van NOS een rol speelt in de pathogenese van migraine. Hiertoe meten we een aantal biomerkers die systemische NO productie reflecteren. Het meten van biomerkers in patiënten wordt in de klinische farmacologie vaak gebruikt om nieuwe behandelingsstrategieën te identificeren. Dezelfde biomerkers worden in een later stadium dan vaak als "surrogaat" gebruikt om snel de klinische werkzaamheid van nieuwe geneesmiddelen te evalueren. Dat NO een rol speelt bij de ontwikkeling van migraine is reeds meermaals aangetoond. Zo lokken NO-donoren in migraine patiënten laattijdig hoofdpijn uit met dezelfde karakteristieken als migrainehoofdpijn. De niet-selectieve NOS remmer, L-NMMA, is bovendien bewezen effectief voor de behandeling van acute migrainehoofdpijn.

In een eerste studie, onderzoeken we of er bij migrainepatiënten meer NO wordt aangemaakt na toediening van L-arginine dan bij gezonde vrijwilligers (**Hoofdstuk 6**). Dit zou immers wijzen op een hyperactiviteit van de NOS enzymen. Wij voeren eerst een studie uit bij 12 gezonde vrijwilligers om de reproduceerbaarheid van de toename in systemische NO productie te evalueren na het toedienen van een oplaaddosis L-arginine. De reproduceerbaarheid hiervan blijkt voldoende om een klinisch relevant verschil in NO productie tussen 20 hoofdpijnvrije migrainepatiënten en 20 gezonde vrijwilligers te kunnen meten. Om de toename in systemische NO productie te kwantificeren meten we een aantal biomerkers die geassocieerd zijn met de omzetting van L-arginine in NO (i.e. nasale en uitgeademde NO, plasma L-citrulline en L-ariginine en urinaire excretie van nitraten en cyclisch guanosine monofosfaat (cGMP)). De resultaten van deze studie wijzen niet in de richting van een toegenomen NOS activiteit bij migrainepatiënten. Integendeel, bij migrainepatiënten is er zelfs een kleinere toename in de plasma-L-citrulline concentraties en de urinaire excretie van nitriet/nitraat en cGMP dan bij gezonde vrijwilligers. Een mogelijke verklaring hiervoor is een lagere endotheliale NOS (eNOS) activiteit als gevolg van endotheeldysfunctie. Aangezien recent heel wat studies verschenen die wijzen in de richting van een verminderde activiteit van eNOS bij migrainepatiënten, bevestigen deze resultaten de idee als zou migraine een aandoening zijn van het endotheel.

De tweede studie in **Deel II** van het proefschrift onderzoekt bij 15 migrainepatiënten de farmacokinetische en farmacodynamische eigenschappen van GW273629, een krachtige remmer van het induceerbaar NOS (iNOS). GW273629 wordt zowel bestudeerd in hoofdpijnvrije omstandigheden en tijdens een acute hoofdpijnaanval om het effect van de migraineaanval te evalueren (**Hoofdstuk 7**). Ook in deze studie maken we gebruik van een aantal biomerkers om een idee te krijgen van de systemische aanmaak van NO (i.e. nasale en uitgeademde NO, plasma nitraat en plasma-3-nitrotyrosine concentraties). Op deze manier is het mogelijk te bestuderen welke effecten zowel acute migraine hoofdpijn als GW273629 hebben op de systemische aanmaak van NO. Onze studie toont duidelijk aan dat tijdens een migraineaanval de vroege absorptie van oraal toegediend GW273629 vermindert. Dit heeft evenwel geen invloed op het tijdsverloop van de vermindering van uitgeademd NO ten gevolge van iNOS inhibitie. Er is met andere woorden een duidelijke invloed van migraine hoofdpijn op de famacokinetische kenmerken van GW273629, maar dit heeft geen farmacodynamische weerslag. Omdat sommige biomerkers voor NO aanmaak hoger lagen tijdens

een acute migraine aanval dan wanneer patiënten hoofdpijnvrij waren, lijken de resultaten van deze studie aan te geven dat migraine hoofdpijn geassocieerd is met overmatige systemische NO aanmaak. Daartegenover staat dat de sterke inhibitie van iNOS door GW273629, die duidelijk wordt bevestigd door NO metingen in de uitgeademde lucht, geen enkel gunstig effect had op migraine hoofdpijn in deze kleine niet geblindeerde studie. Recentere grotere klinische studies konden evenmin een klinische werkzaamheid van iNOS remmers aantonen in zowel de profylactische als acute behandeling van migraine hoofdpijn.

