Microvasculature, the Trigeminal System and Migraine;

A focus on female sex hormones

Khatera Ibrahimi

Microvasculature, the Trigeminal System and Migraine; A focus on female sex hormones

Khatera Ibrahimi

Microvasculature, the Trigeminal System and Migraine;

A focus on female sex hormones

Thesis, Erasmus University, Rotterdam. With summary in English and Dutch.

ISBN 978-94-6203-933-9

Cover: Cure, by Keith Negley

Printing: CPI Koninklijke Wöhrmann

Microvasculature, the Trigeminal System and Migraine;

A focus on female sex hormones

Microvasculatuur, het trigeminale systeem en migraine;
een focus op vrouwelijke geslachtshormonen

Proefschrift

ter verkrijging van de graad van doctor aan de

Erasmus Universiteit Rotterdam

op gezag van de

rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 14 oktober 2015 om 11:30 uur

door

Khatera Ibrahimi

geboren te Kabul, Afghanistan

Erasmus University Rotterdam

Erasmus

Promotiecommissie

Promotor:	Prof.dr. A.H.J. Danser
Overige leden:	Prof.dr. J.S.E. Laven
	Prof.dr. ir. A.P.N. Themmen
	Prof.dr. C.M. Villalón
Copromotoren:	Dr. A. Maassen van den Brink
	Dr. A.H. van den Meiracker

The research described in this thesis was supported by:

The Netherlands Organization for Scientific Research 14 (NWO) [VIDI 917.11.349]

The Dutch Brain Foundation

Printing of this thesis was generously supported by:

Unilever R&D Vlaardingen Nederland

Perimed AB Järfälla Sweden

Nederlandse Hoofdpijn Vereniging

Contents

CHAPTER 1

General Introduction

Migraine impact and clinical presentation

Migraine is a paroxysmal neurovascular disorder with a prevalence of 15% that affects a large proportion of the general population.[1] It is a highly debilitating disease, which has been ranked by the WHO as the 3rd disabling disease in women and the 7th disabler when both sexes are considered together.[2] The 2 most prevalent subtypes of migraine are migraine without aura (MO) and migraine with aura (MA). Patients with MO have migraine attacks that can last up to 72 hours, suffering from headache accompanied by autonomic nervous system dysfunctions such as photophobia, phonophobia and nausea. The attacks in MA patients are similar to those in MO patients, but preceded by an aura phase. During this phase, which can have a duration of 5-60 min, patients mostly experience visual disturbances, although paresthesia, speech disturbance and motor symptom can also occur.[3]

Migraine pathophysiology and CGRP

The pathophysiology of migraine is poorly understood, although there is consensus on the trigeminovascular system as the key structure being involved. Migraine is considered to be a result of the activation of the trigeminovascular system with the primary dysfunction located in brainstem centers regulating vascular tone and pain sensation.[4] During a migraine attack the vasodilatory neuropeptide calcitonin gene-related peptide (CGRP) is released from trigeminal sensory nerves.[5, 6] The resulting vasodilatation of meningeal arteries and activation of the nociceptive system are thought to be the basis of the headache during migraine.[7] The key role of CGRP is emphasized by the finding that plasma concentrations of CGRP are elevated during migraine headache and normalize after treatment with triptans (specific acute anti-migraine drugs) in parallel with alleviation of headache.[8] In view of the key role of CGRP in the pathophysiology of migraine, and evidence that blocking CGRP aborts migraines, CGRP and its receptors have now become a novel drug target for anti-migraine therapy.[9]

Migraine and female sex

Migraine is a predominantly female disease, as it affects women much more than men with a female-to-male ratio of 3:1.[10, 11] Especially hormonal milestones accompanied by fluctuations in estrogen levels such as menarche, pregnancy and menopause seem to have vast effects on migraine prevalence and frequency. The onset of migraine in women usually coincides with menarche and a close relation between migraine occurrence and the menstrual cycle remains during the reproductive years.[12] Approximately 50% of women with migraine are affected by menstrually-related migraine (MRM),[13] i.e. MO that occurs on day 1±2 of the menstrual cycle in at least 2 of 3 consecutive menstrual cycles with additional attacks with or without aura that can occur at other times of the month.[14] Migraine frequency drops during pregnancy,[15, 16] particularly during the second and third trimester, when serum levels of estradiol and progesterone are much higher than peak levels during the menstrual cycle. Postpartum, migraine reoccurs, but less in lactating women. During perimenopause, changes in migraine prevalence have been reported.[17] Major fluctuations in estrogen

levels take place during perimenopause, ultimately leading to dropping levels.[18] The prevalence of migraine headaches during this period seems to be higher in patients who previously had a history of MRM and premenstrual syndrome.[19, 20]

Vascular disease and migraine

Besides the considerable migraine disease burden, migraineurs have an increased ischemic stroke risk. This risk is highest in young female MA patients using oral contraceptives.[21] Imaging studies have confirmed the association between MA and ischemic stroke, by showing an increased risk of silent infarct-like lesions in MA patients.[22] Deep white matter lesions are also increased in women with MO and MA. The risk for both types of lesions increases with rising attack frequency.[22] Migraine has recently been identified as a major cardiovascular risk factor in women (IR = 7.9; 6.2-10.0).[23] A recent meta-analysis of observational studies indicated an increased risk of myocardial infarction (pooled adjusted effect estimate 1.33, 95% confidence interval 1.08–1.64; P = 0.007) and of angina (pooled adjusted effect estimate 1.29, 95% confidence interval 1.17–1.43; P < 0.0001) in migraineurs compared to non-migraineurs. Migraine seems to be an even larger risk factor than well-known risk factors such as diabetes, obesity or smoking.[23] Interestingly, systemic vascular dysfunction in migraine patients, expected based on the increased cardiovascular disease risk, has not been confirmed.[24]

Migraine and monogenic diseases

The rare monogenic microvascular disorders comorbid with migraine, RVCL (autosomal dominant Retinal Vasculopathy with Cerebral Leukodystrophy, caused by a mutation in the *TREX1* gene, encoding for a DNA exonuclease) and CADASIL (Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy, caused by mutations in the *NOTCH3* gene), provide a unique opportunity to study not only the genetic mechanisms involved in migraine but also the vascular mechanisms. In RVCL patients primarily the microvessels in the brain and retina are affected and patients develop retinopathy and in the endstage of the disease, cerebral mass lesions.[25] MO, with 59% of the patients affected, is a prominent feature of RVCL.[26] Endothelial dysfunction in RVCL patients has been recently identified.[27] Approximately 30% of CADASIL patients have MA and migraine is often the presenting symptom.[28] White matter hyperintensities and lacunar infarcts are the most prominent features of CADASIL. The *NOTCH3* gene encodes a cell surface protein that in human adult tissue is only expressed in vascular smooth muscle cells.[29, 30] Functional tests have revealed impaired smooth muscle cell relaxation in skin resistance vessels of CADASIL patients.[27] CADASIL and RVCL mouse models have been developed to further investigate both diseases and their association with migraine.[31, 32]

References

1. Stovner LJ, Andree C. Prevalence of headache in Europe: a review for the Eurolight project. The journal of headache and pain 2010;11:289-299.
2. Steiner TJ, Stovner LJ, Birbeck GL. Migraine: the seventh disabler. Cephalalgia : an international journal of headache 2013;33:289-290.
3. Eriksen MK, Thomsen LL, Olesen J. New international classification of migraine with aura (ICHD-2) applied to 362 migraine patients. European journal of neurology : the official journal of the European Federation of Neurological Societies 2004;11:583-591.
4. Pietrobon D, Moskowitz MA. Pathophysiology of migraine. Annu Rev Physiol 2013;75:365-391.
5. Edvinsson L, Villalón CM, MaassenVanDenBrink A. Basic mechanisms of migraine and its acute treatment. Pharmacol Ther 2012;136:319-333.
6. Villalón CM, Olesen J. The role of CGRP in the pathophysiology of migraine and efficacy of CGRP receptor antagonists as acute antimigraine drugs. Pharmacol Ther 2009;124:309-323.
7. Ho TW, Edvinsson L, Goadsby PJ. CGRP and its receptors provide new insights into migraine pathophysiology. Nature reviews Neurology 2010;6:573-582.
8. Goadsby PJ, Edvinsson L. The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Ann Neurol 1993;33:48-56.
9. Karsan N, Goadsby PJ. Calcitonin gene-related peptide and migraine. Current opinion in neurology 2015;28:250-254.
10. Leonardi M, Steiner TJ, Scher AT, Lipton RB. The global burden of migraine: measuring disability in headache disorders with WHO's Classification of Functioning, Disability and Health (ICF). The journal of headache and pain 2005;6:429-440.
11. Bigal ME, Lipton RB. The epidemiology, burden, and comorbidities of migraine. Neurologic clinics 2009;27:321-334.
12. Lipton RB, Stewart WF, Diamond S, Diamond ML, Reed M. Prevalence and burden of migraine in the United States: data from the American Migraine Study II. Headache 2001;41:646-657.
13. Martin VT. Menstrual migraine: a review of prophylactic therapies. Curr Pain Headache Rep 2004;8:229-237.
14. Headache Classification Committee of the International Headache S. The International Classification of Headache Disorders, 3rd edition (beta version). Cephalalgia : an international journal of headache 2013;33:629-808.
15. Sances G, Granella F, Nappi RE, et al. Course of migraine during pregnancy and postpartum: a prospective study. Cephalalgia : an international journal of headache 2003;23:197-205.
16. Somerville BW. A study of migraine in pregnancy. Neurology 1972;22:824-828.
17. Research on the menopause in the 1990s. Report of a WHO Scientific Group. World Health Organ Tech Rep Ser 1996;866:1-107.
18. Yamada M, Soda M, Fujiwara S. Follicle-stimulating hormone and oestradiol levels during perimenopause in a cohort of Japanese women. Int J Clin Pract 2008;62:1623-1627.
19. Mattsson P. Hormonal factors in migraine: a population-based study of women aged 40 to 74 years. Headache 2003;43:27-35.
20. Wang SJ, Fuh JL, Lu SR, Juang KD, Wang PH. Migraine prevalence during menopausal transition. Headache 2003;43:470-478.
21. Etminan M, Takkouche B, Isorna FC, Samii A. Risk of ischaemic stroke in people with migraine: systematic review and meta-analysis of observational studies. Bmj 2005;330:63.
22. Kruit MC, van Buchem MA, Hofman PA, et al. Migraine as a risk factor for subclinical brain lesions. Jama 2004;291:427-434.
23. Kurth T, Bubes V, Buring JE. Relative Contribution of Migraine with Aura to Cardiovascular Disease Occurrence in Women. . Neurology 2013;80:S40.001.
24. Sacco S, Ripa P, Grassi D, et al. Peripheral vascular dysfunction in migraine: a review. The journal of headache and pain 2013;14:80.
25. Stam AH, Haan J, van den Maagdenberg AM, Ferrari MD, Terwindt GM. Migraine and genetic and acquired vasculopathies. Cephalalgia : an international journal of headache 2009;29:1006-1017.
26. Terwindt GM, Haan J, Ophoff RA, et al. Clinical and genetic analysis of a large Dutch family with autosomal dominant vascular retinopathy, migraine and Raynaud's phenomenon. Brain : a journal of neurology 1998;121 (Pt 2):303-316.
27. Vermeersch S, Stam AH, Zielman R, et al. Trex1-mutation associated with endothelial dysfunction in RVCL patients. Cephalalgia : an international journal of headache 2011;31:13.
28. Dichgans M, Mayer M, Uttner I, et al. The phenotypic spectrum of CADASIL: clinical findings in 102 cases. Ann Neurol 1998;44:731-739.
29. Joutel A, Andreux F, Gaulis S, et al. The ectodomain of the Notch3 receptor accumulates within the

cerebrovasculature of CADASIL patients. The Journal of clinical investigation 2000;105:597-605.
30. Joutel A, Corpechot C, Ducros A, et al. Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. Nature 1996;383:707-710.
31. Eikermann-Haerter K, Yuzawa I, Dilekoz E, Joutel A, Moskowitz MA, Ayata C. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy syndrome mutations increase susceptibility to spreading depression. Ann Neurol 2011;69:413-418.
32. Klever RR, Rutten JW, Labruijere S, et al. Novel Transgenic Mouse Models for Monogenic Cerebral Small Vessel Diseases Related to Migraine. Cephalalgia : an international journal of headache 2013;33:972-972.

CHAPTER 2

Aims of this thesis

Aims of this thesis

In **Part I** the focus is on experimental models of migraine, that are applied in pharmacological as well as in pathophysiological studies. Calcitonin gene-related peptide is the key neuropeptide in migraine pathophysiology. CGRP is being extensively researched, especially as a novel anti-migraine drug target. In **Chapter 3** we review existing experimental models that can be applied to study the effects of the novel CGRP receptor antagonists and the recently developed antibodies directed against CGRP and its receptor. In **Chapter 4** we comment on an existing experimental model to demonstrate its limitations with regard to distinguishing between structures important to migraine, like the intracranial part of the middle meningeal artery. In **Chapter 5** we describe our experimental trigeminal nerve-mediated vasodilatation model. We developed this model to study trigeminovascular pathophysiological and pharmacological mechanisms of migraine. In **Chapter 6** we investigate the effects of the anti-migraine drug sumatriptan with this model, to validate it as a biomarker for studies on future anti-migraine drugs.

Besides its direct burden, migraine has recently also been identified as a major independent risk factor for cardiovascular disease. Yet, cross-sectional studies investigating the vascular dysfunction in migraine patients have reported conflicting results. This may relate to the fact that the experimental models used to identify vascular dysfunction were diverse and often limited. Therefore, in **Chapter 7** we compared different measures of vascular function to assess their reproducibility and their usefulness to identify endothelial dysfunction. In **Chapter 8** we applied one of these measures, the post-occlusive reactive hyperemia (PORH), to characterize the microvascular function in a knock-in mouse (V235fs KI mice) model of migraine.

In **Part II** we evaluate the role of female sex hormones in migraine. The prevalence of migraine is much higher in women than in men. Hormonal milestones in women are accompanied by changes in frequency and prevalence of migraine. In **Chapter 9** we review the effect of the perimenopausal period on migraine. In **Chapter 10** we have performed a detailed pathophysiological study to investigate the influence of the menstrual cycle on trigeminovascular activation in patients with menstrually-related migraine, making use of the trigeminovascular model described in Chapter 5. In **Chapter 11** we performed a similar study on the human forearm, allowing a comparison of the forehead findings with peripheral findings.

Part I

CHAPTER 3

Discovery techniques for calcitonin gene-related peptide receptor antagonists for potential antimigraine therapies

Based on: Expert Opin Drug Discov, 2013, 8:11, 1309-1323

S. Labruijere, K. Ibrahimi, K.Y. Chan & A. MaassenVanDenBrink

Abstract

Introduction: Calcitonin gene-related peptide (CGRP) exerts a key function in migraine pathophysiology through the trigeminovascular system. Influencing this system via CGRP receptor antagonists seems to be an important new option in treating migraine attacks. To characterize new compounds, models are used to study the vascular effects as well as their effects on the central nervous system.

Areas covered: The authors review the clinical trials and many different *in vitro* and *in vivo* experimental models that have been used to investigate effects and side effects in animals, healthy subjects and patients. These experimental models are essential, not only in characterizing new CGRP receptor antagonists, but also to get more insight into the pathophysiological mechanisms behind migraines.

Expert opinion: Although triptans were a major breakthrough in migraine treatment, they are not effective for every patient and contraindicated in patients with cardiovascular disease. There is still a demand for other acute antimigraine acting drugs with CGRP receptor antagonists being the most promising candidates. CGRP plays a role in protection against ischemia, but CGRP receptor antagonists do not seem to affect this protection to a harmful extent, when used incidentally as acute antimigraine treatment. In order for drug specificity to be increased, the site of action needs to be identified; this consequently may lead to a decrease in dosing with fewer side effects.

1. Introduction

Migraine is a disabling neurovascular disorder, characterized by a unilateral throbbing headache that lasts from 4 to 72 hours. The headache is often accompanied by nausea, vomiting, phonophobia and photophobia and worsens by physical exercise.[1] There are different types of migraine where migraine with aura (MA) and migraine without aura (MO) are the most common forms.[2] A migraine attack can have different phases, the prodromal or premonitory phase, the aura phase, the headache phase and the postdromal phase. During the premonitory phase, patients have often symptoms like yawning, nausea and craving for food. The migraine aura is thought to be caused by a cortical spreading depression (CSD). During the postdromal phase, which can last hours to days after the resolution of the headache, patients are often tired.[3] Migraine has a worldwide prevalence of 10% and a prevalence of 15 – 20% in the Western Countries.[4] This shows that migraine is a major health problem, which also involves a high financial burden to society. Migraine pathophysiology is not clearly understood, but the trigeminovascular system is thought to play an important role in it.[5]

Apart from the more general analgesics there are different medicines available to treat migraine. These drugs can be divided into two types: i) drugs aimed at abolishing migraine attacks and ii) drugs aimed at preventing migraine attacks. The latter group is prescribed to patients that have frequent attacks and in which the acute drugs do not have sufficient effect, in order to reduce the severity or the number of attacks.[6]

The first drugs used to treat acute migraine attacks were the ergot alkaloids, which were discovered in the beginning of the 20th century. Ergot alkaloids are

vasoconstrictors that act by binding of 5-hydroxytryptamine (5-HT) receptors, α-adrenoceptors and dopamineD_2 receptors. Due to their multiple receptor binding properties these drugs have many side effects.[7] In the 1990s of the former century, the triptans were introduced. These drugs have shown to be as effective as the ergot alkaloids in treating migraine attacks, but with less sideeffects due to their specificity for 5-$HT_{1B/1D}$ receptors.[8, 9] Triptans are not effective in all migraineurs and contra-indicated in patients with cardiovascular disease, so it is important that more drugs are developed to abolish migraine attacks.[10] In the early nineties, Edvinsson and Goadsby discovered an increase in calcitonin gene related peptide (CGRP) levels in plasma of the jugular vein during a migraine attack and the CGRP levels normalized after treatment with triptans.[11, 12] CGRP is a potent vasodilator and is widely present in the brain (especially the trigeminal system) and the cardiovascular system together with its receptor, which is composed of three parts: receptor activity modifying protein 1 (RAMP1), calcitonin-like receptor (CLR) and receptor component protein (RCP).[13, 14]

A migraine attack is thought to start in the brainstem due to certain triggers, which have not been exactly identified yet, leading to the release of CGRP at trigeminal nerve endings innervating meningeal arteries.[15] CGRP binds to its receptor that is present on the smooth muscle cell membrane, activating adenylate cyclase, causing an increase in intracellular cyclic AMP (cAMP). This leads to activation of different signalling pathways and vasodilatation, where activation of nitric oxide synthase (NOS), leading to an increase in nitric oxide (NO),[16] may also be involved. The vasodilatation is thought to affect sensory neurons which, in turn, activate the trigeminal system, passing on sensory information to higher brain regions, but also leading to other neurological symptoms as phono- and photophobia.[17, 18]

The first potent and selective CGRP receptor antagonist was olcegepant and showed to be effective in the acute treatment of migraine attacks similar to the triptans, but without its cardiovascular side effect profile.[19] Because of its low oral bioavailability, other CGRP receptor antagonists with improved oral bioavailability were developed.[20, 21] Telcagepant was the first improved, orally available CGRP receptor antagonist that was tested in multiple clinical trials, as well as other ones developed later on. These clinical trials are discussed below. It is crucial that various effects and side effects of newly developed CGRP receptor antagonists are characterized to be sure. Furthermore, as migraine is a syndrome involving multiple physiological systems, it is very important to obtain more knowledge about the underlying mechanisms causing migraine. This may eventually lead to more specific treatment for specified migraine patient groups. Since migraine involves multiple organ systems, both vascular and neuronal, there are many different research models, which are used to study different aspects of migraine and antimigraine medication. The aim of this review is to describe the experimental *in vivo* and *in vitro* models that can be used for the discovery and characterization of new CGRP receptor antagonists.

2. Clinical trials with CGRP receptor antagonists

CGRP receptor antagonists have proven to be effective in the acute treatment of migraine in multiple clinical trials (Table 1). Olcegepant (BIBN4096BS) was the first CGRP receptor antagonist to be tested in clinical trials. In phase I trials olcegepant showed good safety and tolerability[19, 22] and adverse events (AE's) reported were only transient and mild paresthesias. In a Phase II trial in 126 migraine patients, olcegepant proved to be an effective drug for the acute treatment of migraine and headache relief after 2 hours was achieved in 66% of the migraineurs.[23] Due to the chemical structure of olcegepant it can only be administered intravenously and is consequently unfeasible in a clinical setting. It was clear that a CGRP receptor antagonist with an improved pharmacokinetic profile was required. Telcagepant (MK-0974) was the first orally bioavailable CGRP receptor antagonist that was discovered. In a Phase II trial, where several doses of telcagepant were compared to rizatriptan, telcagepant proved to be effective in the acute treatment of moderate or severe migraine attacks. Headache relief after 2 hours was achieved in 68% of the migraineurs when treated with the highest dose that was used in this trial (600 mg), compared to 69% when treated with rizatriptan.[24] In Phase III trials telcagepant repeatedly acted effectively as acute treatment of migraine when compared to placebo.[25, 26] However, when raised levels of liver transaminases were detected in a few participants in a Phase II trial (NCT00797667) where telcagepant was assessed as a prophylactic treatment for migraine, the development of telcagepant was suspended.[27] The development of another CGRP receptor antagonist (MK-3207) with higher bioavailability than telcagepant and also effective in the acute treatment of migraine, was discontinued as well after reports of raised levels of liver transaminases.[21, 28] The CGRP receptor antagonist BI 44370 TA has proven to be successful in treatment of acute migraine in a Phase II trial. Headache relief after 2 hours was reached in 27% of the participants for BI 44370 TA (400 mg), compared to in 9% of the participants for placebo and there were very few reports of AE's, with an incidence of $\leq$ 3 %.[29] Finally, in a recent Phase I trial, the CGRP receptor antagonist BMS-927711 showed to be safe and tolerable in eight healthy subjects at single doses up to 1500 mg and multiple doses up to 600 mg. No serious AE's were reported.[30] Though clinical trials provide an excellent opportunity to study the pharmacokinetics and pharmacodynamics of a drug, they provide little information of the underlying mechanisms of action. Consequently, *in vitro* and *in vivo* research models are needed to allow us insight into these mechanisms.

3. Experimental Models

Clinical trials in healthy subjects and patients cannot be performed without prior characterization studies of the potential drug in experimental models. There is no experimental model in which a complete migraine attack can be studied. All available models focus on a specific component of the disease. Different *in vitro* and *in vivo* models are used to investigate effects and side effects of prospective antimigraine drugs (Table 2 and Figure 1).

Table 1. Overview of clinical trials performed with CGRP receptor antagonists.

CGRP receptor antagonist	Administration route	Last trial phase completed (headache relief after 2h)	Antimigraine effective dose	Adverse events reported (>5%)	Status
Olcegepant [23]	i.v	Phase II (66%)	2.5 mg	Mild paresthesias	Discontinued due to low oral availability
Telcagepant [25]	Oral	Phase III (24-27%)	140-400 mg	Dry mouth, nausea, somnolence, dizziness and fatigue	Discontinued due to increased liver enzymes
MK-3207 [28]	Oral	Phase II (36%)	200 mg	Dry mouth, nausea, vomiting, dizziness, fatigue and headache	Discontinued due to increased liver enzymes
BI 44370 TA [29]	Oral	Phase II (27%)	400 mg	none	In development
BMS-927711 [30]	Oral	Phase I (unknown)	unknown	Headache, constipation, dizziness and nausea	In development

3.1 *In vitro* models

Because migraine is a heterogeneous disease and pain is difficult to measure in animals, different *in vitro* models have been developed to study separate components of the disease. Since migraine is a neurovascular disease, various models have been developed that focus on the vasculature. CGRP is a very potent vasodilator[31] so newly developed CGRP receptor antagonists can be studied for their potency in different types of isolated blood vessels. Apart from its effects on the vasculature, CGRP also plays an important role in different brain regions.[14] CGRP has shown to be present in perivascular nerves, but also in the trigeminal system, amygdalae, striatum, colliculi, hypothalamus, cerebellum and brainstem.[13] *In vitro* models focussing on brain tissue can be used to study effects of CGRP receptor antagonists on its neurotransmitter function. In general, *in vitro* models have a number of advantages: i) multiple tissue samples can be studied of the same tissue, making it possible to perform a detailed pharmacological analysis and ii) there is no influence of varying blood pressure, circulating hormones or central and autonomous nervous system, reducing the number of confounding factors when performing pharmacological analyses. However, *in vitro* models are not sufficient to completely predict a therapeutic effect or to predict the pharmacokinetics, including brain penetration, of a compound, and should thus be used complementary to *in vivo* models, which will be described further on in this review.

3.1.1 Isometric tension measurements in blood vessels from experimental animals and humans

Organ baths for isometric tension measurements are experimental models in which vasoconstrictor or vasodilator properties of compounds can be studied in isolated blood vessels. There are different sizes of organ baths, made for blood vessels with different diameter size. Vessel segments with a diameter of 0.05 – 3 mm and a length of 2 – 5 mm can be mounted in wire myographs between two wires. One of the wires is connected to a force transducer and computer and the vessels are stretched to a

Table 2. Overview of experimental models.

Experimental model	In vivo/ In vitro	Description	Advantages	Disadvantages
Isometric tension measurement [31]	*In vitro*	Study of vasoconstrictor or vasodilator properties of compounds on isolated blood vessel segments in organ baths	Multiple segments can be studied at once	No difference can be made between luminal and abluminal side
Pressure myography [35]	*In vitro*	Study of vasoconstrictor or vasodilator properties of compounds on isolated blood vessel segments in organ bath in which compounds can be administrated luminally or abluminally	Effects can be studied on luminal and abluminal side of the blood vessel	Mounting of segments is time consuming so only a few segments can be studied at once
Brain slices [21]	*In vitro*	Binding of CGRP receptor antagonists in the brain can be studied	Binding properties can be studied in detail in the brain	No information on brain access, penetration or functional response
Vascular animal models [37]	*In vivo*	Effects of both exogenous and endogenous CGRP can be studied in living animals	Hemodynamic effects can be studied	No information on brain activity
Intravital microscopy via closed cranial window [40-42]	*In vivo*	Study of dural blood vessel properties via a thinned skull (closed cranial window)	The effect on dural vessel can be studied in a living animal	No information on brain activity
Imaging models [47, 50]	*In vivo*	Real time imaging of blood vessels and brain via MRI, Bold-MRI and PET	Effects in living animals or humans	expensive and time consuming
Laser Doppler perfusion imaging [56]	*In vivo*	Measurement of skin perfusion with laser Doppler scanner	small resistance vessels are studied, non invasive	No measurements of dural blood vessels
Human migraine models [60, 63, 67, 72]	*In vivo*	Infusion of migraine-inducing compounds in either migraine patients or healthy volunteers	Effect of CGRP receptor antagonists can be studied in human	Not all subjects get headache. Headache is not identical to migraine
Fos expression [77]	*In vivo*	Expression of the Fos protein is related to neuronal activation and can be used to study activation of the trigeminovascular system	Central effects of CGRP receptor antagonists can be studied.	Fos is not expressed in all neurons and stimulations necessary to induce Fos expression are sometimes higher than physiological
Electrophysiological recordings [91]	*In vivo*	Measurement of action potential activity in brain of living animal via electrodes	Living animal, physiological	Small area can be measured, no information on vascular system
Transgenic mice models [127]	*In vivo*	Mice in which a gene of another species is inserted and expressed	The physiological role of the CGRP-ergic system can be studied	Difficult to examine migraine symptoms in animals

force that is comparable to a normal blood pressure. Different organ bath studies have been performed on the characterization of the CGRP receptor antagonist olcegepant and telcagepant. As may be expected from their mechanism of action that differs from that of the triptans that are vasoconstrictors, CGRP receptor antagonists have been found to be devoid of vasoconstrictor effects per se.[22, 31] However, it is important to assess their potency to antagonize vasodilator responses to CGRP in isolated blood vessels. In both human and bovine cerebral arteries it was shown that

olcegepant blocked the effect of exogenously administrated CGRP.[32, 33] Furthermore, it was shown in human isolated coronary as well as in cranial arteries that telcagepant, similar to olcegepant, antagonized vasodilator responses to CGRP, and moreover did not induce any contraction or relaxation of the blood vessels (Figure 2).[31, 34] A disadvantage of this model is that the compounds under investigation reach the blood vessels from both the luminal and abluminal side, which is not physiological, because neuropeptides like CGRP are released form nerve afferents on the abluminal side of the artery. In contrast, drugs are transported through the blood and need to cross the blood vessel wall to reach the smooth muscle cells, where for example, CGRP receptors are located.[34] This is especially important in the brain, were the blood brain-barrier is present. Therefore another model was developed, which is described below.

3.1.2 Pressure myography in blood vessels from experimental animals and humans

In the pressure myograph model a segment of a blood vessel is placed over a small glass pipette tip at both sides and securely tied up by small wires. A flow is established through the vessel, leading to a certain pressure, which can be measured. The blood vessel is mounted in an organ bath, with the abluminal side of the vessel completely separated from the luminal side. This model can be used for example to investigate ability of a substance to cross the endothelial layer of a blood vessel (e.g. the blood brain barrier),[35] but also to study receptors that are present on smooth muscle cells and activated by release of proteins through nerve endings, in a more physiological way.[35] It was shown with this model that luminally applied olcegepant could not block the relaxing effect of abluminally applied CGRP in middle cerebral arteries. This indicates that olcegepant possibly cannot cross the blood brain barrier.[36] A disadvantage of this model is that only a few pieces of vessel can be studied at a time resulting in a very time consuming method. Furthermore, because a stable intraluminal pressure is needed, the construction of concentration response curves is difficult from the luminal side of the blood vessel.

3.1.3 Brain slices of experimental animals

Brain slices can be used to study binding of antagonists to a receptor. A study in rhesus monkey brain slices on a new CGRP receptor antagonist, MK-3207, showed a very high density of binding in the meninges, cerebellum and brainstem and a very low binding in the cortex, providing information about the potential central sites of action of the CGRP receptor antagonist.[21] Obviously, results obtained from such methods should be complemented by investigations on the brain penetration of the CGRP receptor antagonists.

3.2 *In vivo* models

Major limitations of the *in vitro* models that are used to study migraine are that they are not physiological and thus cannot be used without complementary *in vivo* studies to predict therapeutic effects and pharmacokinetics of investigational drugs. In the next part, *in vivo* models for migraine that can be used to characterize effects

of CGRP receptor antagonists on vasculature as well as the central nervous system are discussed.

3.2.1 Vascular models in experimental animals

A main part of research performed for the development of the triptans was based on purely vascular models in experimental animals (for review, see Villalón and Gupta[10]). For research on CGRP receptor antagonists, a neuronal component should be implicated as well, since otherwise only effects on exogenously administered CGRP can be studied. Administration of capsaicin induces endogenous CGRP release, which in turn leads to vasodilatation. This model has been applied in anaesthetized vagosympathectomized dogs.[37] An alternative approach to induce endogenous CGRP release is electrical spinal stimulation, for example in pithed rats.[38] These models may well be used to study the hemodynamic effects of CGRP receptor antagonists.

3.2.2 Closed cranial window model in experimental animals

The dilation of blood vessels in the meninges is caused by activation of the trigeminovascular system.[39] The closed cranial window model is a preclinical *in vivo* model, which is based on intravital microscopy for a direct measurement of dural blood vessel diameter through a closed cranial window in anaesthetized rats[40] or mice.[41] In this model the skull is not opened, thus avoiding alteration of vessel reactivity due to brain swelling or change in pressure. Dilation of the dural artery can be induced by intravenous injection of CGRP or capsaicin[40] as well as by electrical stimulation on the surface of the skull.[42] Due to the different stimulations to induce dural vasodilatation, effects of antimigraine drugs can be studied on three levels; postsynaptic at the blood vessels due to exogenous CGRP injection, presynaptic at the transient receptor potential cation channel subfamily V member 1 (TRPV1) channel due to capsaicin injection and in trigeminovascular neurons due to electrical stimulation. Exogenous CGRP exerts its effect by binding to the CGRP receptor located on the vascular smooth muscle cells, capsaicin induces endogenous CGRP release by binding on the TRPV1 channel of TRPV1 channel containing neurons and electrical stimulation induces endogenous CGRP release by depolarization of perivascular trigeminal nerves.

In the past years, different potential antimigraine drugs like the neuronal NOS inhibitor, s-methyl-l-thiocitrulline,[43] the antiepileptic drug, topiramate[44] and different P/Q-,N- and L-type calcium channel blockers[45] have shown to block electrical stimulation-induced neurogenic vasodilatation, but not exogenous CGRP-induced vasodilatation. This suggests that all these compounds inhibit CGRP release from the perivascular nerve via presynaptic blockade of trigeminovascular neurons. In contrast, Chan *et al*. showed that the glutamate receptor antagonist LY466195, which shows antimigraine effects, did not affect CGRP release or its vasodilator effects, suggesting a central mechanism not involving CGRPergic pathways.[46] The cranial window model can be used to study the effect of potential CGRP receptor antagonists on dural vasodilatation *in vivo*. As may be expected from their mechanism

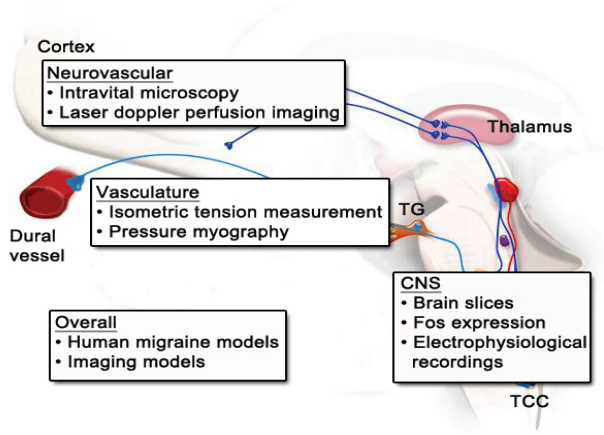


Figure 1. Experimental models in the trigeminovascular system.

of action, CGRP receptor antagonists inhibit vasodilator responses to both exogenous and endogenous CGRP.[41] Furthermore, this model can be used to study differences between exogenous and endogenous CGRP on dural vasodilatation, providing information about (patho)physiological factors affecting either the release of CGRP, or the response mediated by its receptors.

3.2.3 Imaging models in human

In recent years, imaging of the brain and its related vasculature has become important in migraine drug research. Positron emission tomography (PET), a 3D imaging technique that can be used to image functional processes of the body, has successfully been used to study the effects of antimigraine drugs. With PET imaging, rizatriptan was shown to cause a decrease of cerebral blood flow (CBF) and blood volume of around 13%.[47] PET imaging was also used to show that sumatriptan can normalize the migraine-related increase in brain serotonin synthesis.[48] More recently, central CGRP receptor occupancy was investigated with PET imaging in healthy subjects. The investigators showed low receptor occupancy (4 - 10%) after dosing with the lowest clinically effective dose of telcagepant and moderate receptor occupancy (43 - 58%) after dosing with a supra-therapeutic dose of telcagepant. They therefore concluded that central antagonism of CGRP receptors may not be required for a CGRP receptor antagonist to be effective in migraine treatment.[49] Due to the low temporal and spatial resolution of PET imaging, this technique is limited to investigating large areas of the brain. Consequently, magnetic resonance imaging (MRI) and especially, blood oxygenation level-dependent functional MRI (BOLD-fMRI) has become more accepted as the technique to assess altered brain activation and functional connectivity between brain regions.[50] BOLD-fMRI is favored because it is a noninvasive technique with high spatial and temporal resolution.[51] The effect of CGRP infusion and subsequent application of a subcutaneous therapeutic

dose of sumatriptan on the visual cortex BOLD signal response was investigated and no changes in the visual neuronal activity after either CGRP infusion or subcutaneous sumatriptan were detected.[52] Using magnetic resonance angiography, an MRI technique to image blood vessels, Asghar *et al*. showed dilatation of the middle meningeal artery after infusion of CGRP and the reversal of this dilatation by subcutaneous sumatriptan in healthy volunteers.[53] The application of imaging techniques in the study of migraine headache, spontaneous or provoked by infusion of drugs, can provide information on the yet poorly understood underlying mechanism of migraine. In the research of CGRP receptor antagonist, imaging techniques can offer insight into the mode and location of action of the drugs.

3.2.4 Dermal blood flow in response to stimulation with capsaicin as well as electrical stimulation in animal models and humans

Capsaicin, the active ingredient of chili peppers, stimulates the TRPV1 channel and causes release of CGRP from perivascular nerve terminals.[54] Topically applied capsaicin on the human forearm skin is known to increase dermal blood flow (DBF), which can be measured with laser Doppler perfusion imaging.[55] This increase in DBF was inhibited by the CGRP receptor antagonist telcagepant.[56] Endogenous CGRP release can also be accomplished by electrical stimulation.[11, 57] In humans, current-induced vasodilatation can be inhibited by local anesthesia and reduced by desensitization of C-nociceptive fibers.[58] Though not confirmed with the application of a CGRP receptor antagonist, current induced vasodilatation is thought to be mediated mostly by CGRP and substance P. Current-induced vasodilatation may be used to study the release of endogenous CGRP, without the involvement of the TRPV1 channel. A combination of the capsaicin application response and the current-induced vasodilatation provides a suitable model to study CGRP receptor antagonists, especially to elucidate the role of the TRPV1 receptor in migraine headaches.[59] An advantage of this model is that due to its direct translatability between species, this model can be applied in both humans and animal models.

3.2.5 Human migraine models

Human migraine models play an important role in the research for new antimigraine drugs. There are several models in either healthy volunteers or migraine patients. In the latter group, migraine-like attacks can be triggered through infusion of drugs.

3.2.5.1 CGRP infusion

Infusion of CGRP in migraine patients, but not in healthy volunteers can induce delayed (1-12 hours after infusion) headache fulfilling the International Headache Society (IHS) criteria for 67% of MO patients and in 57% of MA patients.[60-62] The mechanism behind migraine after CGRP infusion is still not clear. Recently a study by Asghar *et al*. revealed that CGRP infusion leads to dilation of the middle meningeal artery but not to dilation the middle cerebral artery.[53] It would be interesting to investigate the effects of CGRP receptor antagonists in the CGRP infusion model on the delayed migraine-like headache.

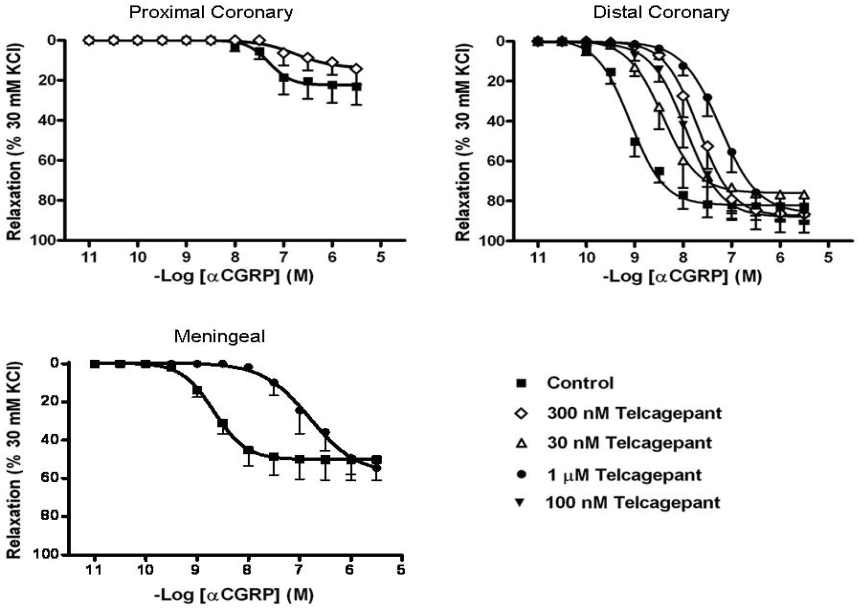


Figure 2. Relaxant effect of αCGRP on human proximal (top left) and distal (top right) coronary arteries and human meningeal arteries (bottom). Concentration–response curves to αCGRP were constructed in the absence or presence of 300 nM telcagepant (proximal), 30 nM to 1 μM telcagepant (distal), and 1 μM telcagepant (meningeal). Values given represent mean ± S.E.M. (n = 4–8). Adapted from: *Chan et al, JPET 2010, 334, 746-752 and Edvinsson et al. Cephalalgia 2010;30:1233-1240.*

3.2.5.2 Glyceryl trinitrate infusion

Glyceryl trinitrate (GTN) infusion in migraine patients also triggers headache attacks, fulfilling the IHS criteria for MO, 4-5 hours after infusion in 50% of patients with MA and in 80% of patients with MO. In healthy volunteers GTN infusion does not cause a delayed migraine attack, although headache during and immediately after infusion does occur.[63, 64] As with the CGRP infusion model, the exact mechanism behind the GTN migraine model is not known. GTN infusion leads to the production of NO, a potent vasodilator, in the body. NO-inhibitors are currently under research as possible new therapy target for migraine.[65] Although more studies are needed to draw categorical conclusions, the GTN migraine model may not be suitable to study the effects of CGRP receptor antagonists, since Tvedskov *et al*. showed that GTN-induced migraine was not prevented by olcegepant.[66] A possible explanation could be that CGRP exerts its effect earlier than NO in the events leading to migraine after GTN infusion.

3.2.5.3 Pituitary adenylate cyclase activating polypeptide infusion

The pituitary adenylate cyclase activating polypeptide (PACAP) migraine model is a model that also makes use of a strong vasodilator. PACAP infusion induces migraine in 58% of patients with MO.[67] Infusion of vasoactive intestinal peptide (VIP) does not cause migraine in migraine patients.[68] VIP and PACAP share receptors, except the PACAP type 1 receptor (PAC_1), which suggests the involvement of PAC_1 in the

migraine attacks seen after PACAP infusion.[69] PACAP and CGRP are hypothesized to share the cAMP signaling pathway and it would be interesting to investigate whether CGRP receptor antagonists can abort PACAP-induced migraine.[70, 71]

3.2.5.4 Prostaglandin infusion

Finally, infusion of prostaglandin E_2 can also induce migraine.[72] After prostaglandin infusion, 75% of patients with MO experience migraine-like attacks.[73] The migraine-like attacks occur during and immediately after infusion, which is different from the other pharmacologically induced migraine models. The immediate onset of the attacks points towards a role for prostaglandins in the late phase of migraine development. This is an interesting model to investigate the effect of CGRP receptor antagonists in this phase of migraine.

3.2.6 Fos expression in experimental animals

Immunoreactivity of the Fos protein, which is a nuclear protein that regulates transcription of other target genes, has been shown to be a marker of neuronal activity.[74, 75] To identify related nociceptive pathways in migraine, activation of Fos expression in the trigeminovascular system has been used.[76] The expression of the Fos protein occurs *in vivo* after a certain stimulus, although obviously the measurement of the protein is performed *in vitro*. Fos expression can be activated by applying mechanical, electrical or chemical stimuli in either extracranial or intracranial tissues innervated by the trigeminal nerves. This includes electrical stimulation of the trigeminal ganglion and superior sagittal sinus (SSS) as well as chemical stimulation of the meninges (dura mater) with capsaicin or other irritant substances.[75]

3.2.6.1 Stimulation of the trigeminal ganglion and meninges

Trigeminal ganglion activation is a well-established method for the activation of the trigeminovascular system. Electrical stimulation of the trigeminal ganglion induces expression of c-Fos mRNA and Fos protein in trigeminal nucleus caudalis (TNC) of pigs[77] and rats.[78] However, trigeminal ganglion stimulation may induce widespread Fos expression by discharging many other afferents than those innervating the meninges, which is a limitation of this method.[75] A more direct activation of the meninges is chemical stimulation using capsaicin or other irritant substances.[75] Primary sensory fibers supplying the meninges are activated by injection of capsaicin or other irritant substances through a small catheter into the cisterna magna through the atlanto-occipital membrane. Fos protein immunoreactivity is detected in both sides of the TNC 2 h after injection and capsaicin increases Fos protein immunoreactivity in a dose-dependent manner.[79] The limitation of chemical stimulation of the meninges is that no comparison can be made between the two sides of the TNC.[75] To provide more information about the site of action in the TNC, stimulation of the SSS to induce Fos expression seems to be the most suitable model.

3.2.6.2 Stimulation of the superior sagittal sinus

Mechanical and electrical stimulation of the SSS has been used to induce Fos immunoreactivity in the TNC of rat[80], cat[81] and non-human primate.[82] Activation of brainstem structures after SSS stimulation, which is observed in different studies, reflects to the neurovascular activation of pain-sensitive structures during a migraine attack.[75] Moreover, neurons in the TNC that are activated through SSS stimulation are primary located in the deeper part of the nucleus caudalis, lamina V which correspond to Aδ-fiber input[81, 83] and SSS stimulation induces the release of neuropeptides like CGRP and VIP in a pattern similar to that observed during migraine attacks.[12, 84]

3.2.6.3 Systemic infusion of stimuli

Systemic infusion of noxious stimuli can also be used to induce Fos protein expression. GTN (an NO donor) infusion induced Fos expression in the trigeminocervical complex (TCC, TNC and C1/2 spinal cords levels),[85] which suggests a role of NO donors in activating the trigeminovascular system. This is supported by the results that the NOS inhibitor N-Nitro-L-Arginine Methyl Ester (L-NAME) decreases capsaicin- as well as electrically-induced Fos expression,[83, 86] Infusion of CGRP has also been shown to induce Fos expression in TNC.[87] The CGRP receptor antagonist, olcegepant inhibits systemically administered capsaicin-induced Fos expression in the spinal trigeminal nucleus, but not the increased phosphorylated extracellular signal-regulated kinase in the trigeminal ganglion when capsaicin is injected unilaterally in the face.[88] This might suggest that olcegepant exerts its effect mainly in the central nervous system rather than periphery including the trigeminal ganglion, which is activated when capsaicin is injected in the face.[88] On the other hand, olcegepant was administered intravenously so it is questionable if the concentration that reached the central nervous system is high enough to exert any effects, because of its incapability of crossing the blood brain barrier (paragraph 3.2.2). More studies with CGRP receptor antagonists comparing central and peripheral effects need to be done to investigate this proposition.

Fos protein expression models are only good models when the stimulus drives the expression of Fos protein.[75] However, to induce a Fos expression to quantifiable levels, a strong, consistent stimulation is required that is often not physiological.[89] Moreover, since Fos is not expressed in all neurons,[74] lack of Fos expression does not mean that there is no neuronal activity.[90] Despite these limitations, the Fos expression model with its different stimulations is a good model to investigate the neuronal activity of the trigeminovascular system on different levels.[75, 90] The different stimulations have allowed the identification of a subpopulation of neurons that is activated in response to noxious stimuli and thus identifying related nociceptive response.[90] The Fos expression model already greatly increased our understanding of the trigeminovascular system[75, 76] and the role of CGRP signaling in this system. In addition, the therapeutic mechanisms of possible CGRP receptor antagonists in the trigeminovascular system can be investigated via determination of neuronal activity.

3.2.7 *In vivo* electrophysiological recording in experimental animals

Electrophysiological recording is a method to measure action potential activity or local field potentials, which represent the net activity of a population of cells, in neurons by placing electrodes into the brain area of interest in a living animal.[76] In migraine research, electrophysiological recordings are performed in different parts of the trigeminovascular system, like the trigeminal ganglion[91] and the TCC.[92, 93] Moreover, since functional imaging studies in spontaneous migraine attacks as well as human models using trigeminal nociceptive stimulation have shown a consistent activation of the thalamus[94-96] and the majority of the secondary neurons in the TCC project to the thalamus,[97] the ventral posteromedial thalamic nucleus (VPM) is also an area of interest.[76] Indeed, electrophysiological studies in experimental models demonstrate that trigeminovascular nociceptive stimulation activates neurons in the VPM.[12, 98] Action potentials in the TCC and VPM, measured by electrophysiological recordings, can be triggered by electrical stimulation of SSS (see Fos protein), electrical stimulation of dura mater (see cranial window) and micro-iontophoresis of $_{L}$-glutamate in either TCC[93] or VPM.[99]

This model can be used to investigate the effect on neuronal activity in the second order neurons of TCC and the third order neurons of VPM for a possible site of action of antimigraine drugs.[76] Moreover, using micro-iontophoresis of $_{L}$glutamate, effects on postsynaptic action of neuronal elements including dendrites and cell bodies distal to the synaptic cleft can be studied more specifically.[99] The great advantage of this model is the real-time resolution of the response to a stimulus or pharmacological intervention. Although the neuronal activity can only be studied in a few neurons at the same time,[76] potential targets of triptans[92, 98] as well as prophylactic antimigraine drugs have been discovered with this model.[99, 100]

CGRP release has been shown in many structures of the trigeminovascular system, including second-order neurons in the TCC.[39] Therefore, CGRP receptor antagonists might also affect the TCC and the VPM. Intravenous administration of olcegepant has been shown to reduce spinal trigeminal activity induced by electrical stimulation of the dura mater[91] and thalamic cell firing in VPM[101] as well as TCC[102] by SSS stimulation. Moreover, the CGRP receptor antagonist, $CGRP_{8-37}$, is able to inhibit cell firing induced by micro-iontophoresis of $_{L}$glutamate and SSS stimulation in the VPM.[101, 102] These results suggest that VPM might be a possible therapeutic target for CGRP receptor antagonists.[102] Because of the positive results obtained with the CGRP receptor antagonists olcegpant and $CGRP_{8-37}$, electrical recording of TCC and VPM seems an excellent experimental model to identify the effect of CGRP receptor antagonists in the second-order neurons of the TCC and the third order neurons of the VPM as a possible site of therapeutic action.

3.2.7.1 Cortical spreading depression

CSD is regarded to be a main pathogenic step in migraine with, and possibly also without, aura. Although the potential role of CGRP in CSD has not yet been examined in detail, a recent study suggests that CGRP is involved in CSD and that

CGRP receptor antagonism reduces CSD. This suggests, in contrast to the low CGRP receptor expression in cortex[21] and the limited CNS penetration of telcagepant[49] mentioned earlier in this review, that central CGRP receptors may be a relevant target for CGRP receptor antagonism.

3.2.8 Transgenic mice

Another model in which the physiological role of the CGRP-ergic system can be studied are transgenic mice. In these animals a gene of another species is inserted and expressed. A recently developed transgenic mouse model is the *nestin/hRAMP1* mouse model. These animals over-express the RAMP1 part of the human CGRP receptor in the central and peripheral nervous system and show an aversive behaviour against light, which increases enormously after injection of intracerebroventricular CGRP.[103] Interestingly, CGRP concentrations in cerebrospinal fluid are increased in these mice, suggesting a positive feedback mechanism that is not yet fully understood.[103] It was shown that this light aversive behaviour in these animals could be blocked by olcegepant. Furthermore some CGRP knockout mice show decreased pain responses and increased blood pressure.[104, 105] These transgenic animals can thus be used to study effects of newly developed CGRP receptor antagonists.

4. Future developments

4.1 CGRP receptor blockade for the prophylactic treatment of migraine

While blockade of CGRP receptors has been demonstrated to be effective and well tolerated in the acute treatment of migraine, this concept has also been proposed as a mechanism of action for prophylactic antimigraine drugs, which are used by patients who suffer from highly frequent attacks, or where acutely acting drugs are contraindicated. Indeed, there is a need for effective preventive antimigraine treatments. Of the current prophylactics, none is specific for migraine (e.g., valproate was developed as an anti-epileptic drug and betablockers were developed to treat high blood pressure), explaining the high number of side effects due to their low specificity.[106] In addition, prophylactic drugs are generally effective in only 50% of patients.