In **Deel III** van het proefschrift, exploreren we de mogelijkheden die moderne beeldvormingstechnieken bieden om met migraine geassocieerde veranderingen in het endocannabinoïd systeem op te sporen. Dankzij de ontwikkeling van radioliganden voor positron emissie tomografie (PET) is het immers mogelijk de beschikbaarheid van de type 1 cannabis receptor (CB1R) *in vivo* bij de mens te bestuderen. Wij vergelijken de CB1R beschikbaarheid in het centraal zenuwstelsel van 20 vrouwelijke migrainepatiënten en 18 gezonde vrouwen. De beschikbaarheid van CB1R was in alle delen van hersenen duidelijk toegenomen bij migrainepatiënten, maar de toename was het meest uitgesproken in de cingulo-frontale cortex en het limbisch systeem. Van deze hersengebiedengebieden wordt aangenomen dat ze een rol spelen bij pijntransmissie door neerwaartse modulatie vanuit de cortex op de lager gelegen neuronale netwerken. Onze studie bevestigt hiermee resultaten van eerdere studies die suggereren dat een dysfunctie van het endocannabinoïd systeem mee aan de basis ligt van het ontstaan van migrainehoofdpijn en ook een rol zou spelen bij het evolueren naar chronische hoofdpijnaandoeningen. Ook deze studie illustreert de verscheidenheid van technieken die in de klinische farmacologie worden aangewend om nieuwe behandelingstrategieën te toetsen. De observatie dat het endocannabinoïd systeem ontregelt is bij migrainepatiënten kan als aanzet dienen voor verder onderzoek naar de rol van dit systeem in de pathofysiologie van migraine en kan op die manier in de toekomst nieuwe medicamenteuze behandelingsperspectieven bieden.

Algemene conclusie

In dit proefschrift wordt aangetoond dat de klinische farmacologie, door gebruik te maken van geavanceerde technieken, kan bijdragen tot het snel identificeren en evalueren van nieuwe medicamenteuze therapieën voor migraine.

Hoewel preklinisch onderzoek een essentiële hoeksteen blijft in onze zoektocht naar efficiëntere en veiligere migraine behandelingen, geven onze bevindingen duidelijk aan dat er grote verschillen bestaan tussen mens en dier op het vlak van mediatoren die verondersteld worden een belangrijke rol te spelen bij het ontstaan van migrainehoofdpijn. Bovendien is migraine een bij uitstek menselijke ervaring waardoor het ontwikkelen en valideren van dierenmodellen in dit domein uiterst moeilijk is.

De klinische ontwikkeling van CGRP receptor antagonisten is reeds ver gevorderd en telcagepant zal dan ook in de nabije toekomst op de markt komen voor de behandeling van acute migraine hoofdpijn. De toekomst van NOS remmers en modulatoren van het endocannabinoïd systeem is nog onzeker. Niet-selectieve NOS inhibitie is dan wel effectief bij het bestrijden van migrainehoofdpijn, maar heeft

een onaantrekkelijk cardiovasculair veiligheidsprofiel. Onze bevindingen suggereren dat nNOS een rol speelt in de pathofysiologie van migraine maar op dit moment zijn er nog steeds onvoldoende klinische gegevens om dit hard te maken. Het remmen van eNOS en iNOS lijkt geen goede behandelingstrategie.

Hoewel de mogelijke betrokkenheid van het endocannabinoïd systeem bij de pathofysiologie van migraine nieuwe behandelingsperspectieven biedt, is voorzichtigheid geboden. De recente terugtrekking van de cannabinoid receptor blokkers toont immers duidelijk aan dat farmacotherapeutische interventies in het cannabinoïd systeem aanleiding kunnen geven tot onaanvaardbare nevenwerkingen.