Chronic CGRP receptor blockade can be reached via different strategies. Firstly, CGRP receptor antagonists can be administered on a (twice) daily basis. As described above in paragraph 3, this approach has been followed for telcagepant, which induced raised levels of liver transaminases and subsequent termination of the development of telcagepant. Alternatively, chronic CGRP receptor blockade may be reached by the administration of antibodies directed against the CGRP receptor. Using this latter approach, the CGRP receptor is blocked for up to 7 days after one single administration.[107] While a permanent blockade of the CGRP receptors could indeed be effective against migraine, it should be born in mind that, under such conditions, the physiological role of CGRP is constantly repressed. CGRP is one of the main contributors to the maintenance of normal vascular tone under ischemic conditions and protects the brain as well as peripheral organs, including the heart, against excessive vasoconstriction.[108-112] These important protective functions of

CGRP would most likely be blocked when CGRP receptor antagonists or antibodies directed against the CGRP receptor are used as a prophylaxis, which could have potentially harmful consequences. On the other hand, in mice and rat no evidence was found that CGRP receptor antagonists have a negative effect on ischemic injury[113] and in rat and pig no effect of CGRP receptor antagonists olcegepant and $CGRP_{8-37}$ was seen on infarct size in ischemia/reperfusion studies.[114, 115] Because these results are still contradictive, it is of crucial importance that extensive preclinical, as well as clinical studies are performed to elucidate whether it is safe to constantly repress the function of CGRP.

An alternative approach to blockade of the CGRP receptor is the use of an antibody directed against the CGRP protein itself.[116] Currently, the antibody LY2951742 is under investigation in a clinical trial. The potential objections mentioned above for repression of the CGRP receptor function obviously also apply for eliminating the function of the CGRP protein itself.

4.2 CGRP receptor antagonists against migraine as well as hot flushes during menopause?

Menopause is known to disturb the secretion of CGRP, resulting in lower levels of plasma CGRP in postmenopausal women compared to fertile women.[117] Vasomotor changes, such as hot flushes, on the other hand, are accompanied by a temporary rise in plasma CGRP levels in menopausal women. Indeed, infusion of CGRP can cause facial flushing.[118] Thus, CGRP may be an important factor in the occurrence of hot flushes.[119] As about one quarter of women visiting menopause clinics also report migraine,[120] a combined treatment of both the vasomotor symptoms and the headaches would seem useful. The vasomotor changes in menopausal women are until now treated with hormone replacement therapy (HRT).[121] However, at the onset of HRT migraine attacks may become more frequent.[122] In addition, HRT is associated with cardiovascular side effects.[123] An alternative treatment for the vasomotor changes during menopause seems to be required. The perimenopausal migraine headaches are treated not differently from any other migraine headaches.[124] CGRP receptor antagonists could be effective against both the perimenopausal migraine, as well as the vasomotor symptoms. However, considering the transient character and short duration of the hot flushes, CGRP receptor antagonists should probably be administered as prophylaxis. Obviously, the same potential drawbacks as described above for the use of CGRP receptor antagonists as prophylactic treatment against migraine apply, and the safety should be well investigated.

5. Conclusion

CGRP receptor antagonists seem to be promising new compounds for the acute treatment of migraine attacks, but no compound has passed clinical trials yet. The advent of novel compounds is awaited with great interest. Before a compound is tested in clinical trials, different preclinical experimental models may be used to characterize its pharmacological properties. As migraine is a neurovascular disease and no integrative model exists encompassing all components of this disease, the

models used to study new CGRP receptor antagonists include both vascular models as well as models of the central nervous system. All these models study specific properties of a compound, and their results should be integrated to get a complete picture of the effects and side-effects. Furthermore, these models can also be applied for more basic studies to obtain a better understanding of the pathophysiology of migraine.

6. Expert Opinion

The most widely used acute antimigraine drugs available are the triptans. Although they provided a major breakthrough in the acute treatment of migraine attacks when they were discovered, they are not effective in all patients and because of their vascular side-effect potential contraindicated in patients with cardiovascular disease. Thus, a major proportion of migraine patients cannot use, or has no profit of the use of triptans. Therefore it is highly relevant to find novel types of antimigraine drugs with fewer side effects. The discovery of the important role of CGRP in the pathophysiology of migraine prompted the search for a new type of acute antimigraine drugs, the CGRP receptor antagonists. The outcome of the first studies with the CGRP receptor antagonists, olcegepant and telcagepant, was positive. Both drugs showed little side effects and their effectivity against migraine was comparable to that of the triptans. CGRP receptor antagonists do not cause any constriction of coronary arteries [31], which may be an advantage compared to the triptans in view of cardiovascular safety. Furthermore, the patients that respond well to CGRP receptor antagonists are not necessarily the same patients that respond well to the triptans.[125] Therefore, CGRP receptor antagonists may fulfil a complementary role in the acute treatment of migraine and may hopefully also be of benefit to non-responders to triptan therapy.

It is still a question where CGRP receptor antagonists exert their therapeutic effects in migraine. Some studies, for example the above-mentioned study showing only moderate brain receptor binding for telcagepant,[49] suggest that a central action may not be essential for the effects of CGRP receptor antagonists. Other studies, however, suggest that vascular changes in migraine are absent or an irrelevant epiphenomenon.[126] While it is still unclear where the therapeutic site of action of the CGRP receptor antagonists is located, possibly the efficacy or lack of efficacy of antibodies directed against the CGRP receptor, that are unlikely to penetrate into the brain in view of their high molecular weight, may shed more light on this discussion. It is important in this light to realize that the trigeminal ganglion is located outside the blood brain barrier, and could also serve as a target for ligands without central penetration.

As described above, it remains to be demonstrated whether continuous blockade of CGRP receptors does not increase the risk for myocardial or cerebral ischemia. Further, since overexpression of the CGRP receptor in mice leads to increased CGRP levels in cerebrospinal fluid,[103] it is important to investigate whether blockade of CGRP receptors would lead to an opposite effect, i.e., decrease the levels of CGRP.

Since CGRP receptor antagonists act via a different mechanism of action than the triptans, it is important that their effects are assessed in adequate models. Currently available experimental migraine models only study a small part of all potentially relevant processes that are going on during a migraine attack. Integrated neurovascular animal as well as human models that measure both vascular and neuronal components simultaneously would increase the knowledge on the mechanism of action of prospective CGRP receptor antagonists. Further, more emphasis should be put on the translation of the human infusion model to animal models, where effects of infusion of migraine triggers may be studied in detail. It would be desirable to perform more studies in awake animals, where behaviour can be studied and results are not confounded by the use of anesthetics. The use of transgenic animals in these models may be of additive value.

References

1. Olesen J, Steiner TJ. The International classification of headache disorders, 2nd edn (ICDH-II). Journal of neurology, neurosurgery, and psychiatry 2004;75:808-811.
2. Kelman L. The aura: a tertiary care study of 952 migraine patients. Cephalalgia 2004;24:728-734.
3. Charles A. The evolution of a migraine attack - a review of recent evidence. Headache 2013;53:413-419.
4. Stovner L, Hagen K, Jensen R, et al. The global burden of headache: a documentation of headache prevalence and disability worldwide. Cephalalgia 2007;27:193-210.
5. Goadsby PJ. Recent advances in understanding migraine mechanisms, molecules and therapeutics. Trends Mol Med 2007;13:39-44.
6. Silberstein SD, Goadsby PJ. Migraine: preventive treatment. Cephalalgia 2002;22:491-512.
7. Silberstein SD, McCrory DC. Ergotamine and dihydroergotamine: history, pharmacology, and efficacy. Headache 2003;43:144-166.
8. MaassenVanDenBrink A, Reekers M, Bax WA, Ferrari MD, Saxena PR. Coronary side-effect potential of current and prospective antimigraine drugs. Circulation 1998;98:25-30.
9. Edvinsson L, Villalon CM, MaassenVanDenBrink A. Basic mechanisms of migraine and its acute treatment. Pharmacol Ther 2012;136:319-333.
10. Gupta S, Villalon CM. The relevance of preclinical research models for the development of antimigraine drugs: focus on 5-HT(1B/1D) and CGRP receptors. Pharmacol Ther 2010;128:170-190.
11. Goadsby PJ, Edvinsson L. The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Ann Neurol 1993;33:48-56.
12. Zagami AS, Goadsby PJ, Edvinsson L. Stimulation of the superior sagittal sinus in the cat causes release of vasoactive peptides. Neuropeptides 1990;16:69-75.
13. Eftekhari S, Edvinsson L. Possible sites of action of the new calcitonin gene-related peptide receptor antagonists. Ther Adv Neurol Disord 2010;3:369-378.
14. Wimalawansa SJ. Calcitonin gene-related peptide and its receptors: molecular genetics, physiology, pathophysiology, and therapeutic potentials. Endocr Rev 1996;17:533-585.
15. Edvinsson L, Ho TW. CGRP receptor antagonism and migraine. Neurotherapeutics 2010;7:164-175.
16. Walker CS, Conner AC, Poyner DR, Hay DL. Regulation of signal transduction by calcitonin gene-related peptide receptors. Trends Pharmacol Sci 2010;31:476-483.
17. Durham PL. CGRP-receptor antagonists--a fresh approach to migraine therapy? N Engl J Med 2004;350:1073-1075.
18. Villalon CM, Centurion D, Valdivia LF, de Vries P, Saxena PR. Migraine: pathophysiology, pharmacology, treatment and future trends. Curr Vasc Pharmacol 2003;1:71-84.
19. Iovino M, Feifel U, Yong CL, Wolters JM, Wallenstein G. Safety, tolerability and pharmacokinetics of BIBN 4096 BS, the first selective small molecule calcitonin gene-related peptide receptor antagonist, following single intravenous administration in healthy volunteers. Cephalalgia 2004;24:645-656.
20. Salvatore CA, Hershey JC, Corcoran HA, et al. Pharmacological characterization of MK-0974 [N-[(3R,6S)-6-(2,3-difluorophenyl)-2-oxo-1-(2,2,2-trifluoroethyl)azepan-3-yl]-4-(2-oxo-2,3-dihydro-1H-imidazo[4,5-b]pyridin-1-yl)piperidine-1-carboxamide], a potent and orally active calcitonin gene-related peptide receptor antagonist for the treatment of migraine. J Pharmacol Exp Ther 2008;324:416-421.
21. Salvatore CA, Moore EL, Calamari A, et al. Pharmacological properties of MK-3207, a potent and orally active calcitonin gene-related peptide receptor antagonist. J Pharmacol Exp Ther 2010;333:152-160.
22. Petersen KA, Birk S, Lassen LH, et al. The CGRP-antagonist, BIBN4096BS does not affect cerebral or systemic haemodynamics in healthy volunteers. Cephalalgia 2005;25:139-147.
23. Olesen J, Diener HC, Husstedt IW, et al. Calcitonin gene-related peptide receptor antagonist BIBN 4096 BS for the acute treatment of migraine. N Engl J Med 2004;350:1104-1110.
24. Ho TW, Mannix LK, Fan X, et al. Randomized controlled trial of an oral CGRP receptor antagonist, MK-0974, in acute treatment of migraine. Neurology 2008;70:1304-1312.
25. Ho TW, Ferrari MD, Dodick DW, et al. Efficacy and tolerability of MK-0974 (telcagepant), a new oral antagonist of calcitonin gene-related peptide receptor, compared with zolmitriptan for acute migraine: a randomised, placebo-controlled, parallel-treatment trial. Lancet 2008;372:2115-2123.
26. Connor KM, Shapiro RE, Diener HC, et al. Randomized, controlled trial of telcagepant for the acute treatment of migraine. Neurology 2009;73:970-977.
27. Han TH, Blanchard RL, Palcza J, et al. Single- and multiple-dose pharmacokinetics and tolerability of telcagepant, an oral calcitonin gene-related peptide receptor antagonist, in adults. J Clin Pharmacol 2010;50:1367-1376.
28. Hewitt DJ, Aurora SK, Dodick DW, et al. Randomized controlled trial of the CGRP receptor antagonist MK-3207 in the acute treatment of migraine. Cephalalgia 2011;31:712-722.
29. Diener HC, Barbanti P, Dahlof C, Reuter U, Habeck J, Podhorna J. BI 44370 TA, an oral CGRP antagonist

for the treatment of acute migraine attacks: results from a phase II study. Cephalalgia 2011;31:573-584.
30. Tong G, Savant I, Jariwala N, et al. Phase I single and multiple dose study to evaluate the safety, tolerability, and pharmacokinetics of BMS-927711 in healthy subjects. The Journal of Headache and Pain 2013;1:P118.
31. Chan KY, Edvinsson L, Eftekhari S, et al. Characterization of the calcitonin gene-related peptide receptor antagonist telcagepant (MK-0974) in human isolated coronary arteries. J Pharmacol Exp Ther 2010;334:746-752.
32. Moreno MJ, Abounader R, Hebert E, Doods H, Hamel E. Efficacy of the non-peptide CGRP receptor antagonist BIBN4096BS in blocking CGRP-induced dilations in human and bovine cerebral arteries: potential implications in acute migraine treatment. Neuropharmacology 2002;42:568-576.
33. Gupta S, Lozano-Cuenca J, Villalon CM, et al. Pharmacological characterisation of capsaicin-induced relaxations in human and porcine isolated arteries. Naunyn Schmiedebergs Arch Pharmacol 2007;375:29-38.
34. Edvinsson L, Chan KY, Eftekhari S, et al. Effect of the calcitonin gene-related peptide (CGRP) receptor antagonist telcagepant in human cranial arteries. Cephalalgia 2010;30:1233-1240.
35. Shahid M, Buys ES. Assessing murine resistance artery function using pressure myography. Journal of visualized experiments : JoVE 2013.
36. Edvinsson L, Nilsson E, Jansen-Olesen I. Inhibitory effect of BIBN4096BS, CGRP(8-37), a CGRP antibody and an RNA-Spiegelmer on CGRP induced vasodilatation in the perfused and non-perfused rat middle cerebral artery. Br J Pharmacol 2007;150:633-640.
37. Marichal-Cancino BA, Gonzalez-Hernandez A, Manrique-Maldonado G, et al. Intrathecal dihydroergotamine inhibits capsaicin-induced vasodilatation in the canine external carotid circulation via GR127935- and rauwolscine-sensitive receptors. Eur J Pharmacol 2012;692:69-77.
38. Gonzalez-Hernandez A, Manrique-Maldonado G, Lozano-Cuenca J, et al. The 5-$HT_{(1)}$ receptors inhibiting the rat vasodepressor sensory CGRPergic outflow: further involvement of 5-$HT_{(1F)}$, but not 5-$HT_{(1A)}$ or 5-$HT_{(1D)}$, subtypes. Eur J Pharmacol 2011;659:233-243.
39. Edvinsson L, Linde M. New drugs in migraine treatment and prophylaxis: telcagepant and topiramate. Lancet 2010;376:645-655.
40. Williamson DJ, Hargreaves RJ, Hill RG, Shepheard SL. Sumatriptan inhibits neurogenic vasodilation of dural blood vessels in the anaesthetized rat--intravital microscope studies. Cephalalgia 1997;17:525-531.
41. Gupta S, Akerman S, van den Maagdenberg AM, Saxena PR, Goadsby PJ, van den Brink AM. Intravital microscopy on a closed cranial window in mice: a model to study trigeminovascular mechanisms involved in migraine. Cephalalgia 2006;26:1294-1303.
42. Williamson DJ, Hargreaves RJ, Hill RG, Shepheard SL. Intravital microscope studies on the effects of neurokinin agonists and calcitonin gene-related peptide on dural vessel diameter in the anaesthetized rat. Cephalalgia 1997;17:518-524.
43. Akerman S, Williamson DJ, Kaube H, Goadsby PJ. Nitric oxide synthase inhibitors can antagonize neurogenic and calcitonin gene-related peptide induced dilation of dural meningeal vessels. Br J Pharmacol 2002;137:62-68.
44. Akerman S, Goadsby PJ. Topiramate inhibits trigeminovascular activation: an intravital microscopy study. Br J Pharmacol 2005;146:7-14.
45. Akerman S, Williamson DJ, Goadsby PJ. Voltage-dependent calcium channels are involved in neurogenic dural vasodilatation via a presynaptic transmitter release mechanism. Br J Pharmacol 2003;140:558-566.
46. Chan KY, Gupta S, de Vries R, et al. Effects of ionotropic glutamate receptor antagonists on rat dural artery diameter in an intravital microscopy model. Br J Pharmacol 2010;160:1316-1325.
47. Okazawa H, Tsuchida T, Pagani M, et al. Effects of 5-HT1B/1D receptor agonist rizatriptan on cerebral blood flow and blood volume in normal circulation. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 2006;26:92-98.
48. Sakai Y, Dobson C, Diksic M, Aube M, Hamel E. Sumatriptan normalizes the migraine attack-related increase in brain serotonin synthesis. Neurology 2008;70:431-439.
49. Vermeersch S, de Hoon J, De Saint-Hubert B, et al. PET imaging in healthy subjects and migraineurs suggests CGRP receptor antagonists do not have to act centrally to achieve clinical efficacy. J Headache Pain 2013;1:P224.
50. Bandettini PA. Seven topics in functional magnetic resonance imaging. Journal of integrative neuroscience 2009;8:371-403.
51. Ogawa S, Lee TM, Kay AR, Tank DW. Brain magnetic resonance imaging with contrast dependent on blood oxygenation. Proceedings of the National Academy of Sciences of the United States of America 1990;87:9868-9872.
52. Asghar MS, Hansen AE, Larsson HB, Olesen J, Ashina M. Effect of CGRP and sumatriptan on the BOLD response in visual cortex. J Headache Pain 2012;13:159-166.
53. Asghar MS, Hansen AE, Kapijimpanga T, et al. Dilation by CGRP of middle meningeal artery and

reversal by sumatriptan in normal volunteers. Neurology 2010;75:1520-1526.
54. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 1997;389:816-824.
55. Van der Schueren BJ, de Hoon JN, Vanmolkot FH, et al. Reproducibility of the capsaicin-induced dermal blood flow response as assessed by laser Doppler perfusion imaging. British journal of clinical pharmacology 2007;64:580-590.
56. Sinclair SR, Kane SA, Van der Schueren BJ, et al. Inhibition of capsaicin-induced increase in dermal blood flow by the oral CGRP receptor antagonist, telcagepant (MK-0974). British journal of clinical pharmacology 2010;69:15-22.
57. Buzzi MG, Carter WB, Shimizu T, Heath H, 3rd, Moskowitz MA. Dihydroergotamine and sumatriptan attenuate levels of CGRP in plasma in rat superior sagittal sinus during electrical stimulation of the trigeminal ganglion. Neuropharmacology 1991;30:1193-1200.
58. Durand S, Fromy B, Bouye P, Saumet JL, Abraham P. Current-induced vasodilation during water iontophoresis (5 min, 0.10 mA) is delayed from current onset and involves aspirin sensitive mechanisms. Journal of vascular research 2002;39:59-71.
59. Ibrahimi K, Danser A, Villalon C, van den Meiracker A, MaassenVanDenBrink A. Influence of varying estrogen levels on trigeminal CGRP release in healthy women. The Journal of Headache and Pain 2013;1:P123.
60. Classification and diagnostic criteria for headache disorders, cranial neuralgias and facial pain. Headache Classification Committee of the International Headache Society. Cephalalgia 1988;8 Suppl 7:1-96.
61. Hansen JM, Hauge AW, Olesen J, Ashina M. Calcitonin gene-related peptide triggers migraine-like attacks in patients with migraine with aura. Cephalalgia 2010;30:1179-1186.
62. Lassen LH, Haderslev PA, Jacobsen VB, Iversen HK, Sperling B, Olesen J. CGRP may play a causative role in migraine. Cephalalgia 2002;22:54-61.
63. Thomsen LL, Kruuse C, Iversen HK, Olesen J. A nitric oxide donor (nitroglycerin) triggers genuine migraine attacks. European Journal of Neurology 1994;1:73-80.
64. Christiansen I, Thomsen LL, Daugaard D, Ulrich V, Olesen J. Glyceryl trinitrate induces attacks of migraine without aura in sufferers of migraine with aura. Cephalalgia 1999;19:660-667; discussion 626.
65. Hoivik HO, Laurijssens BE, Harnisch LO, et al. Lack of efficacy of the selective iNOS inhibitor GW274150 in prophylaxis of migraine headache. Cephalalgia 2010;30:1458-1467.
66. Tvedskov JF, Tfelt-Hansen P, Petersen KA, Jensen LT, Olesen J. CGRP receptor antagonist olcegepant (BIBN4096BS) does not prevent glyceryl trinitrate-induced migraine. Cephalalgia 2010;30:1346-1353.
67. Schytz HW, Birk S, Wienecke T, Kruuse C, Olesen J, Ashina M. PACAP38 induces migraine-like attacks in patients with migraine without aura. Brain : a journal of neurology 2009;132:16-25.
68. Rahmann A, Wienecke T, Hansen JM, Fahrenkrug J, Olesen J, Ashina M. Vasoactive intestinal peptide causes marked cephalic vasodilation, but does not induce migraine. Cephalalgia 2008;28:226-236.
69. Harmar AJ, Arimura A, Gozes I, et al. International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Pharmacological reviews 1998;50:265-270.
70. Jansen-Olesen I, Mortensen A, Edvinsson L. Calcitonin gene-related peptide is released from capsaicin-sensitive nerve fibres and induces vasodilatation of human cerebral arteries concomitant with activation of adenylyl cyclase. Cephalalgia 1996;16:310-316.
71. Emery AC, Eiden LE. Signaling through the neuropeptide GPCR PAC(1) induces neuritogenesis via a single linear cAMP- and ERK-dependent pathway using a novel cAMP sensor. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2012;26:3199-3211.
72. Wienecke T, Olesen J, Oturai PS, Ashina M. Prostaglandin E2(PGE2) induces headache in healthy subjects. Cephalalgia 2009;29:509-519.
73. Antonova M, Wienecke T, Olesen J, Ashina M. Prostaglandin E(2) induces immediate migraine-like attack in migraine patients without aura. Cephalalgia 2012;32:822-833.
74. Hunt SP, Pini A, Evan G. Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. Nature 1987;328:632-634.
75. Mitsikostas DD, Sanchez del Rio M. Receptor systems mediating c-fos expression within trigeminal nucleus caudalis in animal models of migraine. Brain research 2001;35:20-35.
76. Andreou AP, Summ O, Charbit AR, Romero-Reyes M, Goadsby PJ. Animal models of headache: from bedside to bench and back to bedside. Expert review of neurotherapeutics 2010;10:389-411.
77. Clayton JS, Gaskin PJ, Beattie DT. Attenuation of Fos-like immunoreactivity in the trigeminal nucleus caudalis following trigeminovascular activation in the anaesthetised guinea-pig. Brain Res 1997;775:74-80.
78. Bohar Z, Fejes-Szabo A, Tar L, et al. Evaluation of c-Fos immunoreactivity in the rat brainstem nuclei relevant in migraine pathogenesis after electrical stimulation of the trigeminal ganglion. Neurol Sci 2013.
79. Mitsikostas DD, Sanchez del Rio M, Waeber C, Moskowitz MA, Cutrer FM. The NMDA receptor

antagonist MK-801 reduces capsaicin-induced c-fos expression within rat trigeminal nucleus caudalis. Pain 1998;76:239-248.
80. Strassman AM, Potrebic S, Maciewicz RJ. Anatomical properties of brainstem trigeminal neurons that respond to electrical stimulation of dural blood vessels. The Journal of comparative neurology 1994;346:349-365.
81. Kaube H, Keay KA, Hoskin KL, Bandler R, Goadsby PJ. Expression of c-Fos-like immunoreactivity in the caudal medulla and upper cervical spinal cord following stimulation of the superior sagittal sinus in the cat. Brain Res 1993;629:95-102.
82. Goadsby PJ, Hoskin KL. The distribution of trigeminovascular afferents in the nonhuman primate brain Macaca nemestrina: a c-fos immunocytochemical study. Journal of anatomy 1997;190 (Pt 3):367-375.
83. Hoskin KL, Bulmer DC, Goadsby PJ. Fos expression in the trigeminocervical complex of the cat after stimulation of the superior sagittal sinus is reduced by L-NAME. Neuroscience letters 1999;266:173-176.
84. Edvinsson L, Goadsby PJ. Neuropeptides in migraine and cluster headache. Cephalalgia 1994;14:320-327.
85. Tassorelli C, Joseph SA. Systemic nitroglycerin induces Fos immunoreactivity in brainstem and forebrain structures of the rat. Brain Res 1995;682:167-181.
86. Offenhauser N, Zinck T, Hoffmann J, et al. CGRP release and c-fos expression within trigeminal nucleus caudalis of the rat following glyceryltrinitrate infusion. Cephalalgia 2005;25:225-236.
87. Chatchaisak D, Srikiatkhachorn A, Maneesri-le Grand S, Govitrapong P, Chetsawang B. The role of calcitonin gene-related peptide on the increase in transient receptor potential vanilloid-1 levels in trigeminal ganglion and trigeminal nucleus caudalis activation of rat. Journal of chemical neuroanatomy 2013;47:50-56.
88. Sixt ML, Messlinger K, Fischer MJ. Calcitonin gene-related peptide receptor antagonist olcegepant acts in the spinal trigeminal nucleus. Brain : a journal of neurology 2009;132:3134-3141.
89. Bullitt E, Lee CL, Light AR, Willcockson H. The effect of stimulus duration on noxious-stimulus induced c-fos expression in the rodent spinal cord. Brain Res 1992;580:172-179.
90. Bergerot A, Holland PR, Akerman S, et al. Animal models of migraine: looking at the component parts of a complex disorder. The European journal of neuroscience 2006;24:1517-1534.
91. Koulchitsky S, Fischer MJ, Messlinger K. Calcitonin gene-related peptide receptor inhibition reduces neuronal activity induced by prolonged increase in nitric oxide in the rat spinal trigeminal nucleus. Cephalalgia 2009;29:408-417.
92. Storer RJ, Goadsby PJ. Microiontophoretic application of serotonin (5HT)1B/1D agonists inhibits trigeminal cell firing in the cat. Brain : a journal of neurology 1997;120 (Pt 12):2171-2177.
93. Shields KG, Storer RJ, Akerman S, Goadsby PJ. Calcium channels modulate nociceptive transmission in the trigeminal nucleus of the cat. Neuroscience 2005;135:203-212.
94. Bahra A, Matharu MS, Buchel C, Frackowiak RS, Goadsby PJ. Brainstem activation specific to migraine headache. Lancet 2001;357:1016-1017.
95. DaSilva AF, Becerra L, Makris N, et al. Somatotopic activation in the human trigeminal pain pathway. J Neurosci 2002;22:8183-8192.
96. Kobari M, Meyer JS, Ichijo M, Imai A, Oravez WT. Hyperperfusion of cerebral cortex, thalamus and basal ganglia during spontaneously occurring migraine headaches. Headache 1989;29:282-289.
97. Percheron G. Thalamus. In: Paxinos G, May J, editors. The Human Nervous System. Amsterdam: Elsevier; 2003. p. 592-675.
98. Shields KG, Goadsby PJ. Serotonin receptors modulate trigeminovascular responses in ventroposteromedial nucleus of thalamus: a migraine target? Neurobiology of disease 2006;23:491-501.
99. Shields KG, Goadsby PJ. Propranolol modulates trigeminovascular responses in thalamic ventroposteromedial nucleus: a role in migraine? Brain : a journal of neurology 2005;128:86-97.
100. Andreou AP, Shields KG, Goadsby PJ. GABA and valproate modulate trigeminovascular nociceptive transmission in the thalamus. Neurobiology of disease 2010;37:314-323.
101. Summ O, Charbit AR, Andreou AP, Goadsby PJ. Modulation of nocioceptive transmission with calcitonin gene-related peptide receptor antagonists in the thalamus. Brain : a journal of neurology 2010;133:2540-2548.
102. Storer RJ, Akerman S, Goadsby PJ. Calcitonin gene-related peptide (CGRP) modulates nociceptive trigeminovascular transmission in the cat. Br J Pharmacol 2004;142:1171-1181.
103. Recober A, Kuburas A, Zhang Z, Wemmie JA, Anderson MG, Russo AF. Role of calcitonin gene-related peptide in light-aversive behavior: implications for migraine. J Neurosci 2009;29:8798-8804.
104. Zhang L, Hoff AO, Wimalawansa SJ, Cote GJ, Gagel RF, Westlund KN. Arthritic calcitonin/alpha calcitonin gene-related peptide knockout mice have reduced nociceptive hypersensitivity. Pain 2001;89:265-273.
105. Gangula PR, Zhao H, Supowit SC, et al. Increased blood pressure in alpha-calcitonin gene-related

peptide/calcitonin gene knockout mice. Hypertension 2000;35:470-475.
106. Barbanti P, Aurilia C, Egeo G, Fofi L. Future trends in drugs for migraine prophylaxis. Neurol Sci 2012;33 Suppl 1:S137-140.
107. Zhu D, Zhang J, Zhou L, et al. A human CGRP receptor antagonist antibody, AA95, is effective in inhibiting capsaicin-induced increase in dermal blood flow in cynomolgus monkeys. Abstract of 54th Annual Scientific Meeting of the American Headache Society. Headache 2012;52:862-914.
108. Chai W, Mehrotra S, Jan Danser AH, Schoemaker RG. The role of calcitonin gene-related peptide (CGRP) in ischemic preconditioning in isolated rat hearts. Eur J Pharmacol 2006;531:246-253.
109. Li D, Li NS, Chen QQ, et al. Calcitonin gene-related peptide-mediated cardioprotection of postconditioning in isolated rat hearts. Regul Pept 2008;147:4-8.
110. Li YJ, Peng J. The cardioprotection of calcitonin gene-related peptide-mediated preconditioning. Eur J Pharmacol 2002;442:173-177.
111. Song S, Liu N, Liu W, Shi R, Guo KJ, Liu YF. The effect of pretreatment with calcitonin gene-related peptide on attenuation of liver ischemia and reperfusion injury due to oxygen free radicals and apoptosis. Hepatogastroenterology 2009;56:1724-1729.
112. Schebesch KM, Herbst A, Bele S, et al. Calcitonin-gene related peptide and cerebral vasospasm. Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia 2013;20:584-586.
113. Wang L, Wang DH. TRPV1 gene knockout impairs postischemic recovery in isolated perfused heart in mice. Circulation 2005;112:3617-3623.
114. Kallner G, Gonon A, Franco-Cereceda A. Calcitonin gene-related peptide in myocardial ischaemia and reperfusion in the pig. Cardiovasc Res 1998;38:493-499.
115. Wu DM, van Zwieten PA, Doods HN. Effects of calcitonin gene-related peptide and BIBN4096BS on myocardial ischemia in anesthetized rats. Acta Pharmacol Sin 2001;22:588-594.
116. Zeller J, Poulsen KT, Sutton JE, et al. CGRP function-blocking antibodies inhibit neurogenic vasodilatation without affecting heart rate or arterial blood pressure in the rat. Br J Pharmacol 2008;155:1093-1103.
117. Valentini A, Petraglia F, De Vita D, et al. Changes of plasma calcitonin gene-related peptide levels in postmenopausal women. Am J Obstet Gynecol 1996;175:638-642.
118. Gennari C, Nami R, Pecchi S, De Franco V, Panza F, Pavese G. Plethysmographic evaluation of the vascular effects of human calcitonin gene-related peptide in man. Angiology 1991;42:462-467.
119. Hay DL, Poyner DR. Calcitonin gene-related peptide, adrenomedullin and flushing. Maturitas 2009;64:104-108.
120. MacGregor EA. Migraine, the menopause and hormone replacement therapy: a clinical review. J Fam Plann Reprod Health Care 2007;33:245-249.
121. The 2012 hormone therapy position statement of: The North American Menopause Society. Menopause 2012;19:257-271.
122. Nappi RE, Cagnacci A, Granella F, Piccinini F, Polatti F, Facchinetti F. Course of primary headaches during hormone replacement therapy. Maturitas 2001;38:157-163.
123. Rossouw JE, Anderson GL, Prentice RL, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA 2002;288:321-333.
124. Evers S, Afra J, Frese A, et al. EFNS guideline on the drug treatment of migraine--revised report of an EFNS task force. European journal of neurology : the official journal of the European Federation of Neurological Societies 2009;16:968-981.
125. Ho TW, Olesen J, Dodick DW, Kost J, Lines C, Ferrari MD. Antimigraine efficacy of telcagepant based on patient's historical triptan response. Headache 2011;51:64-72.
126. Amin FM, Asghar MS, Hougaard A, et al. Magnetic resonance angiography of intracranial and extracranial arteries in patients with spontaneous migraine without aura: a cross-sectional study. Lancet neurology 2013;12:454-461.
127. Zhang Z, Winborn CS, Marquez de Prado B, Russo AF. Sensitization of calcitonin gene-related peptide receptors by receptor activity-modifying protein-1 in the trigeminal ganglion. J Neurosci 2007;27:2693-2703.

CHAPTER 4

Intracranial and extracranial arteries

Based on: Lancet Neurol, 2013, 12:9, 847-848

A. MaassenVanDenBrink, K. Ibrahimi & L. Edvinsson

After reading the interesting Article by Faisal Mohammad Amin and colleagues,[1] we were confused about the conclusion made by the authors, namely that "future migraine research should focus on the peripheral and central pain pathways rather than simple arterial dilatation". This conclusion was based on their observation that during a migraine attack, "no statistically significant dilatation was seen of the extracranial arteries on the pain side" and "only a slight dilatation" of cerebral arteries was detected.

Although the authors correctly comment that they could not exclude possible dilatation of dural branches of the middle meningeal artery (MMA) given that these small branches could not be analyzed due to technical limitations, they still felt justified to draw the above-mentioned conclusion. We perceive this limitation as an important issue and fear that their interpretation is premature. Amin and colleagues disregard the fact that an intracranial branch of an artery, such as the intracranial dural branches of the MMA, can differ from its extracranial branches.[2, 3] In particular, they did not measure intracranial MMA circumference in any of their human magnetic resonance angiography studies, but instead based their findings on assessments of only the extracranial MMA. By 1961, Hassler[3] showed structural changes in the basilar artery wall between the open and closed skull after craniotomy. In the open skull preparation the basilar artery had profoundly changed its vessel wall properties; hence we argue that the structure (and consequently also function) of the extracranial MMA might differ substantially from the intracranial MMA.

We are further surprised by a previous report by the same group, in which they concluded that "migraine without aura is associated with dilatation of extra- and intracerebral arteries and that the headache location is associated with the location of vasodilatation".[4] Admittedly, the migraine attacks observed in this study[4] were induced by systemic infusion of calcitonin gene-related peptide (CGRP). Their finding that spontaneous migraine attacks are not associated with dilation of extracranial arteries suggests that provocation with CGRP infusion is a flawed migraine model.

Alternatively, this discrepancy might be related to some concerns regarding the study population and the procedures applied in the study by Amin and colleagues.[1] The migraine attacks reported were of moderate severity and time between attack and attack-free scans was 5-274 days, as opposed to only a few hours in their CGRP infusion study.[4] The arterial circumference changes measured between attack and attack-free scans were slight and had large SD. These limitations could have resulted in an underpowered study measuring minor changes from variable baseline values owing to the long period between scans.

We acknowledge the sheer complexity of doing magnatic resonance angiography scans during spontaneous migraine attacks and value the study by Amin and colleagues.[1] However, we contest their, in our view, premature conclusion. We sincerely approve of findings that help our understanding of migraine and migraine pain, but these should be based on solid arguments and data.

References

1. Amin FM, Asghar MS, Hougaard A, et al. Magnetic resonance angiography of intracranial and extracranial arteries in patients with spontaneous migraine without aura: a cross-sectional study. The Lancet Neurology 2013;12:454-461.
2. MaassenVanDenBrink AM, Duncker DJ, Saxena PR. Migraine headache is not associated with cerebral or meningeal vasodilatation--a 3T magnetic resonance angiography study. Brain 2009;132:e112; author reply e113.
3. Hassler O. Morphological studies on the large cerebral arteries, with reference to the aetiology of subarachnoid haemorrhage. Acta Psychiatr Scand Suppl 1961;154:1-145.
4. Asghar MS, Hansen AE, Amin FM, et al. Evidence for a vascular factor in migraine. Ann Neurol 2011;69:635-645.

CHAPTER 5

Development of an experimental model to study trigeminal nerve-mediated vasodilation on the human forehead

Based on: Cephalalgia, 2014, 34:7, 514-522

K. Ibrahimi, S. Vermeersch, A.H.J. Danser, C.M. Villalón,
A.H. van den Meiracker, J. de Hoon & A. MaassenVanDenBrink

Abstract

Background: During migraine, trigeminal sensory nerve terminals release calcitonin gene-related peptide (CGRP), inducing nociception and vasodilatation. Applied on the skin, capsaicin activates the transient receptor potential vanilloid type 1 (TRPV1) channel and releases CGRP from sensory nerve terminals, thus increasing dermal blood flow (DBF). Using capsaicin application and electrical stimulation of the forehead skin, a trigeminal nerve-innervated dermatome, we aimed to develop a model to measure trigeminal nervemediated vasodilatation in humans. **Methods:** Using laser Doppler imaging, forehead DBF responses to application of capsaicin (0.06 mg/ml and 6.0 mg/ml) and saline, with and without iontophoresis, were studied in healthy subjects. The within-subject coefficient of variation (WCV) of repeated DBF measurements was calculated to assess reproducibility. **Results:** Maximal DBF responses to 6.0 mg/ml capsaicin with and without iontophoresis did not differ (E_{max} 459±32 and 424±32 arbitrary units (a.u.), WCV 6±4%). In contrast, DBF responses to 0.06 mg/ml capsaicin were significantly larger with than without iontophoresis (E_{max} 307±60 versus 187±21 a.u., WCV 21±13%). Saline with iontophoresis significantly increased DBF (E_{max}: 245±26 a.u, WCV 11±8%), while saline application without iontophoresis did not affect DBF. **Conclusion:** Topical application of capsaicin and electrical stimulation induce reproducible forehead DBF increases and therefore are suitable to study trigeminal nervemediated vasodilatation in humans.

Introduction

Migraine is a neurovascular disorder[1] involving: (i) activation of the trigeminovascular system with the primary dysfunction located in brainstem centres regulating vascular tone and pain sensation;[1] (ii) release of vasoactive neuropeptides from trigeminal sensory nerves including calcitonin gene-related peptide (CGRP);[2, 3] and (iii) vasodilatation of intracranial arteries and of the extracranial branches of the external carotid artery.[4, 5] Indeed, it has been reported that plasma concentrations of CGRP are elevated during migraine headache and they diminish to normal levels after treatment with triptans in parallel with alleviation of headache.[6] In view of the key role of CGRP in the pathophysiology of migraine, CGRP and its receptors have now become a novel drug target for antimigraine therapy.[7]

CGRP is predominantly located in sensory neurons and perivascular (including trigeminal sensory) nerves.[2, 3, 8-10] Upon release in the cranial circulation, CGRP dilates cranial blood vessels and is involved in nociception.[8] Release of endogenous CGRP from trigeminal nerves can experimentally be induced by electrical stimulation[6, 11, 12] or by chemical stimulation with capsaicin,[13-15] the latter through activation of the transient receptor potential vanilloid type 1 (TRPV1) cation channel.[16, 17] Administration of capsaicin in the carotid artery has been reported to induce vasodilatation of the extracranial circulation of pigs[18] and dogs.[19] These capsaicin-induced vasodilator responses can be blocked by CGRP receptor antagonist.[18, 20] Similarly, both electrically and capsaicin-induced intracranial dural vasodilatation in rats[21] and mice[22] are mediated by trigeminal CGRP release. Likewise, topically applied capsaicin on the human forearm skin increases dermal blood flow (DBF),

and this response can be prevented by CGRP receptor antagonists[23] as well as by monoclonal antibodies preventing the binding of CGRP with its receptor.[24, 25] This "peripheral" human capsaicin model has been succesfull as a biomarker in early clinical drug development by providing proof of target engagement in phase I clinical trials and guiding dose selection for phase II clinical trials. Although the capsaicin-induced increase in DBF of the human forearm was shown to be reproducuble and user friendly, its major limitation remains the uncertainty about the translation of peripheral versus cranial vascular responses induced by capsaicin.

Until now straightforward and reproducible human methods for the analysis of trigeminal nerve-mediated vasodilatation with minimal demands of the test subject have been lacking. Since the dermatome of the forehead is innervated by the trigeminal nerve, the forehead seems well suited to investigate trigeminal mechanisms. Gazerani et al. have developed a capsaicin model for trigeminal sensitization and have applied the model to investigate for example gender-specific differences and the effect of Botulinum Toxin type A.[26-29] However, this is an invasive model where human subjects receive intradermal capsaicin injections to the forehead, resulting in unilateral, throbbing, headache and symptoms of general trigeminal sensitization. Similarly, May et al. applied subcutaneous capsaicin injections in an experimental pain model to examine the neurovascular mechanisms in cluster headache.[30] Participants of this study reported "terrible pain" after the subcutaneous capsaicin injections. We primarily aimed with our study to investigate whether measurement of local blood flow in the forehead region, using laser Doppler imaging, in response to either electrical stimulation, or capsaicin application could be used as a novel model to assess local trigeminal nerve-mediated vasodilation in humans. Whereas application of capsaicin induces CGRP release via activation of TRPV1 channels[23] electrical stimulation induces neuropeptide release without direct activation of these channels.[31] In recent years interest in transdermal drug delivery techniques that enhance permeation of drugs into the skin has increased.[32] One such technique is iontophoresis, where a solution of a compound or drug is put in an electrode reservoir bearing the same charge as the compound or drug of interest. When an electric current is applied to the reservoir, ions set in motion toward the electrode of opposite charge and more easily penetrate into the skin.[32] In this study, as a secondary aim, we wanted to investigate whether iontophoresis of capsaicin might improve its delivery on the human forehead, resulting in a higher response and/or a shorter response latency.

Materials and methods

Subjects

Twenty-four healthy human volunteers (14 women and 10 men) were studied (Table 1). The study protocol was reviewed and approved by the Independent Ethics Committee of Erasmus MC, Rotterdam, The Netherlands and all participants gave written informed consent after explanation of the purpose of the study. The study was conducted in accordance with local laws, the ethical principles of the Declaration of Helsinki, as well as the principles of Good Clinical Practice.

Experimental setup

Experiments were performed in a quiet, temperature-controlled room. Participants were not allowed to use any drugs for 48 hours preceding the experiment and they fasted for at least three hours before the start of the experiment. To investigate short term reproducibility, two research visits with three to four days in between were scheduled for the participants to study potential neuropeptide depletion and receptor desensitization in this period. Some of the subjects participated in multiple experiments; in these cases the time between two subsequent experiments was at least 14 days to exclude any potential interference of previous experiments. The forehead DBF responses induced by topical application of capsaicin, vehicle and

	Male	Female	All
Population, *n*	10	14	24
Age, years	32 (11)	33 (10)	33 (10)
BMI, kg/m^2	23.5 (2.4)	24.0 (2.6)	23.8 (2.5)
BP, mmHg			
Systolic	115 (7)	110 (8)	112 (8)
Diastolic	69 (6)	68 (9)	68 (7)
HR , bpm	68 (6)	64 (10)	66 (9)

Table 1. Demographics of the study population. BMI, body mass index. BP, blood pressure. HR, heart rate. Mean (SD).

physiological saline with and without iontophoresis were measured with a laser Doppler perfusion imaging device (LDPI; Periscan PIM 3 system, Perimed AB, Järfälla, Sweden). Subjects received topical applications of capsaicin (0.06 mg/ml and 6.0 mg/ml), vehicle (a mixture of ethanol 100%, Tween 20 and distilled water; 3:3:4) or physiological saline (0.9% NaCl, 0.5 ml in each case) on the forehead in electrodes with reservoirs specifically designed for this purpose (Perimed AB, Järfälla, Sweden). For electrical stimulation of the forehead skin, iontophoresis of physiological saline was applied.

Capsaicin was obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). We decided to use a methodology and dose similar to that previously reported by Van der Schueren *et al.*[33] In addition, since we intend to use our model also for studies aimed at observing trigeminal hyperreactivity, we furthermore decided to analyze the effect produced by a two log unit lower concentration in an attempt to be able to detect low responses around the activation threshold.

Trigeminal nerve-mediated vasodilatation model

For the trigeminal nerve-mediated vasodilatation model, we used two electrodes with 0.06 and 6.0 mg/ml capsaicin solution and a cumulative successive rate of currents applied to an electrode containing physiological saline (0.2, 0.4, 0.6, 0.8 and 1.0 mA, each lasting one min, with an interval of six min between successive current

intensities). To assess reproducibility these experiments were performed twice at an interval of three to 10 days. The trigeminal nerve-mediated vasodilatation model was evaluated in 12 subjects.

In-depth characterization of the trigeminal nerve-mediated vasodilatation model

Capsaicin iontophoresis was performed on the forehead by application of a small current (0.4 mA, lasting one min) to two electrode chambers, filled with either 0.06 mg/ml or 6.0 mg/ml capsaicin in seven subjects. In these seven subjects, a third electrode was placed on the forehead for iontophoresis of vehicle (0.4 mA, lasting one min).

To evaluate potential effects of electrode location, iontophoresis was performed on two electrodes placed on either the left or right side of the forehead during the same experiment. To investigate iontophoresis-independent effects of vehicle, vehicle was topically applied on the forehead without iontophoresis in four subjects.

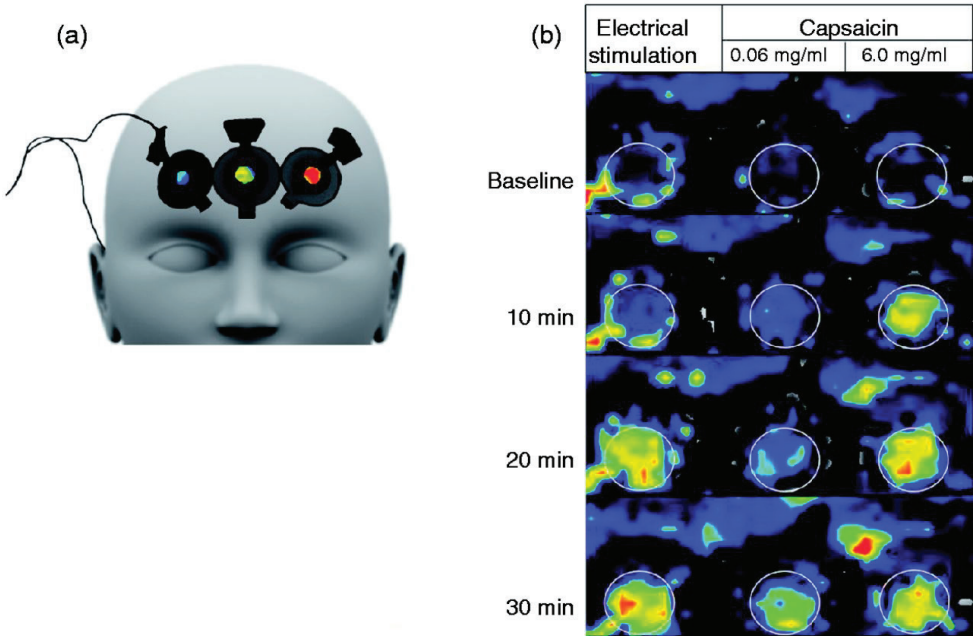


Figure 1. (a) Illustration of electrode placement. From left to right: electrical stimulation, application without iontophoresis 0.06 mg/ml capsaicin and application without iontophoresis of 6.0 mg/ml capsaicin. **(b)** Color-coded perfusion images of forehead dermal bloodflow measured at baseline and 10, 20 and 30 minutes. In the white circles from left to right: electrical stimulation, application without iontophoresis of 0.06 mg/ml capsaicin and application without iontophoresis of 6.0 mg/ml capsaicin. Perfusion is indicated in color code that ranges from black (no perfusion) to red (high perfusion).

Data presentation and statistical evaluation

The DBF values are presented in arbitrary units (a.u.) as means ± S.E.M. Student's *t* test (paired or unpaired where appropriate) was used to compare maximal responses

(E_{max}) to application and iontophoresis of different solutions for groups with $n \geq 7$. Wilcoxon matched pairs test or Mann-Whitney test was applied where appropriate for groups with $n<7$ to compare means. E_{max} was defined as the maximum DBF response in a.u. after subtraction of baseline DBF. Within-subject coefficient of variation (WCV) and intraclass correlation coefficient (ICC) were calculated as measures of reproducibility and agreement. We also performed correlation analysis using the Pearson correlation coefficient and Bland-Altman analysis. The between subject coefficient of variation (BCV) was also calculated. Sample size calculations, given a type I error probability (α) of 0.05 and a power of 80%, were performed without assumptions on an eventual increase in statistical power because of a paired nature of measurements (unpaired model). Calculations were based on the application without iontophoresis of the 6.0 mg/ml capsaicin solution to detect a DBF response difference of 10%, 20%, and 30%. A p value <0.05 was considered to indicate statistical significance.

	Without iontophoresis	With iontophoresis
	DBF E_{max} in a.u.	DBF E_{max} in a.u.
Saline	10 ± 5 * (n=4)	245 ± 26 * (n=12)
Vehicle	153 ± 45 * (n=4)	356 ± 53 * (n=11)
Capsaicin 0.06 mg/ml	187 ± 21 * (n=12)	306 ± 60 * (n=12)
Capsaicin 6.0 mg/ml	459 ± 32 (n=12)	424 ± 43 (n=7)

Table 2. Maximal forehead dermal blood flow responses to physiological saline, vehicle, 0.06 mg/ml capsaicin and 6.0 mg/ml capsaicin with and without iontophoresis.
Iontophoresis with 0.4 mA during 1 min. Grey shaded columns depict parameters of our trigeminal nerve-mediated vasodilatation model. *: Significant difference in Emax between application only and application with iontophoresis.

Results

Forehead application of the capsaicin solutions (0.06 mg/ml and 6.0 mg/ml) with and without iontophoresis and forehead electrical stimulation were well tolerated and side effects other than local redness were not encountered. None of the subjects reported pain.

Trigeminal nerve-mediated vasodilatation model

Application without iontophoresis of the 0.06 mg/ml and the 6.0 mg/ml capsaicin solutions resulted in fast increases in DBF (Figure 1) with an E_{max} of respectively 187±21 and 459±32 a.u. (Table 2). The DBF responses at 30 min, averaged for both visits, were 123±10 a.u. for the low and 370±28 a.u. for the high capsaicin concentration (Figure 2(a)). The time to reach E_{max} was 22±3 and 11±1 min for 0.06 and 6.0 mg/ml capsaicin, respectively. The DBF response to electrical stimulation differed from baseline for each applied current ranging from 0.2 to 1.0 mA (Figure 2(b)). The between-visit DBF responses for either of the two capsaicin solutions or electrical stimulation did not differ (Figure 2(a) and (b)).

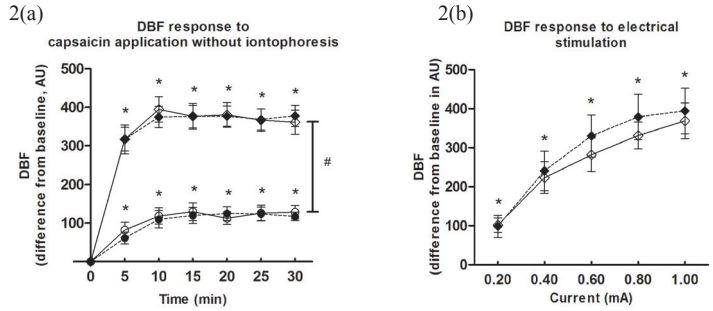


Figure 2. (a) Time course of forehead dermal blood flow responses to 0.06 mg/ml (○) and 6.0 mg/ml (◊) application of capsaicin without iontophoresis during visit 1 (open) and during visit 2 (filled). *n=12*. *: Difference from baseline, t-test: p<0.05. **(b)** Response of forehead dermal blood flow to increasing currents of electrical stimulation of a physiological saline solution during visit 1 (open) and visit 2 (filled). *n=7*.*: Significantly different from baseline.

The application of 6.0 mg/ml capsaicin without iontophoresis and electrical stimulation with 1.0 mA at the end of the cumulative successive rate of currents were the best reproducible experimental approaches of the trigeminal nerve-mediated vasodilatation model, whereas the reproducibility of the DBF response to

Experiment	ICC	WCV (%) (95% CI)
Electrical stimulation (E_{max})		
0.2 mA	0.68	28 (10, 122)
0.4 mA	0.74	33 (0, 48)
0.6 mA	0.84	24 (0,34)
0.8 mA	0.80	15 (0,22)
1.0 mA	0.91	11 (0, 16)
Application capsaicin (E_{max})		
0.06 mg/ml	0.35	40 (20, 53)
6.0 mg/ml	0.96	6 (3, 8)

Table 3. Reproducibility. ICC, Intraclass correlation coefficient indicating strength of agreement; WCV, Within-subject coefficient of variation.