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Curriculum Vitae

Bart Van der Schueren started his medical training in 1995 at the Faculty of Medicine of the Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium. He completed his medical studies at the Catholic University of Leuven (KULeuven), Leuven, Belgium, and became a Medical Doctor magna cum laude in 2002. During his medical training the author worked as a student researcher at the departments of Morphology of the Catholic University of Leuven (under supervision of Prof. Dr. K. Geboes), Experimental Therapy of the Netherlands Cancer Institute (under supervision of Prof. Dr. A. Begg) and Molecular and Cardiovascular Research of the Catholic University Leuven (under supervision of Prof. Dr. P. Carmeliet), where he studied vascular perfusion in tumours. After graduation, he started a training in Internal Medicine under supervision of Prof. Dr. J. Fevery and Prof. Dr. W. Peetermans. In 2004, he embarked on a clinical PhD project entitled “New Targets in Migraine Therapy” at the Centre for Clinical Pharmacology, University Hospital Leuven, Belgium, under supervision of Prof. Dr. J. de Hoon and Prof. Dr. K. Van Laere. To support his research, the work received a grant from the Research Foundation-Flanders (FWO-Vlaanderen). In addition he received unrestricted grants from GlaxoSmithKline (GSK) and Merck Imaging group. During his stay at the Centre for Clinical Pharmacology he completed his training in clinical pharmacology and became registered as a clinical pharmacologist in the Netherlands in June 2008. In September 2008, the author started his residency in Endocrinology at the University Hospital Leuven, headed by Prof. Dr. R. Bouillon.

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List of abbreviations

5-HT	5-hydroxytriptamine, serotonin
ADBP	Aortic Diastolic Blood Pressure
AIx	Augmentation Index
ANOVA	Analysis of Variance
ASBP	Aortic Systolic Blood Pressure
AU	Arbitrary Units
AUC	Area Under the Curve
BAD	Brachial Artery Diameter
BMI	Body Mass Index
BP	Blood Pressure
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
cAMP	cyclic Adenosine MonoPhosphate
CB1R	the type 1 Cannabinoid Receptor
CB2R	the type 2 Cannabinoid Receptor
CCC	Concordance Correlation Coefficient
cGMP	cyclic Guanosine MonoPhosphate
CGRP	Calcitonin Gene-Related Peptide
CI	Confidence Interval
CLR	Calcitonin-Like Receptor
CSD	Cortical Spreading Depression
DBF	Dermal Blood Flow
DBP	Diastolic Blood Pressure
eNO	exhaled Nitric Oxide
eNOS	endothelial Nitric Oxide Synthase
FHM	Familial Hemiplegic Migraine
GPCR	G-Protein-Coupled Receptor
HR	Heart Rate
IA	Infused Arm
IHS	International Headache Society
iNOS	inducible Nitric Oxide Synthase
L-NMMA	N^G-MonoMethyl-L-Arginine MonoAcetate
MAP	Mean Arterial Pressure
MIDAS	Migraine Disability Assessment Score
MNI	Montreal Neurological Institute
MR	Magnetic Resonance
NIA	Non-Infused Arm
NKA	NeuroKinin A

nNO	nasal Nitric Oxide
nNOS	neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NTG	NiTroGlycerin
PACAP-38	Pituitary Adenylate Cyclase Activating Peptide
PET	Positron Emission Tomgraphy
PPB	Parts Per Billion
PWF	Pressure Waveform
RAMP	Receptor Activity-Modifying Protein
RC	Repeatability Coefficient
SBP	Systolic Blood Pressure
SD	Standard Deviation
SEM	Standard Error of the Mean
SP	Substance P
SPM	Statistical Parametric Mapping
SSC	Sample Size Calculation
SUV	Standardized Uptake Values
TGVS	TriGeminoVascular System
TRPV1	Transient Receptor Potential Vanilloid type 1 Receptor
VIP	Vasoactive Intestinal Polypeptide
VOI	Volume of Interest
WCV	Within-subject Coefficient of Variation