0.06 mg/ml capsaicin application without iontophoresis and electrical stimulation with currents lower than 1.0 mA was fair to good (Table 3 and Figure 3).

The BCV was 38% for 0.06 mg/ml capsaicin, 24% for the 6.0 mg/ml capsaicin and 35% for electrical stimulation with 1.0 mA. We performed sample size calculations based on the application of 6.0 mg/ml capsaicin without iontophoresis. The required group sizes to detect differences in DBF response resulted in groups of *n*=91, 23 and 11 subjects to detect a 10%, 20% or 30% shift, respectively.

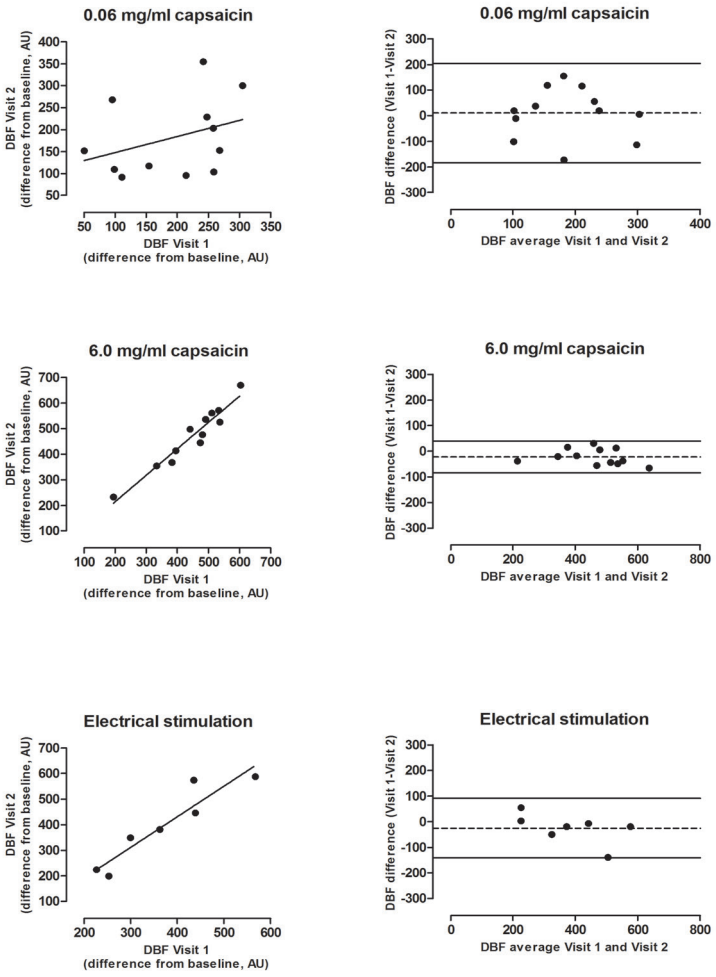


Figure 3. Scatter plots (left-hand side) show correlation between Visit 1 and Visit 2 calculated for DBF responses to 0.06 mg/ml capsaicin application without iontophoresis (upper panel), 6.0 mg/ml capsaicin application without iontophoresis (middle panel) and electrical stimulation (lower panel). 0.06 mg/ml capsaicin: r = 0.35, p = 0.26; 6.0 mg/ml capsaicin: r = 0.96, p <0.0001; electrical stimulation: r = 0.93, p <0.005. **Bland-Altman plots (right-hand side)** show bias (dotted lines) and limits of agreement (solid lines). *n=7-12.*

In-depth characterization of the trigeminal nerve-mediated vasodilatation model

Compared to application only, iontophoresis (0.4 mA) of 0.06 mg/ml capsaicin resulted in a marked increase in forehead DBF (Table 2). In contrast, compared to application only, iontophoresis of the high dose of capsaicin did not cause a further increase in DBF (Table 2). In comparison to application without iontophoresis, the response latency was significantly shorter for application with iontophoresis of 0.06 mg/ml capsaicin (time to E_{max} of 13±2 min), but not for application with iontophoresis of 6.0 mg/ml capsaicin (time to E_{max} of 10±2 min).

Interestingly, application of vehicle, but not saline, without iontophoresis also

caused a modest increase in DBF comparable to the DBF response to application without iontophoresis of 0.06 mg/ml capsaicin, whereas iontophoresis of vehicle and saline caused a further marked increase in DBF (Table 2). Electrode placement site had no influence on the DBF response (E_{max} at 0.4 mA right side of the forehead: 430 ± 58. E_{max} at 0.4 mA left side of the forehead: 456 ± 91).

Discussion

We here present a novel human model for measuring trigeminal nerve-mediated vasodilatation in the forehead skin. With our results we show reproducible DBF responses to topical capsaicin application and electrical stimulation. We have demonstrated that iontophoresis of capsaicin does not result in higher DBF responses or shorter response latency in comparison to application of capsaicin without iontophoresis. In addition to the chemical stimulation with capsaicin, electrical stimulation allows us to stimulate the trigeminal afferents without direct activation of the TRPV1 channel. Advantages of the model we developed are its noninvasiveness, its relative simplicity and its suitability to be used in humans. Furthermore, the topical application of a high and low concentration of capsaicin allows one to study not only high responses, but probably also to distinguish between subjects with a lower or higher threshold response to TRPV1 channel activation. Altogether, the developed model has the potential to be applied for studying trigeminal-nerve-mediated disorders as well as for the evaluation of (new) drugs for these conditions.

Analysis of our data revealed that the DBF responses to the highest intensity of electrical stimulation (1 mA) and the application without iontophoresis of the high concentration of capsaicin (6.0 mg/ml) were well reproducible, with an ICC that indicated an almost perfect strength of agreement for the maximal measured effects. These are also the two experimental approaches of our trigeminal nerve-mediated vasodilatation model with the lowest WCV and the highest (maximally) observed values. The high maximal values, combined with the lower variability, suggest that in these experiments responses were approaching a physiological maximum response. In contrast, the application without iontophoresis of 0.06 mg/ml capsaicin had a low ICC and a high WCV. Electrical stimulation with current intensities lower than 1.0 mA also resulted in lower ICC values and higher WCV values. With the 0.06 mg/ml capsaicin the maximal DBF response was low, indicating an incomplete response. While acknowledging the higher variation of the low capsaicin concentration and electrical stimulation with currents lower than 1.0 mA, we included these parameters into the model as potential methods to detect individual response threshold differences. For example, capsaicin induces gender-specific sensory and vasomotor responses,[26] indicating that individual threshold response differences may exist.

In our experiments we observed no significant difference in DBF responses between male and female subjects (results not shown). Obviously, the number of subjects in our experiment (three males and nine females) is too small to discern potentially small sex differences. Furthermore, we did not select female participants based on

the phase of their menstrual cycle, pre- or postmenopausal status or use of hormonal contraceptives. Sex differences should be explored in future experiments as previous studies have shown that estradiol blood levels may influence vasodilatory responses of capillary blood flow in women.[26, 34]

The DBF response to vehicle application with and without iontophoresis and saline iontophoresis requires discussion. The vehicle consists of a mixture of ethanol 100%, Tween 20 and distilled water (3:3:4). Since distilled water can be considered harmless and Tween 20 has been shown to have a neutralizing effect on toxicity when mixed with skin irritants,[35] the DBF response to application of vehicle without iontophoresis is most likely induced by ethanol. Indeed, ethanol is known to induce TRPV1 channel activation.[36-38] Thus, it seems likely that a part of the blood flow responses to capsaicin in our study were caused by ethanol in its vehicle. This is especially relevant in the case of 0.06 mg/ml capsaicin, where the response was not significantly different from that to vehicle. Notwithstanding, we decided to include 0.06 mg/ml capsaicin solution in our model to have a mode of stimulation (ethanol plus capsaicin) of the TRPV1 channel with a low intensity, which, as described above, may be useful in detecting trigeminal hyperreactivity as observed in disease states.[36] In this light, we have decided to express our responses relative to the baseline responses at the start of the experiment and not relative to the response to the vehicle, since this, as described above, also includes activation of the TRPV1 channel.

The application of vehicle with iontophoresis resulted in considerable increases in DBF. This could be explained by additional current-induced effects of iontophoresis that have been reported previously[31] on top of the DBF response to ethanol. Our exploration of the effects of iontophoresis on capsaicin revealed that DBF responses were higher and the response latency shorter only when applying 0.06 mg/ml capsaicin with iontophoresis to the forehead skin. Given that the DBF responses to 0.06 mg/ml capsaicin were not significantly different to the DBF responses to vehicle, the higher response and shorter latency could be attributed to the current induced vasodilatation. Thus, we conclude that the addition of iontophoresis to the application of the capsaicin had no advantages in our model.

With iontophoresis of physiological saline the current-induced DBF effects were used as an electrical stimulation parameter in our model, complementary to the effects of chemical TRPV1-mediated trigeminal activation by capsaicin. Since the current-induced vasodilatation can be inhibited by local anaesthesia and is reduced by desensitization of C-nociceptive fibres, as demonstrated by Durand et al., we assume that these effects are largely mediated by CGRP and substance P.[31] Contrary to the effects of capsaicin, CGRP release without direct activation of the TRPV1 channel may be studied with electrical stimulation. Thus, similar as described above for the two different concentrations of capsaicin, the application of several current intensities allows to discern both differences in threshold for stimulation, as well as in maximal response.

Admittedly, the involvement of CGRP in the responses to capsaicin, vehicle, as well as electrical stimulation could not unequivocally be proven in the current study since this would require the administration of a CGRP receptor antagonist, as was performed previously in the capsaicin-induced vasodilatation model in the human forearm.[23, 33] Unfortunately, we do not have a CGRP receptor antagonist available that we can administer to human subjects. Similarly, an orally available TRPV1 antagonist would be required to unequivocally assess the involvement of this channel in DBF responses to capsaicin and electrical stimulation.

Comparison with capsaicin-induced dermal vasodilatation in the human forearm

It is interesting to compare the properties of our model with those of the previously described model measuring capsaicin-induced dermal vasodilatation in the human forearm.[33] In the forearm model, maximal vasodilator responses to capsaicin (1000 µg, dose per mm^2 of skin corresponding to our 6.0 mg/ml solution) are observed after 30-40 min. In contrast, in our model maximal responses to application without iontophoresis of this concentration were observed after an average of 11 min. Applying iontophoresis to the 6.0 mg/ml capsaicin solution did not result in a shorter response latency in our model, since E_{max} of 6.0 mg/ml with iontophoresis was reached after 10 min.

Intriguingly, in the forearm model, the vehicle of capsaicin did not induce a discernible increase in vasodilatation, while in our model, as described above, vehicle-induced effects were considerable. These differences between forearm and forehead skin responses to capsaicin are probably related to the well-established variation in skin response to topical irritants that exists throughout the body.[39] Furthermore, evidence exists that especially the face is more sensitive to capsaicin compared to other dermatomes of the body.[40]

In conclusion, using capsaicin topical application and electrical stimulation of the forehead skin, we have developed a noninvasive, reproducible model that can evaluate trigeminal-nerve mediated vasodilation. Future investigations should learn whether the model is suitable to differentiate between subjects with and without trigeminal nerve-mediated conditions including migraine and whether it is useful for the evaluation of novel potential anti-migraine drugs.

References

1. Pietrobon D, Moskowitz MA. Pathophysiology of migraine. Annu Rev Physiol 2013;75:365-391.
2. Edvinsson L, Villalón CM, MaassenVanDenBrink A. Basic mechanisms of migraine and its acute treatment. Pharmacol Ther 2012;136:319-333.
3. Villalón CM, Olesen J. The role of CGRP in the pathophysiology of migraine and efficacy of CGRP receptor antagonists as acute antimigraine drugs. Pharmacol Ther 2009;124:309-323.
4. Olesen J, Burstein R, Ashina M, Tfelt-Hansen P. Origin of pain in migraine: evidence for peripheral sensitisation. Lancet Neurol 2009;8:679-690.
5. Shevel E. The extracranial vascular theory of migraine--a great story confirmed by the facts. Headache 2011;51:409-417.
6. Goadsby PJ, Edvinsson L. The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Ann Neurol 1993;33:48-56.
7. Edvinsson L, Linde M. New drugs in migraine treatment and prophylaxis: telcagepant and topiramate. Lancet 2010;376:645-655.
8. Arulmani U, Maassenvandenbrink A, Villalón CM, Saxena PR. Calcitonin gene-related peptide and its role in migraine pathophysiology. Eur J Pharmacol 2004;500:315-330.
9. Eftekhari S, Salvatore CA, Calamari A, Kane SA, Tajti J, Edvinsson L. Differential distribution of calcitonin gene-related peptide and its receptor components in the human trigeminal ganglion. Neuroscience 2010;169:683-696.
10. Edvinsson L, Ho TW. CGRP receptor antagonism and migraine. Neurotherapeutics 2010;7:164-175.
11. Buzzi MG, Carter WB, Shimizu T, Heath H, 3rd, Moskowitz MA. Dihydroergotamine and sumatriptan attenuate levels of CGRP in plasma in rat superior sagittal sinus during electrical stimulation of the trigeminal ganglion. Neuropharmacology 1991;30:1193-1200.
12. Chan KY, Gupta S, de Vries R, et al. Effects of ionotropic glutamate receptor antagonists on rat dural artery diameter in an intravital microscopy model. Br J Pharmacol 2010;160:1316-1325.
13. Dux M, Santha P, Jancso G. Capsaicin-sensitive neurogenic sensory vasodilatation in the dura mater of the rat. J Physiol 2003;552:859-867.
14. Hou M, Uddman R, Tajti J, Kanje M, Edvinsson L. Capsaicin receptor immunoreactivity in the human trigeminal ganglion. Neurosci Lett 2002;330:223-226.
15. Potenza MA, De Salvatore G, Montagnani M, Serio M, Mitolo-Chieppa D. Vasodilatation induced by capsaicin in rat mesenteric vessels is probably independent of nitric oxide synthesis. Pharmacol Res 1994;30:253-261.
16. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 1997;389:816-824.
17. Kim KS, Yoo HY, Park KS, Kim JK, Zhang YH, Kim SJ. Differential effects of acute hypoxia on the activation of TRPV1 by capsaicin and acidic pH. J Physiol Sci 2012;62:93-103.
18. Kapoor K, Arulmani U, Heiligers JP, et al. Effects of the CGRP receptor antagonist BIBN4096BS on capsaicin-induced carotid haemodynamic changes in anaesthetised pigs. Br J Pharmacol 2003;140:329-338.
19. Villalón CM, Galicia-Carreon J, Gonzalez-Hernandez A, Marichal-Cancino BA, Manrique-Maldonado G, Centurion D. Pharmacological evidence that spinal alpha(2C)- and, to a lesser extent, alpha(2A)-adrenoceptors inhibit capsaicin-induced vasodilatation in the canine external carotid circulation. Eur J Pharmacol 2012;683:204-210.
20. Kapoor K, Arulmani U, Heiligers JP, et al. Effects of BIBN4096BS on cardiac output distribution and on CGRP-induced carotid haemodynamic responses in the pig. Eur J Pharmacol 2003;475:69-77.
21. Williamson DJ, Hargreaves RJ, Hill RG, Shepheard SL. Intravital microscope studies on the effects of neurokinin agonists and calcitonin gene-related peptide on dural vessel diameter in the anaesthetized rat. Cephalalgia 1997;17:518-524.
22. Gupta S, Akerman S, van den Maagdenberg AM, Saxena PR, Goadsby PJ, van den Brink AM. Intravital microscopy on a closed cranial window in mice: a model to study trigeminovascular mechanisms involved in migraine. Cephalalgia 2006;26:1294-1303.
23. Sinclair SR, Kane SA, Van der Schueren BJ, et al. Inhibition of capsaicin-induced increase in dermal blood flow by the oral CGRP receptor antagonist, telcagepant (MK-0974). Br J Clin Pharmacol 2010;69:15-22.
24. De Hoon J, Montieth D, Vermeersch S, et al. Safety, pharmacokinetics, and pharmacodynamics of LY2951742: a monoclonal antibody targeting CGRP. Cephalalgia 2013;33:247.
25. Vermeersch S, Van Hecken A, Abu-Raddad E, et al. Translational pharmacodynamics of CGRP monoclonal antibody LY2951742 in capsaicin-induced dermal blood flow model. Cephalalgia 2013;33:249-250.
26. Gazerani P, Andersen OK, Arendt-Nielsen L. A human experimental capsaicin model for trigeminal sensitization. Gender-specific differences. Pain 2005;118:155-163.

27. Gazerani P, Staahl C, Drewes AM, Arendt-Nielsen L. The effects of Botulinum Toxin type A on capsaicin-evoked pain, flare, and secondary hyperalgesia in an experimental human model of trigeminal sensitization. Pain 2006;122:315-325.
28. Gazerani P, Andersen OK, Arendt-Nielsen L. Site-specific, dose-dependent, and sex-related responses to the experimental pain model induced by intradermal injection of capsaicin to the foreheads and forearms of healthy humans. J Orofac Pain 2007;21:289-302.
29. Gazerani P, Pedersen NS, Staahl C, Drewes AM, Arendt-Nielsen L. Subcutaneous Botulinum toxin type A reduces capsaicin-induced trigeminal pain and vasomotor reactions in human skin. Pain 2009;141:60-69.
30. May A, Bahra A, Buchel C, Frackowiak RS, Goadsby PJ. PET and MRA findings in cluster headache and MRA in experimental pain. Neurology 2000;55:1328-1335.
31. Durand S, Fromy B, Bouye P, Saumet JL, Abraham P. Current-induced vasodilation during water iontophoresis (5 min, 0.10 mA) is delayed from current onset and involves aspirin sensitive mechanisms. J Vasc Res 2002;39:59-71.
32. Alexander A, Dwivedi S, Ajazuddin, Giri TK, Saraf S, Tripathi DK. Approaches for breaking the barriers of drug permeation through transdermal drug delivery. J Control Release 2012;164:26-40.
33. Van der Schueren BJ, de Hoon JN, Vanmolkot FH, et al. Reproducibility of the capsaicin-induced dermal blood flow response as assessed by laser Doppler perfusion imaging. Br J Clin Pharmacol 2007;64:580-590.
34. Gerhardt U, Hillebrand U, Mehrens T, Hohage H. Impact of estradiol blood concentrations on skin capillary Laser Doppler flow in premenopausal women. Int J Cardiol 2000;75:59-64.
35. Benassi L, Bertazzoni G, Magnoni C, Rinaldi M, Fontanesi C, Seidenari S. Decrease in toxic potential of mixed tensides maintained below the critical micelle concentration: an in vitro study. Skin Pharmacol Appl Skin Physiol 2003;16:156-164.
36. Blednov YA, Harris RA. Deletion of vanilloid receptor (TRPV1) in mice alters behavioral effects of ethanol. Neuropharmacology 2009;56:814-820.
37. Trevisani M, Smart D, Gunthorpe MJ, et al. Ethanol elicits and potentiates nociceptor responses via the vanilloid receptor-1. Nat Neurosci 2002;5:546-551.
38. Nicoletti P, Trevisani M, Manconi M, et al. Ethanol causes neurogenic vasodilation by TRPV1 activation and CGRP release in the trigeminovascular system of the guinea pig. Cephalalgia 2008;28:9-17.
39. Liu M, Max MB, Robinovitz E, Gracely RH, Bennett GJ. The human capsaicin model of allodynia and hyperalgesia: sources of variability and methods for reduction. J Pain Symptom Manage 1998;16:10-20.
40. Green BG. Measurement of sensory irritation of the skin. American Journal of Contact Dermatitis 2000;11:170-180.

CHAPTER 6

A trigeminovascular biomarker for antimigraine drugs; randomized trial with sumatriptan

Submitted

K. Ibrahimi, A.H.J. Danser, G.M. Terwindt, A.H. van den Meiracker & A. MaassenVanDenBrink

Abstract

Current antimigraine drugs like sumatriptan are believed, besides their direct vasoconstrictive effect, to inhibit calcitonin gene-related peptide (CGRP) release from trigeminal nerve endings during a migraine attack. **Objective:** To establish a biomarker for the CGRP-interfering effect of antimigraine drugs. **Methods:** We quantified the effect of sumatriptan on the trigeminal nerve-mediated rise of dermal blood flow (DBF) of the forehead, induced by capsaicin application (0.6 mg/ml) and electrical stimulation (0.2-1.0 mA), in a randomized, double-blind, placebo-controlled cross-over study in healthy male (n=11, age±SD: 29±8 yrs) and female (n=11, 32±7 yrs) subjects. **Results:** DBF responses to capsaicin were significantly attenuated by sumatriptan (ΔDBF, mean±SEM: 82±18 A.U., p=0.0002), but not by placebo (ΔDBF: 21±12 A.U., p=0.1026). **Conclusion:** We demonstrated that sumatriptan inhibits increases in dermal blood flow, induced by the release of CGRP. Thus, our model may be used as a biomarker to establish the trigeminovascular effects of (future) antimigraine drugs, such as CGRP receptor antagonists or antibodies directed against CGRP or its receptor.

Introduction

Understanding the mechanism of action of drugs helps to unravel underlying disease pathophysiology. Although the antimigraine drug sumatriptan, one of 7 triptans currently available, was introduced about two decades ago, its precise action remains elusive. Besides direct vasoconstriction, mediated by 5-HT_{1B} receptors,[1] triptans may also presynaptically inhibit release of the key neuropeptide in migraine, calcitonin gene-related peptide (CGRP),[1] most likely via $5HT_{1D(/1F)}$ receptors.[1] Despite its success, 14-30% of patients do not respond to subcutaneous sumatriptan.[2] To understand the mechanism behind therapeutic nonresponse in migraine, a non-invasive biomarker, e.g., based on sumatriptan's effect on trigeminal CGRP release, is needed, especially now that antagonists and antibodies directed against CGRP or its receptor are developed.[3] Early trials indicate that they are not effective in all patients, reinforcing the importance of investigating their trigeminovascular effects.[3] To identify such a biomarker, we performed a randomized, double-blind, placebo-controlled cross-over study in healthy volunteers to investigate the blocking effect of sumatriptan, the most established acute antimigraine therapy,[1] on the CGRP-mediated rise of capsaicin-induced dermal blood flow (DBF). We hypothesized that sumatriptan inhibits the capsaicin-induced DBF increases. To exclude physiological antagonism by direct vasoconstriction to sumatriptan, we used electrical stimulation (ES) of trigeminal afferents, increasing DBF partly via CGRP, but also via other mechanisms such as substance P and prostaglandin synthesis.[4]

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

The study protocol was reviewed and approved by the Ethics Committee of Erasmus MC, Rotterdam, and registered at the Netherlands Trial Register (ID:NTR4171). All participants gave written informed consent and the study was conducted in

accordance with local laws, the ethical principles of the Declaration of Helsinki and the principles of Good Clinical Practice.

Design and procedures

Healthy non-smoking male and female subjects, aged 18-50 years (BMI 18-30 kg/m^2), without history of cardiovascular disease or use of medication, were eligible. Female subjects were using oral contraceptives and continued their use without the "stop week" to avoid the confounding influence of varying steroid levels.[5] The validated LUMINA questionnaire[6] was used to exclude migraineurs.
Subjects were investigated between September and November 2013 in a quiet, temperature-controlled room. The subjects rested supine and did not speak. For all subjects, two visits were scheduled with a one-week washout in between and during the same time of the day. Participants refrained from using vasoactive drugs (including NSAIDs) for >48 hours, and from consuming alcohol, caffeine-containing beverages and chocolate for at >3 hours prior to the start of experiments. A light meal 3 hours before the start of the experiments was allowed.

Blood pressure and DBF responses to capsaicin (0.6 mg/ml) and ES (0.2 mA-1.0 mA) were measured before and 30 min after (different locations on the forehead used for each measurement) either placebo or sumatriptan during the two research visits. Capsaicin- and ES-induced dermal forehead vasodilatation, measured with a laser Doppler perfusion imager, is described in detail in our model validation paper.[4] Subjects were injected subcutaneously with sumatriptan (6 mg; a gift of GlaxoSmithKline, Zeist, The Netherlands) or placebo (saline) at the lateral side of the upper thigh. Randomization, allocation and blinding was coordinated by the Erasmus MC pharmacy. 'Insulin syringes' with placebo or sumatriptan were prepared and labelled on the experiment day in a room inaccessible to the researchers. With a questionnaire at the end of each experiment, side effects were registered.

Statistical analysis

Sample size was based on detection of a 25% decrease in capsaicin-induced DBF after sumatriptan,[4] at 5% significance (two-tailed) with 80% power. Baseline and maximal DBF (E_{max}) response, expressed in Arbitrary Units (A.U.), to capsaicin and ES, was calculated before and after sumatriptan or placebo. Differences in DBF responses to capsaicin (our primary endpoint) and ES during sumatriptan and placebo were calculated for each subject. Responses to sumatriptan and placebo were compared within subjects using Student's paired t-test. Current response curve of the ES sequence (0.2 mA-1.0 mA) were analyzed with repeated measures ANOVA. $P<0.05$ was considered to indicate significance.

Results

Twenty-two healthy volunteers (11 males) participated (Figure 1 and Table 1). Since results were similar between sexes, data were pooled.
Systolic and diastolic blood pressure significantly increased after sumatriptan, while

	Male	Female	All
Population, n	11	11	22
Age, years	29 (8)	32 (7)	30 (8)
BMI, kg/m^2	23.0 (1.6)	23.8 (3.4)	23.4 (2.6)
BP, mmHG			
Systolic	114 (13)	110 (8)	112 (11)
Diastolic	66 (9)	66 (6)	66 (7)
HR, bpm	58 (10)	55 (7)	57 (8)

Table 1. Demographics of the study population. BMI, body mass index. BP, blood pressure measured recumbent. HR, heart rate. Mean ± (SD).

diastolic blood pressure slightly increased after placebo (Table 2).

Baseline DBF decreased after sumatriptan (ΔDBF: 11±4 A.U., p=0.0126), but not after placebo. The DBF response to capsaicin decreased after sumatriptan (ΔDBF: 82±18 A.U., p=0.0002) but not after placebo (ΔDBF: 21±12 A.U., p=0.1026. Figure 2(A)). ΔDBF sumatriptan was different from ΔDBF placebo (p=0.0036). A sub-analysis showed that in 5/22 volunteers the DBF response to capsaicin did not decrease after sumatriptan. The DBF response to ES was not affected by either sumatriptan or placebo (Figure 2(B)).
Pain at the injection site was the most reported side effect after sumatriptan (15/22) compared to placebo (6/22), followed by a general heavy pressing feeling (14/22 after sumatriptan versus 1/22 after placebo). 8/22 volunteers reported stiffening of

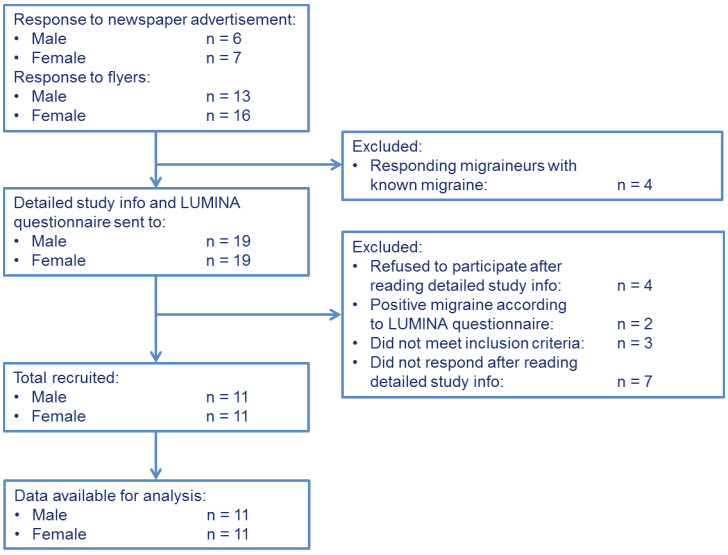


Figure 1. Recruitment flow diagram

	Pre s.c. inject.	Post s.c. inject.	Change	p
Placebo, BP, mmHg				
Systolic	111 (9)	110 (9)	-1	0.7707
Diastolic	66 (7)	68 (6)	2	0.0369
HR, bpm	56 (8)	55 (9)	-1	0.3646
Sumatriptan, BP, mmHg				
Systolic	112 (10)	118 (7)	6	0.0009
Diastolic	65 (7)	76 (6)	11*	< 0.0001
HR, bpm	58 (9)	56 (11)	- 2	0.1349

Table 2. Blood pressure during experiments. BP, blood pressure measured recumbent after 15 min acclimatization. HR, heart rate. Mean ± (SD). * Significant increase in diastolic BP after sumatriptan compared to increase in diastolic BP after placebo.

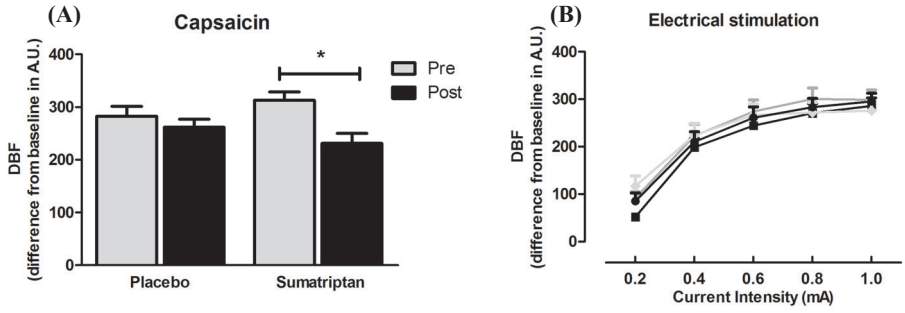


Figure 2. Forehead dermal blood flow responses to capsaicin and ES. (A): maximal DBF response to capsaicin before (grey bars, Pre) and after (black bars, Post) placebo and sumatriptan. **(B):** maximal DBF responses to ES with an increasing stimulation current before placebo (grey triangle), after placebo (black circle), before sumatriptan (grey diamond) and after sumatriptan (black square). Data are presented as mean ± SEM. * Significant decrease in DBF response to capsaicin after sumatriptan.

the neck muscles after sumatriptan, this was not reported after placebo. Anxiety was reported by 4/22 volunteers after sumatriptan and by none after placebo.

Discussion

Our major finding is the inhibition of capsaicin-induced vasodilatory DBF responses after sumatriptan but not after ES, confirming our hypothesis that sumatriptan inhibits the release of CGRP, the key neuropeptide in migraine. Blood pressure changes[1] and side effects were as expected. While side effects may have reduced the blinding efficiency in some subjects during recording, a potential limitation of our study, it is important to highlight that data analysis was performed completely blinded. Although our study was performed in subjects without migraine, the proportion (5/22, i.e., 23%) that did not display inhibition of capsaicin-induced DBF increases after sumatriptan, is in the same range as the number of non-responders to subcutaneous sumatriptan.[2] Future studies should elucidate whether clinical non-

responders to subcutaneous sumatriptan do correlate to the non-responders in our model, although the design and execution of such a trial is complicated due to the 7 different triptans now available.[7]

The decrease in baseline DBF after sumatriptan indicates direct vasoconstriction, in agreement with several preclinical[8] and clinical (magnetic resonance angiography, MRA) studies.[9, 10] Nevertheless, not all MRA studies are consistent. A study with provoked migraine in patients demonstrated sumatriptan-induced constriction of the middle meningeal but not the middle cerebral artery, while sumatriptan constricted both arteries in healthy volunteers.[9] In spontaneous migraine attacks, sumatriptan constricted extracranial arteries and the cavernous portion of the internal carotid artery.[10] The constriction of the cranial vasculature in these studies did not uniformly coincide with the resolution of pain, suggesting involvement of additional mechanisms in the efficacy of triptans. Indeed, several studies suggest that part of the action of triptans is mediated via inhibition of neuropeptide release. For example, elevated serum levels of CGRP were normalized after sumatriptan.[11] As DBF responses to capsaicin are almost entirely inhibited by CGRP receptor blockade,[12] the inhibition of capsaicin-induced DBF responses by sumatriptan in our experiments clearly shows that triptans may inhibit CGRP release. The 26% decrease in DBF response after sumatriptan in our study may seem modest, but is similar to the 33% decrease in jugular CGRP in migraineurs using subcutaneous sumatriptan as reported earlier.[11]

Hypothetically, the decrease of capsaicin-induced DBF could have been due to physiological antagonism (direct vasoconstrictor effect of sumatriptan), as described above. However, the responses to ES should then have been affected by sumatriptan as well, which was not the case (Figure 1B). Thus, we conclude that the inhibition of capsaicin-induced DBF after sumatriptan is due to its effects on neuropeptide release, most likely of CGRP.

In conclusion, trigeminovascular effects of future antimigraine drugs, such as antagonists or antibodies directed against CGRP or its receptor, can be investigated with our human model.

References

1. Chan KY, Vermeersch S, de Hoon J, Villalón CM, MaassenVanDenBrink A. Potential mechanisms of prospective antimigraine drugs: a focus on vascular (side) effects. Pharmacology & therapeutics 2011;129:332-351.
2. Plosker GL, McTavish D. Sumatriptan. A reappraisal of its pharmacology and therapeutic efficacy in the acute treatment of migraine and cluster headache. Drugs 1994;47:622-651.
3. Karsan N, Goadsby PJ. CGRP mechanism antagonists and migraine management. Curr Neurol Neurosci Rep 2015;15:547.
4. Ibrahimi K, Vermeersch S, Danser A, et al. Development of an experimental model to study trigeminal nerve-mediated vasodilation on the human forehead. Cephalalgia 2014;34:514-522.
5. Ibrahimi K, van Oosterhout WP, van Dorp W, et al. Reduced trigeminovascular cyclicity in patients with menstrually related migraine. Neurology 2015;84:125-131.
6. van Oosterhout WP, Weller CM, Stam AH, et al. Validation of the web-based LUMINA questionnaire for recruiting large cohorts of migraineurs. Cephalalgia 2011;31:1359-1367.
7. Viana M, Genazzani AA, Terrazzino S, Nappi G, Goadsby PJ. Triptan nonresponders: do they exist and who are they? Cephalalgia 2013;33:891-896.
8. MaassenVanDenBrink A, Reekers M, Bax WA, Ferrari MD, Saxena PR. Coronary side-effect potential of current and prospective antimigraine drugs. Circulation 1998;98:25-30.
9. Ashina M, Hansen JM, Olesen J. Pearls and pitfalls in human pharmacological models of migraine: 30 years' experience. Cephalalgia 2013;33:540-553.
10. Amin FM, Asghar MS, Hougaard A, et al. Magnetic resonance angiography of intracranial and extracranial arteries in patients with spontaneous migraine without aura: a cross-sectional study. Lancet Neurol 2013;12:454-461.
11. Goadsby PJ, Edvinsson L. The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Ann Neurol 1993;33:48-56.
12. Sinclair SR, Kane SA, Van der Schueren BJ, et al. Inhibition of capsaicin-induced increase in dermal blood flow by the oral CGRP receptor antagonist, telcagepant (MK-0974). Br J Clin Pharmacol 2010;69:15-22.

CHAPTER 7

Local thermal hyperaemia and flow-mediated dilatation as measures of endothelial function are unrelated

Work in progress

K. Ibrahimi, Y. de Graaf, R. Draijer, A.H.J. Danser,
A. MaassenVanDenBrink & A.H. van den Meiracker

Abstract

Background: Flow-mediated dilatation (FMD) of the brachial artery to determine endothelial function is a well-accepted, but investigator-demanding method. Local thermal hyperaemia (LTH) of the skin measured with a laser Doppler flow imager is less demanding and may be a suitable alternative to determine endothelial function. We investigated the reproducibility and NO-dependency of LTH and its correlation with FMD. **Methods:** FMD and LTH were measured in healthy non-smoking (n=14) and smoking (n=7) men on two occasions, one week apart. Local dermal application of L-NMMA by means of iontophoresis was used to measure the nitric oxide (NO)-dependency of LTH. **Results:** The coefficient of variation of the LTH peak and plateau response was 16% and 21% and of the FMD response 12%. The LTH peak response tended to be lower, while the LTH-plateau response was significantly lower in smokers than non-smokers, whereas The FMD response in smokers and non-smokers was similar. Both in non-smokers and smokers the LTH-peak and plateau response was inhibited by L-NMMA, but the inhibition was much less pronounced in smokers. **Conclusions:** 1) The reproducibility of LTH is not better than that of FMD. 2) Both the LTH peak and plateau response are in part mediated by increased NO release. 3) This NO-dependency of the LTH response apparently is diminished in smokers. 4) Information about endothelial function obtained by FMD and LTH measurements may not be interchangeable.

Introduction

The microcirculation is embedded in all organs and serves to provide nutrients and oxygen and to remove metabolic end products e.g. CO_2. In addition, it is essential for fluid exchange and delivery of hormones. Flow-mediated vasodilation (FMD) is a common, clinically applied tool to assess endothelial vascular function. FMD is usually assessed at the brachial artery and represents the ability of this artery to dilate in response to an increase in blood flow induced by an ischemic period of 5 minutes. Evidence indicates that release of nitric oxide (NO) is largely responsible for flow-induced vasodilatation in human peripheral conduit arteries.[1] This accords well with studies showing that low FMD values associate with cardiovascular risk factors that are known to impair NO bioavailability.[2] Conversely, cholesterol-lowering and anti-hypertensive treatment improve FMD,[3] but also nutritional interventions like consumption of cocoa, tea, and beetroot juice appear to be effective in improving FMD.[4-6]

The relationship between vascular function in the relatively large conduit brachial artery and vascular function in the microcirculation has been studied occasionally, yielding divergent results.[7-9] The association between the muscarinic receptor-stimulated increase in microvascular muscle perfusion in the forearm and FMD is weak, despite the fact that NO-dependent vasodilation underlies both measurements.[7, 8] The correlation between FMD and peripheral artery tonometry (EndoPat) values is moderate.[9] In contrast, FMD and increases in skin perfusion caused by iontophoretic application of acetylcholine appear to be closely related.[10] Local thermal hyperaemia (LTH) causes a temperature-dependent sustained increase in skin blood flow that

is largely NO-dependent and therefore may be considered as the microvascular counterpart of FMD.[11] Combined with the Periscan PIM 3 system, which measures skin blood flow by a laser Doppler technique, the reproducibility of this technique has been reported to be excellent.[11] Compared to FMD measurements, measurement of the thermally-induced increase in dermal blood flow with the Periscan PIM 3 system is easier to perform and less operator-dependent. It may therefore be a welcome alternative of FMD. Dermal post-occlusive reactive hyperaemia (PORH) is also a measure that can be used to assess microvascular function, and like FMD, it is measured after a period of ischemia.[12] In the present study we compared FMD with LTH and PORH in healthy non-smoking and smoking men. We included a group of smokers in order to obtain a relatively wide range of FMD values. In addition we investigated to what extent the increase in skin blood flow in response to local heating is NO-dependent by locally applying the NO synthase inhibitor N^G-monomethyl-l-arginine (L-NMMA), using iontophoresis.[13-17] We expected a close correlation between LTH and FMD responses since both techniques are presumed to be highly NO-dependent. We also hypothesized that the PORH response would correlate with FMD as well as with LTH. PORH may correlate with FMD as ischemia-induced increases in blood flow underlie both measurements. The expected correlation between PORH and LTH would be based on the involvement of endothelium-derived hyperpolarizing factor (EDHF) pathway activation during both PORH and LTH.[18, 19]

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

The study protocol was reviewed and approved by the Independent Ethics Committee of the Erasmus MC, Rotterdam, The Netherlands. All participants gave written informed consent after explanation of the study, which was conducted in accordance with local laws, the ethical principles of the Declaration of Helsinki, as well as the principles of Good Clinical Practice.

Design and procedures

For our study we included healthy male non-smokers and smokers. Women were excluded to avoid confounding due to the influence of hormonal changes related to the menstrual cycle on vascular function. All participants were recruited via flyers distributed in the Erasmus MC, the recruitment agency Link2Trials (Hilversum, The Netherlands) or via advertisements in local (Rotterdam, The Netherlands) newspapers. Men in general good health, aged between 25-65, with a body mass index (BMI) between 18-30 kg/m^2 and with a systolic blood pressure <160 mmHg were included. Non-smokers that had been non-smoking for at least 5 years and smokers that had been smoking for at least 5 years were eligible to be included. A history of cardiovascular disease, use of vasoactive drugs and drug and/or alcohol abuse were exclusion criteria. Subjects were not allowed to drink wine or eat chocolate 24 hours prior to the study and caffeine-containing food and beverages were not allowed 6 hours prior to the study. To investigate reproducibility, 2 research visits with 7 days in-between were scheduled for all test subjects. For each individual

subject measurements were performed at the same time of the day, in a temperature controlled room, while subjects had fasted for 3 hours. All measurements were performed with the subjects in the supine position.

LTH Measurement

We determined the skin LTH response at the volar site of the right forearm using the PeriScan PIM 3 system (Perimed AB Sweden) and SHP3 heating probes (Moor Instruments Ltd) to induce local heating. A drug delivery electrode containing a 0.5 ml reservoir and a control electrode (Perimed AB Sweden) were placed on the forearm at least 15 cm apart. The drug delivery electrode was filled with a 2% solution of L-NMMA (Clinalfa, Laufelfingen, Switzerland) in sterile water (Fresenius Kabi, Schelle, Belgium). With the positive lead of the iontophoresis device (Periont 382b, Perimed, Sweden) connected to the electrode containing L-NMMA, subsequently a current (100 μA for 15 minutes) was applied to this electrode. After iontophoresis, the electrodes were removed and the two heat probes were attached to the skin using two-sided adhesive tape and filled with water. One heat probe was placed exactly over the area of the L-NMMA iontophoresis electrode and one on a non-treated area. Before the start of the heating protocol, the heater was set to 33°C for 5 minutes to standardize the skin temperature. Subsequently, baseline skin blood flow was recorded for 5 minutes at 33°C followed by a recording at 40°C for 35 minutes.

FMD measurement

The FMD was measured with the SONIX TOUCH ultra-sound system (Ultrasonix, Richmond, Canada) with the L14-5/38 transducer (Ultrasonix, Richmond, Canada). A blood pressure cuff was placed around the left forearm immediately below the antecubital crease. The echo probe was positioned on the dorsal site of the upper arm approximately 5 cm from elbow in a way that a clear longitudinal section of the brachial artery remained visible. The baseline blood vessel diameter was recorded for 1 minute. Subsequently, the cuff was rapidly inflated to 260-280 mmHg. This cuff pressure was maintained for 5 minutes. After 5 minutes occlusion, the cuff pressure was released and the diameter of the brachial artery was recorded for 4 minutes. The FMD data were processed with Cardiovascular Suite™ version 2.0, Quipu, Pisa, Italy.

PORH measurement

Simultaneously with the FMD measurements we also measured the PORH response that occurred after the release of the pressure cuff used for the FMD measurements. PORH was measured at the volar site of the left forearm just under the pressure cuff, using a laser speckle imager (FLPI, Moor Instruments Ltd, Axminster, United Kingdom).

Statistical analysis

LTH was expressed as change in a.u. from baseline. LTH peak was defined as the maximal dermal blood flow (DBF) response within the first 10 minutes of heating

to 40°C. The LTH plateau phase was defined as the average DBF response in a.u. during the last 5 minutes of the 35 minutes-lasting 40°C heating period. FMD was expressed as the maximal percentage increase in vessel diameter during the reactive hyperaemia relative to the diameter before occlusion. PORH, quantified in a.u., was expressed as the DBF percentage change from baseline. Values are expressed as mean ± SEM unless indicated otherwise. Correlations between FMD, LTH and PORH were determined by Pearson's coefficient of correlation (r). FMD, LTH and PORH response differences between smoking and non-smoking subjects were compared with Student's unpaired t-test, while differences within groups were examined by Student's paired t-test. Coefficient of variation (CV) and intraclass correlation coefficients (ICC) were calculated as measures of reproducibility and agreement. Two tailed P-values < 0.05 were considered to indicate a statistically significant difference.

Results

Subjects

A total of 14 non-smoking and 7 smoking volunteers were included. The smoking subjects had been smoking for an average of 23 ± 3 years. Their current smoking amounted to 14 ±1 cigarettes per day. Demographic characteristics such as age, BMI, waist-to-hip ratio, blood pressure and heart rate were similar in non-smokers and smokers. Baseline values of LTH, FMD and PORH did not differ between non-smokers and smokers (Table 1).

	Non-Smokers	Smokers
Population, n	14	7
Age, years	38(13)	40(9)
BMI, kg/m^2	24(2)	23(3)
WHR	0.89(0.09)	0.88(0.05)
BP, mmHg		
Systolic	113(4)	115(4)
Diastolic	66(5)	69(4)
MAP	83(4)	87(4)
HR, bpm	52(6)	57(5)
LTH BL (a.u.)	27(9)	37(18)
FMD BL (pre-occlusion diameter, mm)	4.30(0.43)	4.26(0.40)
PORH BL (a.u.)	70(37)	85(29)

Table 1. Demographics of the study population and baseline values of measurements. BMI, body mass index. WHR, waist to hip ratio. BP, blood pressure. MAP, mean arterial pressure. HR, heart rate. BL, baseline. Mean (± SD).

LTH measurement

Both the LTH peak and the LTH plateau response were well reproducible, with the lowest CV (16%) and highest ICC (0.80) for the peak LTH response (Table 2 and Figure 1A-1D). The LTH peak DBF response was inhibited by L-NMMA administration in non-smokers and in smokers (% inhibition non-smokers vs. smokers: 43 ±3% . vs. 23 ± 4%, P<0.0001). Likewise the LTH plateau DBF response was inhibited in non-smokers by 73 ±2 % and in smokers by 60 ± 3% (P<0.0001, Figure 1E and 1F). Compared to non-smokers the LTH plateau phase, but not the LTH peak response, in smokers was significantly lower with respective values of 158 ± 12 and 109 ± 22 a.u., P=0.045 (Figure 1E and 1F).

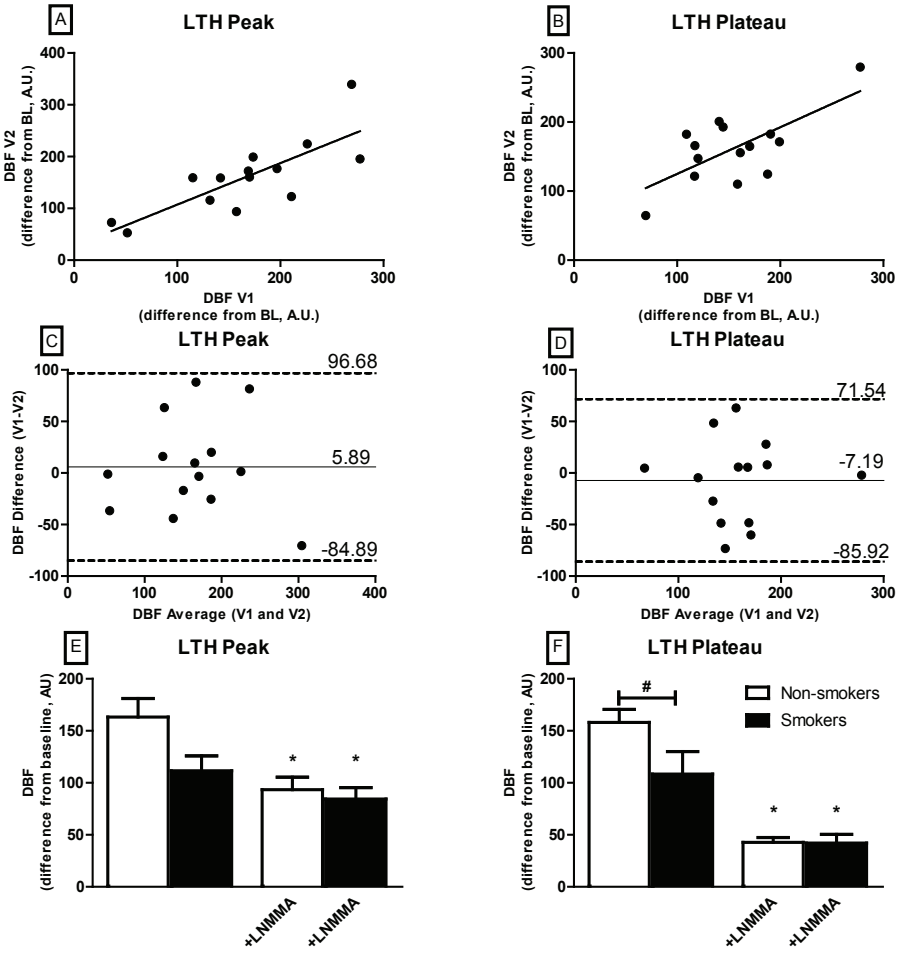


Figure 1. LTH reproducibility and LTH response non-smokers vs. smokers. Reproducibility assessed in non-smokers (*n*=14). Correlation between visit 1 (V1) and visit 2 (V2) LTH peak **(A)** and plateau **(B)** DBF responses (Peak: r = 0.79, p = 0.0006; plateau: r = 0.68, p = 0.007). Bland-Altman plots **(C and D)** show bias (solid line) and 95% limits of agreement (dashed lines) for peak **(C)** and plateau **(D)** LTH DBF responses. The effect of smoking and LNMMA administration on peak **(E)** and plateau **(F)** LTH response. * Significant decrease in LTH response after LNMMA administration. # Significantly lower LTH response smokers (*n*=7) compared to non-smokers.

FMD measurement

Like LTH, FMD was well reproducible with a CV of 12% and an ICC of 0.87 (Table 2). The FMD of non-smokers (4.8 ± 0.6%) did not differ from the FMD of smokers (6.1 ± 1.3%, P=0.283, Figure 2).

	Reproducibility		Correlation (r)		
	CV(%)	ICC	FMD	PORH Peak	PORH AUC
LTH Peak	16	0.80*	0.22	0.27	0.03
LTH Plateau	21	0.63*	0.12	0.56*	0.38
FMD	12	0.87*	-	0.36	0.64*
PORH Peak	11	0.90*	-	-	-
PORH AUC	10	0.89*	-	-	-

Table 2. Reproducibility and Correlation. Reproducibility of the different measurements in non-smokers (n=14). Correlation between different measurements in nonsmokers (n=14) and smokers (n=7). * Statistically significant intraclass correlation coefficient (ICC) or correlation coefficient.

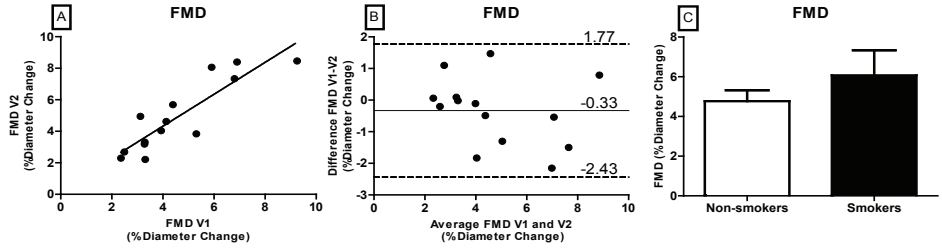


Figure 2. FMD reproducibility and FMD of non-smokers vs. smokers. FMD (% increase in vessel diameter during hyperaemia relative to the diameter before occlusion) reproducibility assessed in non-smokers (n=14). Correlation **(A)** between visit 1 (V1) and visit 2 (V2) FMD r = 0.88, p <0.0001. Bland-Altman plot **(B)** shows bias (solid line) and 95% limits of agreement (dashed lines) between FMD V1 and V2. FMD responses **(C)** of non-smokers (*n*=14) and smokers (*n*=7).

PORH measurement

PORH was also well reproducible with the lowest CV for PORH AUC (CV= 10%) and the highest level of agreement for PORH peak (ICC 0.90, Table 2 and Figure 3A-3D). The results of the PORH contained 1 outlier that we could not exclude based on the other study measures, as they were normal. The outlier response did not have a significant effect on the PORH outcome measures. The PORH peak response or PORH AUC between non-smokers and smokers did not differ.

Correlations between LTH, FMD and PORH measurements

The correlations between different measures of vascular function for all 21 volunteers are displayed in Table 2. For the analysis of correlation between measures we pooled the data from non-smokers and smokers, because there was only significant difference in LTH response between both groups. By pooling the data we also increased our range of values, benefitting the correlation analysis. The LTH plateau phase correlated with the PORH peak response (r = 0.56, P=0.008), whereas FMD

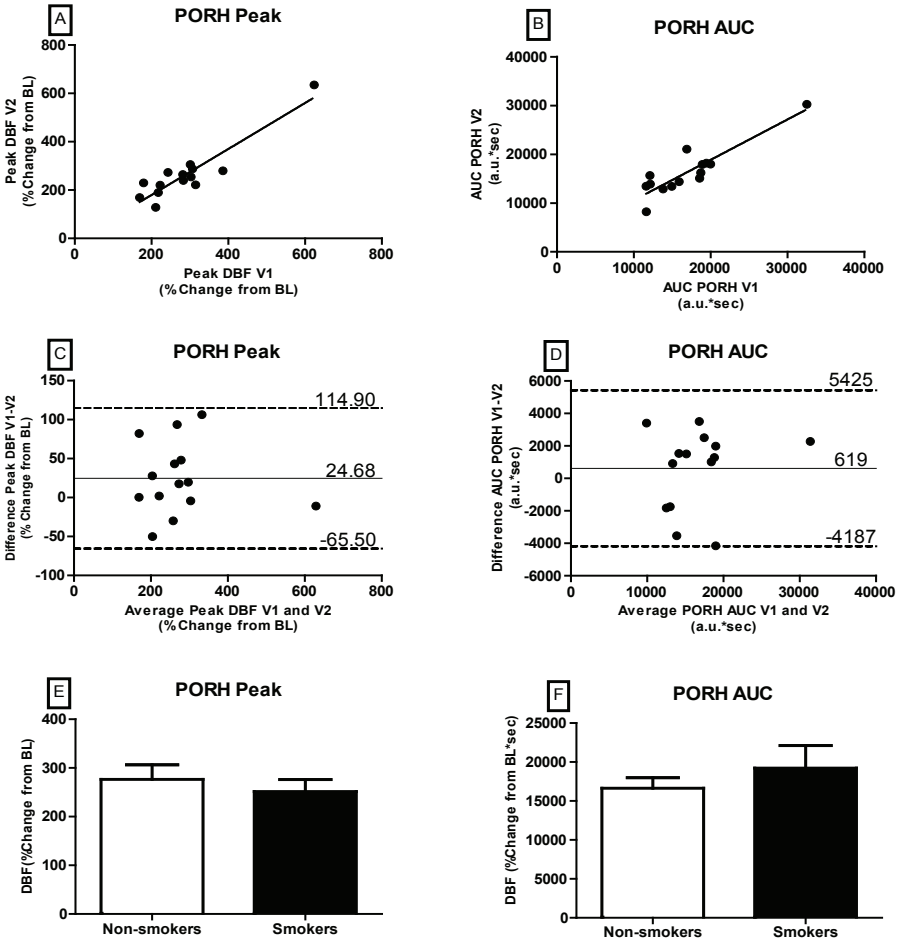


Figure 3. PORH reproducibility and PORH of non-smokers vs. smokers. Reproducibility assessed in non-smokers (*n*=14). Correlation between visit 1 (V1) and visit 2 (V2) PORH peak **(A)** and AUC **(B)** DBF responses (Peak: r = 0.93, p < 0.0001; AUC: r = 0.89, p < 0.0001). Bland-Altman plots **(C and D)** show bias (solid line) and 95% limits of agreement (dashed lines) for peak **(C)** and AUC **(D)** AUC DBF responses. The effect of smoking on peak **(E)** and AUC **(F)** of PORH DBF responses.

and PORH AUC were closely correlated (r = 0.64, P = 0.002), but all other measures of vascular function were unrelated.

Discussion

The main findings of our study are that 1) all measurements of vascular function are well reproducible with the best reproducibility of PORH, 2) the LTH and FMD measurements are unrelated, 3) LTH and FMD measurements are both correlated with PORH measurements and 4) of the three measurements only the LTH measurement shows a distinction in microvascular function between smokers and non-smokers, with a lower response in smokers.

It has been well accepted that endothelial dysfunction, reflected by diminished NO-dependent vasodilation, is an early marker of vascular damage, preceding the process of atherosclerosis and cardiovascular disease.[1, 20] Hence several non-invasive techniques with the potential to measure endothelial dysfunction have been developed. Of these techniques FMD has been most frequently applied and its value for predicting vascular disease is well established.[20] A disadvantage of FMD is its operator-dependency, requiring an experienced technician to obtain reliable results [21]. LTH may be an alternative measurement of NO-dependent endothelial function at the level of the microcirculation.[10, 22] LTH is characterised by 2 phases; the initial peak blood flow during the first 10 minutes followed by a plateau phase after approximately 30 minutes. The peak DBF response appears to be largely mediated by the skin sensory nerves and the plateau phase by NO.[23, 24] Iontophoresis with L-NMMA in our study indicates that over 40% of the LTH peak response and over 70% of the LTH-plateau phase is NO-dependent. Because LTH measurements are relatively easy to perform and the relative magnitude of the response is much larger than for FMD, on forehand we had expected a better reproducibility of LTH. This appeared not be the case. With a CV of 16% for the LTH peak and a CV of 21% for the LTH plateau phase the reproducibility of LTH was somewhat lower than the reproducibility of FMD (CV 12%). It must be emphasized that in the current study FMD was measured by a highly qualified investigator (YdG).

Contrary to our hypothesis neither the LTH peak nor the LTH plateau responses correlated with the FMD response. Given that both responses are largely NO-dependent, this absent association, although in agreement with previous work applying a similar group size,[25] is surprising. Is should be remarked that the dynamic range of LTH and FMD is quite different; while with LTH the DBF response can increase by 900% from baseline DBF, with FMD only diameter changes of up to 12% can be achieved. Furthermore, the LTH measurement is restricted to the skin superficial microcirculation, while FMD is measured in a conductance artery.

In line with our hypothesis, because post-ischemic hyperaemia underlies both measurements, FMD and PORH responses were correlated. Confirming our hypothesis, the LTH plateau phase and PORH peak response were also correlated. Indeed, during both peak PORH and PORH time course as well as during LTH peak response and LTH plateau phase the EDHF pathway is activated.[4, 7] Although the PORH response depends on multiple mechanisms, it has been demonstrated that this response is diminished in patients with coronary artery disease, indicating an association between coronary macrovascular and skin microvascular disease.[12]

Importantly, LTH responses in our study were lower in smokers than in non-smokers. This was mainly due to a diminished NO-dependent vasodilation in smokers as the difference in response between smokers and non-smokers almost completely abolished by local application of L-NMMA (Fig. 1). The FMD and PORH measurements did not detect a difference in vascular function between smokers and non-smokers. Our findings therefore suggest that LTH is more sensitive than FMD or

PORH measurements to detect changes in (NO-dependent) vascular function. With LTH measurements impaired skin microvascular function has also been previously demonstrated in smokers.[22, 26] In accordance with our study, Fujii et al., using local skin heating, showed a decreased cutaneous vascular conductance (both peak response and area under the curve) in response to intradermal microdialysis of L-N-Arginine-Methyl-Ester (L-NAME) in non-smokers, but not in smokers, implying decreased NO-bioavailability in smokers.[27] Conversely, Dreyfuss et al. showed that L-NAME iontophoresis-mediated inhibition of skin thermal hyperaemia was greater in smokers than in non-smokers, suggesting increased bioavailability of NO in smokers.[22] The reason for these discrepant findings is not clear. The smokers in our study, although healthy, were considerably older than those included in the study of Dreyfuss et al.[6] However, in the study of Fujii et al. only young smokers were included.[21]

In the present study no difference in FMD between smokers and non-smokers was observed. Studies investigating the effect of smoking on FMD have provided inconsistent results.[28-30] These variable findings are not easy to explain but, in part, may be related to the age and general health of the smokers included. With advancing age endothelial and vascular function deteriorate; as a consequence the negative effect of smoking op FMD may no longer be detectable.

In conclusion, the dermal LTH response is in part NO dependent and a well reproducible measure that can be applied to assess the microvascular endothelial function. The absent correlation between LTH and FMD measurements may imply that these measurements provide complementary information about endothelium-dependent vascular function.

References

1. Joannides R, Haefeli WE, Linder L, et al. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. Circulation 1995;91:1314-1319.
2. Ras RT, Streppel MT, Draijer R, Zock PL. Flow-mediated dilation and cardiovascular risk prediction: A systematic review with meta-analysis. Int J Cardiol 2013;168:344-351.
3. Charakida M, Masi S, Loukogeorgakis SP, Deanfield JE. The role of flow-mediated dilatation in the evaluation and development of antiatherosclerotic drugs. Curr Opin Lipidol 2009;20:460-466.
4. Hooper L, Kay C, Abdelhamid A, et al. Effects of chocolate, cocoa, and flavan-3-ols on cardiovascular health: a systematic review and meta-analysis of randomized trials. Am J Clin Nutr 2012;95:740-751.
5. Ras RT, Zock PL, Draijer R. Tea consumption enhances endothelial-dependent vasodilation; a meta-analysis. PLoS One 2011;6:e16974.
6. Joris PJ, Mensink RP. Beetroot juice improves in overweight and slightly obese men postprandial endothelial function after consumption of a mixed meal. Atherosclerosis 2013;231:78-83.
7. Lind L, Hall J, Larsson A, Annuk M, Fellstrom B, Lithell H. Evaluation of endothelium-dependent vasodilation in the human peripheral circulation. Clin Physiol 2000;20:440-448.
8. Lind L, Hall J, Johansson K. Evaluation of four different methods to measure endothelium-dependent vasodilation in the human peripheral circulation. Clin Sci (Lond) 2002;102:561-567.
9. Onkelinx S, Cornelissen V, Goetschalckx K, Thomaes T, Verhamme P, Vanhees L. Reproducibility of different methods to measure the endothelial function. Vasc Med 2012;17:79-84.
10. Debbabi H, Bonnin P, Ducluzeau PH, Leftheriotis G, Levy BI. Noninvasive assessment of endothelial function in the skin microcirculation. Am J Hypertens 2010;23:541-546.
11. Roustit M, Millet C, Blaise S, Dufournet B, Cracowski JL. Excellent reproducibility of laser speckle contrast imaging to assess skin microvascular reactivity. Microvasc Res 2010;80:505-511.
12. Keymel S, Sichwardt J, Balzer J, et al. Characterization of the non-invasive assessment of the cutaneous microcirculation by laser Doppler perfusion scanner. Microcirculation 2010;17:358-366.
13. Spilk S, Herr MD, Sinoway LI, Leuenberger UA. Endothelium-derived hyperpolarizing factor contributes to hypoxia-induced skeletal muscle vasodilation in humans. Am J Physiol Heart Circ Physiol 2013;305:H1639-1645.
14. Wray DW, Witman MA, Ives SJ, et al. Does brachial artery flow-mediated vasodilation provide a bioassay for NO? Hypertension 2013;62:345-351.
15. Gamboa A, Okamoto LE, Raj SR, et al. Nitric oxide and regulation of heart rate in patients with postural tachycardia syndrome and healthy subjects. Hypertension 2013;61:376-381.
16. Bellien J, Iacob M, Remy-Jouet I, et al. Epoxyeicosatrienoic acids contribute with altered nitric oxide and endothelin-1 pathways to conduit artery endothelial dysfunction in essential hypertension. Circulation 2012;125:1266-1275.
17. Virdis A, Ghiadoni L, Qasem AA, et al. Effect of aliskiren treatment on endothelium-dependent vasodilation and aortic stiffness in essential hypertensive patients. Eur Heart J 2012;33:1530-1538.
18. Cracowski JL, Gaillard-Bigot F, Cracowski C, Sors C, Roustit M, Millet C. Involvement of cytochrome epoxygenase metabolites in cutaneous postocclusive hyperemia in humans. J Appl Physiol (1985) 2013;114:245-251.
19. Brunt VE, Minson CT. KCa channels and epoxyeicosatrienoic acids: major contributors to thermal hyperaemia in human skin. J Physiol 2012;590:3523-3534.
20. Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. Arterioscler Thromb Vasc Biol 2003;23:168-175.
21. Holowatz LA, Thompson-Torgerson CS, Kenney WL. The human cutaneous circulation as a model of generalized microvascular function. J Appl Physiol (1985) 2008;105:370-372.
22. Dreyfuss C, Wauters A, Adamopoulos D, et al. L-NAME iontophoresis: a tool to assess NO-mediated vasoreactivity during thermal hyperemic vasodilation in humans. Journal of cardiovascular pharmacology 2013;61:361-368.
23. Arildsson M, Asker CL, Salerud EG, Stromberg T. Skin capillary appearance and skin microvascular perfusion due to topical application of analgesia cream. Microvasc Res 2000;59:14-23.
24. Kellogg DL, Jr., Liu Y, Kosiba IF, O'Donnell D. Role of nitric oxide in the vascular effects of local warming of the skin in humans. J Appl Physiol (1985) 1999;86:1185-1190.
25. Hansell J, Henareh L, Agewall S, Norman M. Non-invasive assessment of endothelial function - relation between vasodilatory responses in skin microcirculation and brachial artery. Clinical physiology and functional imaging 2004;24:317-322.
26. Edvinsson ML, Andersson SE, Xu CB, Edvinsson L. Cigarette smoking leads to reduced relaxant responses of the cutaneous microcirculation. Vascular health and risk management 2008;4:699-704.
27. Fujii N, Reinke MC, Brunt VE, Minson CT. Impaired acetylcholine-induced cutaneous vasodilation in young smokers: roles of nitric oxide and prostanoids. Am J Physiol Heart Circ Physiol 2013;304:H667-673.

28. McEvoy JW, Nasir K, DeFilippis AP, et al. Relationship of cigarette smoking with inflammation and subclinical vascular disease: the Multi-Ethnic Study of Atherosclerosis. Arterioscler Thromb Vasc Biol 2015;35:1002-1010.
29. Nordskog BK, Brown BG, Marano KM, Campell LR, Jones BA, Borgerding MF. Study of cardiovascular disease biomarkers among tobacco consumers, part 2: biomarkers of biological effect. Inhal Toxicol 2015;27:157-166.
30. Celermajer DS, Sorensen KE, Georgakopoulos D, et al. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. Circulation 1993;88:2149-2155.

CHAPTER 8

Vascular characterization of a *TREX1* knock-in mouse model for Retinal Vasculopathy with Cerebral Leukodystrophy

Work in progress

R. R. Klever*, K. Ibrahimi*, K.Y. Chan, S. Labruijere, N. Rieff,
L.A.M. Broos, S.H. van Heiningen, M.D. Ferrari, E.A. Tolner,
G.M. Terwindt, A. MaassenVanDenBrink &
A.M.J.M. van den Maagdenberg
*shared first authors

Abstract

Retinal vasculopathy with cerebral leukodystrophy (RVCL) is an autosomal dominant microvascular vasculopathy caused by heterozygous C-terminal frameshift mutations in the *TREX1* gene that encodes the major mammalian 3' to 5' DNA exonuclease. It is currently unknown how mutations lead to RVCL pathology, which is characterised by retinal telangiectasias, microaneurysms, cerebral white matter lesions, ischemic strokes and migraine attacks. We generated the first transgenic knock-in (KI) mouse model for RVCL by introducing the human pathogenic V235fs *TREX1* frameshift mutation in the endogenous mouse *Trex1* gene using a targeting approach. Molecular analyses confirmed the correct introduction of the *TREX1* mutation and expression at the RNA and protein level. Moreover, Western blotting confirmed that the V235fs mutation resulted in a truncated protein of the expected size. In view of the vascular dysfunction in RVCL patients, we set out to characterize the vascular function of homozygous V235fs KI mice at various ages (13-100 weeks). To this end, we executed *in vivo* microvascular characterization by post-occlusive reactive hyperaemia (PORH) measurements of the hindleg with laser Doppler flowmetry. In addition, a detailed pharmacological characterization of the macrovascular function of V235fs KI mice *in vitro*, using Mulvany myographs was performed. The PORH responses were significantly attenuated in V235fs KI animals across all age groups [$F(1,65)=5.7$, $p=0.02$]. In *in vitro* studies, acetylcholine induced lower relaxations in the aortae of 100-week-old V235fs mice (E_{max}:37±8%, n=7) than in the wild-type mice (E_{max}:65±6%, n=7, $p=0.01$). In accordance with preliminary observations in patients, our analysis suggests vascular abnormalities in this RVCL mouse model that may be assigned to decreased endothelial function. Our data suggest that this RVCL mouse model can be used to further our understanding of Trex1 pathology, and more specifically, can help us to unravel the pathophysiology of RVCL, and perhaps migraine.

Introduction

Retinal vasculopathy with cerebral leukodystrophy (RVCL) is a systemic microvascular vasculopathy with middle-age onset and predominantly retinal and neurovascular features leading to visual loss, cognitive disturbances, depression, ischemic strokes and migraine attacks, generally without aura.[1-3] Other symptoms can include renal and liver dysfunction, gastro-intestinal bleedings and Raynaud's phenomenon.[2] The vascular retinopathy is characterized by telangiectasias, microaneurysms and retinal capillary obliteration starting in the macula.[4] MRI imaging and histopathological analysis of cerebral tissue show cerebral mass lesions mainly in the white matter, thrombosis of microvessels, as well as inflammatory gliosis.[5] The presence of Raynaud's phenomenon in a proportion of RVCL patients points towards vascular dysfunction, but functional studies are limited. Preliminary data suggest that endothelial dysfunction and vascular stiffness may underlie RVCL in patients.[6]

RVCL is caused by heterozygous C-terminal frameshift mutations in the *TREX1* gene.[3] *TREX1* encodes the protein Trex1, which is the major mammalian 3' to 5'

DNA exonuclease, thought to be ubiquitously expressed.[3, 7] Frameshift mutations causing RVCL all lead to truncation of the Trex1 protein. This truncated protein lacks the endoplasmatic reticulum (ER)-binding domain, but the exonuclease function of the protein is still intact.[3, 8] In contrast, *TREX1* missense mutations in the N-terminus of the protein lead to diminished exonuclease function and have been found, among others, in patients with the Aicardi-Goutierres Syndrome (AGS)[9] and Systemic Lupus Erythematosus (SLE).[10-12] It remains an enigma how mutant Trex1 leads to disease. One possibility is that Trex1 has a preventive role in immune responses. The Trex1 protein is specific for single-stranded DNA and a key regulator of the interferon-stimulatory DNA (ISD) response[13] and is therefore believed to play an important role in the body's response to viral pathogens.[14] Another possibility is that Trex1 may play a role in the cellular response to DNA damage. Trex1 is part of a SET-protein complex, a multimeric 270-420 kDa endoplasmic reticulum associated complex, that is involved in DNA degradation and the response to oxidative stress by translocating to the nucleus.[15]

Trex1 knock-out (KO) mice develop severe inflammatory myocarditis leading to a drastically reduced lifespan.[16] This Trex1 deficiency model may be more suitable as a model for AGS, as mutations cause a loss-of-function of exonuclease activity, while in RVCL a stable functioning Trex1, but mislocated protein is believed to be present. Therefore, we generated the first knock-in mouse model for RVCL by introducing the human pathogenic V235fs frameshift mutation in the endogenous *Trex1* gene. Details on the generation and molecular characterization of this KI model will be published elsewhere; here we will only describe the essentials of the mouse model.

To investigate whether there is vascular dysfunction in V235fs KI mice that may eventually lead to vascular pathology, as seen in RVCL patients, we performed a vascular characterization of homozygous V235fs KI mice compared with wild-type (WT) mice. Given the fact that RVCL is predominantly a microvascular disease, we assessed the *in vivo* skin microvascular function of V235fs KI mice with post occlusive reactive hyperaemia (PORH) measurements. In addition detailed pharmacological characterization of macrovascular function was determined in vitro.

Material & methods

Generation and molecular characterisation of transgenic TREX1 V235fs mice

To generate TREX1 V235Fs mice, the *Trex1* gene was modified using a gene-targeting approach, in such a manner that the single-exon *Trex1* gene contained the human V235fs frameshift mutation that had been introduced in the targeting construct by site-directed mutagenesis (Figure 1).[17] In the targeting construct, a PGK-driven neomycin selection cassette, flanked by LoxP sites, had been introduced downstream of *Trex1* and upstream of neighbouring *Scotin*, between the two polyA sequences of the two genes to avoid disruption of RNA expression. Chimeras were obtained by injecting correctly targeted E14 ES cells into C57BL/6J blastocysts. In mice that were used for the experiments, the selection cassette was deleted by crossing the original transgenic TREX1 V235Fs mice with transgenic mice of the

EIIA-Cre delete strain,[18] which express Cre recombinase driven by the EIIA early promoter. Offspring that contained V235Fs, but no longer the selection cassette, was subsequently interbred to provide litters containing homozygous mutant and WT genotypes that were used for the experiments. Mice were genotyped by PCRs specific for the mutation using standard molecular methods. Semi-quantitative Western blot analysis of cortical protein extracts revealed 26 KDa mutant (as predicted from the truncation) and 32 KDa WT Trex1 protein bands. In this experiment, actin was used as a loading control. Animals were kept under standard housing conditions in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals. All experiments were approved by the Animal Experiment Ethics Committee of both the Leiden University Medical Centre and the Erasmus University. For all experiments, the investigator was blinded to the genotype.

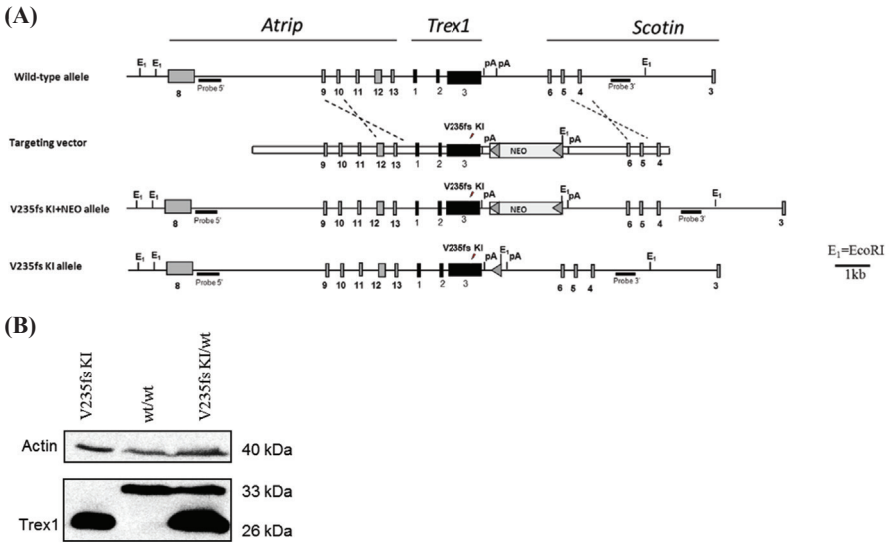


Figure 1. Generation of V235fs *TREX1* Knock-in Mice. (A) Relevant part of the genomic structure of the WT *Trex1* allele, targeting vector and predicted structure after homologous recombination (V235fs KI NEO allele), and after Cre-mediated deletion of the Neo-cassette (depicted as gray box) (V235fs KI allele). LoxP sites are indicated by triangles. Black boxes indicate respective exons, with the V235fs mutation in exon 1. Black horizontal lines indicate probes for Southern and Northern. Primers used for genotyping and confirmation of the V235fs mutation are depicted schematically by a pair of arrows. Restriction sites: EI, EcoRI; EV, EcoRV; K, KpnI; A, ApaI; X, XbaI. **(B)** Western blot of cortical protein extracts isolated from WT and homozygous V235fs KI mice probed with Trex1 or actin antibody. Higher levels of Trex1 protein are present in homozygous V235fs KI mice compared with WT.

Animals

Twenty-four hours before dermal blood flow (DBF) measurements, the hair of the left hind leg of homozygous V235fs KI mice (background C57BL/6j, male and female, age 13, 26, 52 and 100 weeks, n=7-17) and WT mice (male and female) of corresponding ages, was removed with a hair removal cream (Veet®, ReckittBenckiser Inc., Berkshire, UK). On the day of measurement, mice were anesthetized using 4% isoflurane/O_2 ventilation, and kept on a heating pad regulated by a rectal thermometer to keep the body temperature stable between 36.4°C and

37.0°C. After PORH measurements, mice were sacrificed by decapitation and aortas were collected for *in vitro* measurements.

Post-occlusive reactive hyperaemia measurements

In vivo microvascular characterization was obtained by PORH measurements of the hindleg using laser Doppler flowmetry. With PORH the increase of the hindleg perfusion after a transient occlusion of the blood flow (reactive hyperaemia) is measured. In brief, after 5 min of equilibration, 10 min of baseline perfusion was recorded. Subsequently, the hindleg circulation was occluded for 2 min with a tourniquet. After release of the tourniquet, blood flow was monitored till return of blood flow to baseline values (max. 10 min).

Mulvany myograph organ baths

For the *in vitro* macrovascular pharmacological characterization, segments of the mouse aorta (inner diameter 0.5-1 mm) were mounted in a Mulvany myograph with separated 6-mL organ baths containing carbonated (5% CO_2 and 95% O_2) Krebs-Henseleit buffer (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, H_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4) at 37°C. Tension was normalized to 90% of the estimated diameter at 100 mmHg of transmural pressure. A reference contractile response of vessel segments was determined with 100 mM KCl. For the acetylcholine (ACh) and sodium nitroprusside (SNP) concentration response curves (CRCs), aorta segments were pre-contracted with the thromboxane A_2 analogue U46619 (10-100 nM). Relaxant effects of ACh (endothelium-dependent) and SNP (endothelium- independent) and contractile effects of 5-hydroxytryptamine (5-HT) were examined with cumulative application of increasing concentrations of the drugs.

Statistical analysis

For the analysis of PORH, the results were expressed as relative values compared to baseline. Results were expressed as the area under the response curve (AUC), maximal hyperaemia response (Max) and time-to-maximal hyperaemia (TTM). One-way ANOVA followed by Bonferroni post hoc tests were used to compare V235fs KI mice with WT. For the *in vitro* experiments, the vasorelaxant responses to ACh and SNP were expressed as the percentage of maximum contraction induced by U46619. The contractile responses to 5-HT were expressed as percentage of the response to 100 mM KCl. CRCs were analysed using 1-way ANOVA followed by Bonferroni post hoc tests. Data are expressed as mean ± standard error of the mean (SEM). P-values <0.05 were considered to indicate statistical significance.

Results

Animals

There was no difference in weight between male WT and V235fs KI (33 ± 1 g vs. 31 ± 2 g) or between female WT and V235fs KI (26 ± 1 g vs. 24 ± 1 g) mice. Male and female microvascular function and macrovascular pharmacological properties were

similar (however, our study was not powered to detect gender differences), therefore data obtained from both sexes were pooled.

Microvascular reactivity in V235fs *TREX1* KI mice: in vivo PORH measurements

The analysis of the PORH curves revealed that the area under the PORH curve was significantly lower in V235fs KI mice across all age groups (Figure 2A, [$F(1, 65)=5.7$, $p=0.02$]). The max PORH responses were similar between WT and homozygous V235fs KI mice (Figure 2B). Parameters such as baseline DBF and TTM were not different between V235fs KI mice and WT (Table 1).

	13 wk WT	13 wk TG	26 wk WT	26 wk TG	52 wk WT	52 wk TG	100 wk WT	100 wk TG
BL (AU)	39±5	58±7	63±13	40±7	85±31	57±10	70±14	52±4
TTM (Sec)	83±38	111±57	114±17	150±23	77±13	47±9	64±10	45±10

Table 1. Skin baseline DBF and time max hyperemia for V235fs Trex1 Knock-in and WT mice. Data expressed as mean±SEM. TG = homozygous V235fs KI mice, BL = Baseline DBF and TTM = time to max.

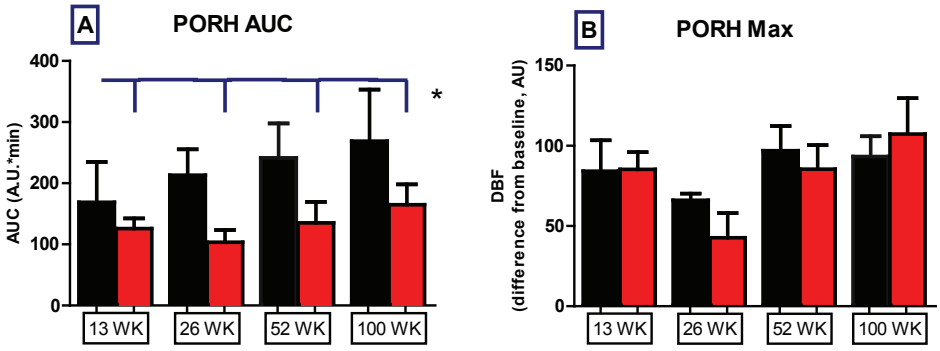


Figure 2. PORH of V235fs *TREX1* Knock-in (red bars) and WT Mice (black bars). (A) The area under the reactive hyperemia curve for 13- to 100- week-old mice (*n*=4-13). * Statistically significant difference between WT and V235fs KI mice across all age groups. **(B)** Maximal reactive hyperemia response for 13-100 week old mice (*n*=4-13).

Macrovascular reactivity in V235fs *TREX1* KI mice: Mulvany myograph studies

Contraction to 100 mM KCl was not significantly different between WT and V235fs KI mice at (13 weeks: 7±1 vs. 8±0 mN, 26 weeks: 9±1 vs. 9±1 mN, 52 weeks: 10±1 vs. 10±1 mN, 100 weeks: 8±1 vs. 8±1 mN).Maximal relaxation induced by ACh was attenuated in 100-week-old V235fs KI mice compared to 100-week-old WT mice (E_{max}: 65±6% vs. 37±8%, respectively, $p=0.011$), however the ACh CRCs between the 13- to 52-week-old V235fs KI mice and the 13-52 week old WT mice did not differ from the 13- to 52-week-old WT mice (Figure 3A-3D). SNP-induced relaxations were not significantly different between V235fs KI and WT mice (Figure 3E-3H). The maximal contraction induced by 5-HT (Figure 3I-3L) tended to be lower in the 52- and 100-week-old V235fs KI mice (respectively E_{max}: 26±8% and E_{max}: 50±13%) than in the 52- and 100-week-old WT mice (respectively E_{max}: 48±9%, $p=0.06$ and E_{max}: 73±11%, $p=0.19$).

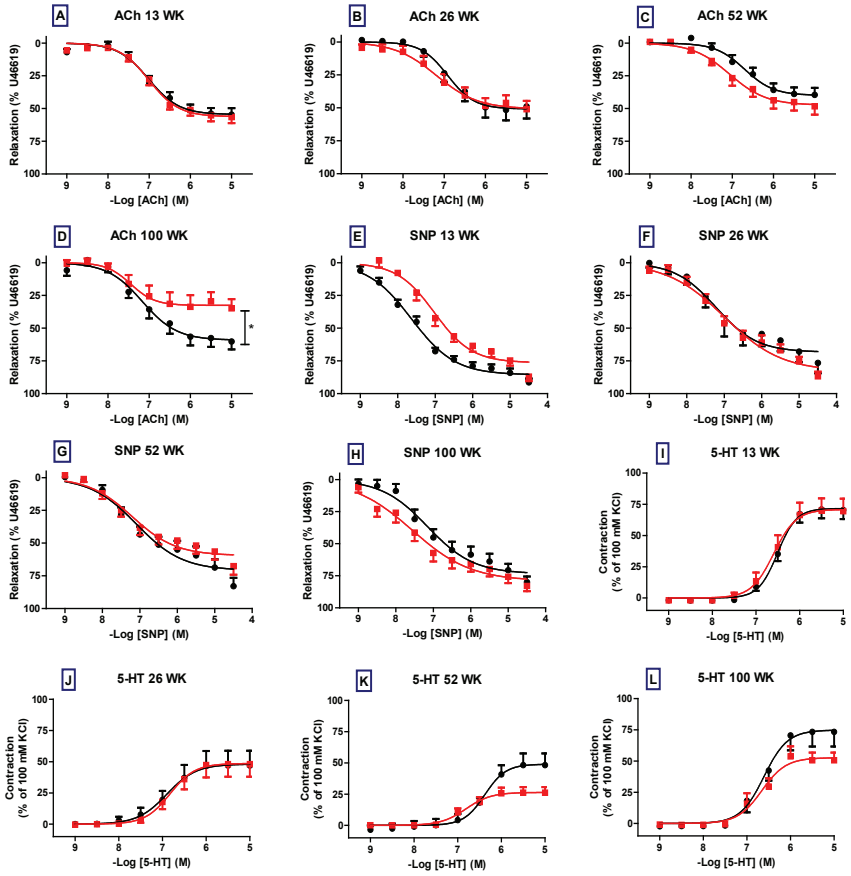


Figure 3. *In vitro* macrovascular characterization of V235fs *TREX1* Knock-in (red squares) and WT (black circles) mice. *In vitro* macrovascular characterization of isolated aorta obtained from homozygous V235fs KI and WT mice. Panels depict concentration response curves to acetylcholine (ACh, **A-D**, *n*=7-16), sodium nitroprusside (SNP, **E-H**, *n*=7-17) and serotonin (5-HT, **I-L**, *n*=5-15). *Statistically significant attenuated relaxant responses to ACh in 100 week-old V235fs KI mice compared to WT mice.

Discussion

We performed an *in vivo* microvascular and *in vitro* macrovascular pharmacological characterization of the first knock-in mouse model for RVCL in which the human pathogenic V235fs *TREX1* mutation was introduced by a gene targeting approach. With our study we identified microvascular dysfunction in 13- to 100-week-old V235fs KI mice. Detailed pharmacological macrovascular (aorta) characterization showed endothelial dysfunction only in mice aged 100 weeks.

The functional PORH test measures the cutaneous microvascular function. The PORH response is caused by interplay of different vasodilatory factors. During the time course of the PORH response, important for the PORH parameter AUC, the endothelium-derived hyperpolarizing factor (EDHF) pathway is activated and causes the resulting vasodilation.[19] During the hyperaemia peak the release of neuropeptides

from perivascular nerves plays a major role.[20] Our results show that in V235fs KI mice the PORH peak is not different from WT, while the AUC is lower in V235fs KI mice across all ages that we studied (13- to 100-week-old mice) compared with WT. The lower PORH AUC points toward endothelial dysfunction in the microvasculature of the V235fs KI mice. Although studies assessing vascular function in RVCL patients are scarce, the preliminary results of the study by Vermeersch et al., where the FMD response was lower in the RVCL patients compared to healthy controls, are in line with our results.[6]

The macrovascular *in vitro* characterization shows that the aortic endothelial function, evaluated by the vasorelaxant response of the aortic rings to ACh in Mulvany organ baths,[21] is decreased in the 100-week-old V235fs KI mice. The CRCs of the aortic rings to SNP do not differ between the V235fs KI mice and WT. Notably, in the aortae of the 100 week old mice the relaxation to SNP even tended to be greater in the V235s KI mice. Thus, we can conclude that the vascular smooth muscle cell (VSMC) function is intact and the decrease in vasodilator response to ACh in the 100 week old mice is probably due to the effect of endothelial dysfunction. With the diminished PORH AUC in the V235fs KI mice we demonstrate the involvement of an affected EDHF response. As EDHF is known to contribute more to vasorelaxation in microvessels than in larger vessels,[22, 23] extensive endothelial dysfunction in the aortae of the V235fs KI mice was not to be expected based on our *in vivo* findings. Therefore the results of our *in vitro* studies are not in contrast with the microvascular PORH results.

The 5-HT system is involved in migraine pathophysiology and for decades now 5-HT_1 receptor agonists have been applied successfully in migraine therapy.[24] As migraine is a prominent feature of RVCL, we also assessed the function of the 5HT receptor present on VSMC using the a selective natural ligand, 5-HT.[25] CRCs to 5-HT tended to be lower in the 52- and 100-week-old V235fs KI mice compared with WT, however, this was not statistically significant.

The results of the present study clearly indicate a microvascular dysfunction, evidenced by the lower AUC of the PORH, in the V235fs KI mice that is present from as early as 13 weeks. This microvascular, endothelial dysfunction is present in the V235fs KI mice up to the age of 100 weeks. Macrovascular endothelial dysfunction revealed by the diminished relaxant responses to ACh in the V235fs KI mice is only present in the 100-week-old animals. The V235fs mutation does not seem to affect endothelium-independent relaxant or contractile vascular responses.

In conclusion, our vascular characterization indicates microvascular abnormalities, most likely due to endothelial dysfunction, in the V235fs KI mice. This seems in accordance with the clinical presentation in the RVCL patients.

References

1. Jen J, Cohen AH, Yue Q, et al. Hereditary endotheliopathy with retinopathy, nephropathy, and stroke (HERNS). Neurology 1997;49:1322-1330.
2. Terwindt GM, Haan J, Ophoff RA, et al. Clinical and genetic analysis of a large Dutch family with autosomal dominant vascular retinopathy, migraine and Raynaud's phenomenon. Brain 1998;121 (Pt 2):303-316.
3. Richards A, van den Maagdenberg AM, Jen JC, et al. C-terminal truncations in human 3'-5' DNA exonuclease TREX1 cause autosomal dominant retinal vasculopathy with cerebral leukodystrophy. Nat Genet 2007;39:1068-1070.
4. Cohn AC, Kotschet K, Veitch A, Delatycki MB, McCombe MF. Novel ophthalmological features in hereditary endotheliopathy with retinopathy, nephropathy and stroke syndrome. Clin Exp Ophthalmol 2005;33:181-183.
5. Stam AH, Haan J, van den Maagdenberg AM, Ferrari MD, Terwindt GM. Migraine and genetic and acquired vasculopathies. Cephalalgia 2009;29:1006-1017.
6. Vermeersch S, Stam AH, Zielman R, et al. Trex1-mutation associated with endothelial dysfunction in RVCL patients. Cephalalgia 2011;31:13.
7. Mazur DJ, Perrino FW. Structure and expression of the TREX1 and TREX2 3' --> 5' exonuclease genes. J Biol Chem 2001;276:14718-14727.
8. Orebaugh CD, Fye JM, Harvey S, Hollis T, Wilkinson JC, Perrino FW. The TREX1 C-terminal region controls cellular localization through ubiquitination. J Biol Chem 2013;288:28881-28892.
9. Crow YJ, Hayward BE, Parmar R, et al. Mutations in the gene encoding the 3'-5' DNA exonuclease TREX1 cause Aicardi-Goutieres syndrome at the AGS1 locus. Nat Genet 2006;38:917-920.
10. Lee-Kirsch MA, Gong M, Chowdhury D, et al. Mutations in the gene encoding the 3'-5' DNA exonuclease TREX1 are associated with systemic lupus erythematosus. Nat Genet 2007;39:1065-1067.
11. de Vries B, Steup-Beekman GM, Haan J, et al. TREX1 gene variant in neuropsychiatric systemic lupus erythematosus. Ann Rheum Dis 2010;69:1886-1887.
12. Pelzer N, Stam AH, Haan J, Ferrari MD, Terwindt GM. Familial and sporadic hemiplegic migraine: diagnosis and treatment. Curr Treat Options Neurol 2013;15:13-27.
13. Stetson DB, Ko JS, Heidmann T, Medzhitov R. Trex1 prevents cell-intrinsic initiation of autoimmunity. Cell 2008;134:587-598.
14. Serra M, Forcales SV, Pereira-Lopes S, Lloberas J, Celada A. Characterization of Trex1 induction by IFN-gamma in murine macrophages. J Immunol 2011;186:2299-2308.
15. Chowdhury D, Beresford PJ, Zhu P, et al. The exonuclease TREX1 is in the SET complex and acts in concert with NM23-H1 to degrade DNA during granzyme A-mediated cell death. Mol Cell 2006;23:133-142.
16. Morita M, Stamp G, Robins P, et al. Gene-targeted mice lacking the Trex1 (DNase III) 3'-->5' DNA exonuclease develop inflammatory myocarditis. Mol Cell Biol 2004;24:6719-6727.
17. Klever RR, Rutten JW, Labruijere S, et al. Novel Transgenic Mouse Models for Monogenic Cerebral Small Vessel Diseases Related to Migraine. Cephalalgia 2013;33:972-972.
18. Lakso M, Pichel JG, Gorman JR, et al. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc Natl Acad Sci U S A 1996;93:5860-5865.
19. Cracowski JL, Gaillard-Bigot F, Cracowski C, Sors C, Roustit M, Millet C. Involvement of cytochrome epoxygenase metabolites in cutaneous postocclusive hyperemia in humans. J Appl Physiol (1985) 2013;114:245-251.
20. Larkin SW, Williams TJ. Evidence for sensory nerve involvement in cutaneous reactive hyperemia in humans. Circ Res 1993;73:147-154.
21. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980;288:373-376.
22. Hwa JJ, Ghibaudi L, Williams P, Chatterjee M. Comparison of acetylcholine-dependent relaxation in large and small arteries of rat mesenteric vascular bed. Am J Physiol 1994;266:H952-958.
23. Danser AH, Tom B, de Vries R, Saxena PR. L-NAME-resistant bradykinin-induced relaxation in porcine coronary arteries is NO-dependent: effect of ACE inhibition. Br J Pharmacol 2000;131:195-202.
24. Goadsby PJ, Lipton RB, Ferrari MD. Migraine--current understanding and treatment. N Engl J Med 2002;346:257-270.
25. Gupta S, Lozano-Cuenca J, Villalon CM, et al. Pharmacological characterisation of capsaicin-induced relaxations in human and porcine isolated arteries. Naunyn Schmiedebergs Arch Pharmacol 2007;375:29-38.

Part II

CHAPTER 9

Migraine and perimenopause

Based on: Maturitas, 2014, 78:4, 277-280

K. Ibrahimi, E.G.M. Couturier & A. MaassenVanDenBrink

Abstract

Perimenopause and migraine are closely linked. The hormonal instability during the perimenopausal period not only causes vasomotor symptoms and mood disturbances, but also increases migraine incidence. Women do report new onset migraine during this period, but the increased incidence is reported by women with menstrually related migraine (MRM). The hormonal fluctuations can be stabilized with hormone replacement therapy (HRT), while simultaneously improving the migraine in some patients. The increased stroke risk in women with migraine with aura (MA) should be taken into consideration when intending to treat perimenopausal women with migraine with HRT.

Introduction

The onset of the perimenopausal period, the period of 2 to 8 years prior the menopause as well as the year after the end of menses, is usually characterized by a sequence of symptoms caused by an estrogen deficit that occurs due to a decline in ovarian function. These symptoms consist foremost of night sweats, hot flushes, joint pain and vaginal dryness, however sleep disturbances and irritability are also prevalent. There is a link between the menopausal transition and migraine as well. Preexisting migraine can remain unchanged, improve, but may also worsen during perimenopause.[1, 2] Consequently, perimenopause is a turbulent period in women's life, not only with regard to migraine. Unfortunately, prospective studies documenting the course of migraine during and after the menopausal transition remain rare.

Migraine pathophysiology

Migraine is a paroxysmal disorder of a neurovascular origin, with great adverse effects on the quality of life. According to the Global Burden of Disease Survey 2010 conducted by the World Health Organization, migraine is ranked as seventh highest cause of disability in the world.[3] The distinctive pulsating headaches manifest as attacks that can last from 4 up to 72 h and are located unilaterally. The headaches worsen with physical activity and/or are associated with photophobia and phonophobia. When the headache is preceded and in some cases accompanied by gradually developing visual, sensory or other central nervous system symptoms (aura), then it is diagnosed as migraine with aura (MA) otherwise it is considered migraine without aura (MO).[4] Migraine is thought to originate from neural events that results in dilation of cranial blood vessels, inducing headache and further nerve activation.[5] Calcitonin gene related peptide (CGRP) is a key neuropeptide in migraine pathophysiology. Indicative of its importance, CGRP levels are elevated in jugular blood and saliva during migraine attacks and normalize after the use of antimigraine drugs.[6, 7] CGRP receptor antagonists and antibodies against CGRP or its receptor are currently being developed as acute migraine therapy.[8, 9]

Estrogen fluctuations and migraine

The prevalence of migraine is according to the recently published American Migraine Prevalence and Prevention study 17% in women and 6% in men.[11]

Especially hormonal milestones accompanied by fluctuations in estrogen levels such as menarche, pregnancy and menopause seem to have vast effects on migraine prevalence and frequency (Figure 1). The onset of migraine in women usually coincides with menarche and a close relation between migraine occurrence and the menstrual cycle remains during the reproductive years.[12] Approximately 50% of women with migraine are affected by MRM;[13] migraine without aura that occurs on day 1±2 of the menstrual cycle in at least 2 of 3 consecutive menstrual cycles with additional attacks with or without aura that can occur at other times of the month.[4] During perimenopause changes in migraine prevalence have been reported.[14] Major fluctuations in estrogen levels take place during perimenopause, ultimately leading to dropping levels.[15] The prevalence of migraine headaches during this period seems to be higher in patients who previously had a history of MRM and premenstrual syndrome.[16, 17]

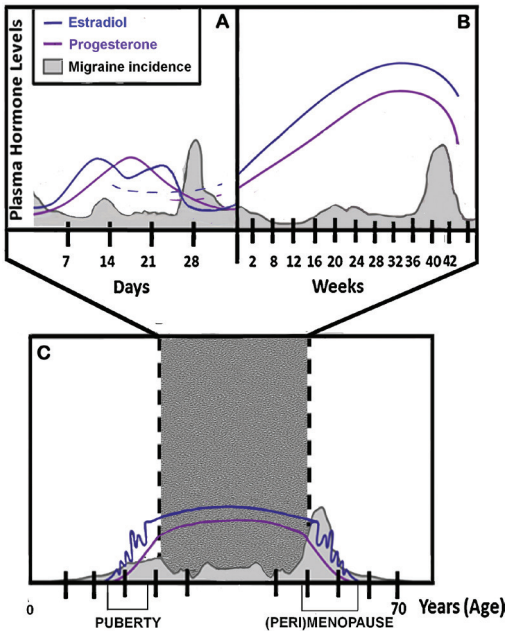


Figure 1. Migraine incidence and female hormones during the menstrual cycle, pregnancy and a women's life. Adapted from Saco et al.[10]

Perimenopausal migraine

During the perimenopausal period serum estradiol levels are low (10-20 pg/ml). Nevertheless, 8-13% of women with migraine report the onset of their migraine during this period.[18, 19] Interestingly, the type of menopause (surgical or spontaneous) influences the effect of the menopausal transition on pre-existing migraine. Improvement of migraine occurs in two-thirds of women after spontaneous menopause, but migraine worsens in two-thirds after surgical menopause.[20] With the increase of time after the start of the menopausal transition, there is a decrease

of migraine prevalence,[17, 21] which is most pronounced in MO.[16] It is not surprising that, given the improvement of migraine in women after spontaneous menopause, oophorectomy has been considered as preventive migraine therapy in perimenopausal women. Pharmacological oophorectomy induced by a gonadotropin-releasing hormone agonist with estrogen add-back therapy seems to improve headache pain severity, but not the headache frequency.[22] Anecdotal evidence suggests that total hysterectomy with bilateral oophorectomy and add-back estrogen therapy being successful in treatment of migraine.[23] This kind of drastic surgical intervention is considered by some doctors in cases of women suffering from severe and debilitating migraine that has improved after pharmacologically induced menopause. These claims have an insufficient scientific base, so gynecological operations like this should be discouraged.

Treatment/management

During perimenopause, HRT may be prescribed to women to fight the symptoms of dropping estrogen levels.[24] HRT can have an improving or worsening effect on migraine in perimenopausal women.[25] Transdermal estrogen patches or gel are preferred in treating women with migraine during this period, since oral treatment can lead to estrogen fluctuations and consequently worsening of the migraine.[26] Estrogen replacement in perimenopausal patients with MA has been associated with an increase in headache severity and visual auras.[27] Prescribing the lowest effective dose of estrogen seems to prevent this undesired effect.[28] This is also prudent considering MA is a risk factor for ischemic stroke, see below.[29-31] When systemic estrogen is used, endometrial protection is needed in perimenopausal women with an intact uterus. Progesterone, preferably micronized as this may be safer with regard to breast cancer risk,[32] should be prescribed next to estrogen. Independent of the HRT to regulate estrogen levels, (pre)menopausal migraine can be treated with prophylactics or acute antimigraine therapy according to standard treatment strategy. While guidelines differ between internationally and even nationally,[33] generally migraine attacks are initially treated with acutely acting medication. According to a treatment ladder, therapy is usually started with an oral analgesic (acetaminophen or NSAID's) combined with an anti-emetic. When migraine relief is not achieved triptans are prescribed. Migraine prophylaxis consisting of beta-blockers, topiromate, depakine or other prophylactics are prescribed in patinets with frequent attacks or in case of insufficient efficacy or overuse of acutely acting drugs.[34]

Future therapeutic options

CGRP seems to be involved in the development of vasomotor changes such as hot flushes, which occur during menopause.[35] A combined therapy of migraine and vasomotor changes during perimenopause is very likely to be a therapeutic development in the future. The CGRP receptor antagonist telcagepant was the most promising amongst the 'gepants', until raised liver enzymes detected during a Phase II clinical trial led to suspension of its development.[36] CGRP antibodies are likely the next chapter in blocking CGRP and are now undergoing clinical trials.[29] Considering the unpredictability of hot flushes, prophylactic treatment with CGRP receptor

antagonists or antibodies is probably required. CGRP receptor antagonists are likely the safest choice as in case of adverse events they can be stopped without the long term effect that would be present with CGRP antibodies.

Long term effects and conditions associated with migraine

Over the last years, migraine has been linked with stroke. More specifically, meta-analysis of observational studies indicates that patients with migraine have a two-fold increased risk of ischemic stroke.[30, 31, 37] This risk is apparently linked to migraine with aura (MA)[30, 31] and seems to increase with attack frequency.[38] However, the recently presented cardiovascular disease (CVD) incidence rate (IR) data from the Women's Health Study during the American Academy of neurology meeting[39] indicates an even higher CVD IR for MA (IR = 7.9; 6.2-10.0) than for example a more well-known CVD risk factors as current smoking (IR = 5.4; 95% CI; 4.6-6.4) and BMI higher than 35 kg/m^2 (IR = 5.3; 95% CI; 4.0-7.2). Since there is evidence of continuation of MA after the menopause, postmenopausal women with migraine requiring HRT should be treated with caution while bearing in mind their increased stroke risk and possible additional risk factors. Moreover, some studies indicate that younger menopausal age increases the risk of ischemic stroke further.[31]

Migraine has also been associated with increased prevalence and progression of clinically silent brain lesions,[40] with the increased progression evident in women, but not in men.[41] Combined with the increased stroke risk, a link between cognitive impairment and migraine would be expected. Nevertheless, results from longitudinal studies show no association between cognitive decline over time and migraine.[41, 42]

On the other hand, there seems to be some positive long term effect of migraine on the risk of developing specific types of postmenopausal breast cancer. A study by Li et al. suggests that women with a history of migraine had a lower risk of breast cancer (hazard ratio [HR], 0.89; 95% CI, 0.80-0.98) than women without a migraine history.[43] This risk reduction was not influenced by use of medication prescribed for migraine.[43]

Summary

Strong hormonal fluctuations, as occurring during menopause, are associated with migraine. However, the pathogenic mechanism behind this association remains to be clarified. With the recent advancement in the research of migraine, there is hope for better understanding of these mechanisms and eventually leading to a better treatment of patients suffering from this debilitating disorder.

References

1. Mueller L. Predictability of exogenous hormone effect on subgroups of migraineurs. Headache 2000;40:189-193.
2. Hodson J, Thompson J, al-Azzawi F. Headache at menopause and in hormone replacement therapy users. Climacteric 2000;3:119-124.
3. Vos T, Flaxman AD, Naghavi M, et al. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 2012;380:2163-2196.
4. Headache Classification Committee of the International Headache S. The International Classification of Headache Disorders, 3rd edition (beta version). Cephalalgia 2013;33:629-808.
5. May A, Goadsby PJ. The trigeminovascular system in humans: pathophysiologic implications for primary headache syndromes of the neural influences on the cerebral circulation. J Cereb Blood Flow Metab 1999;19:115-127.
6. Cady RK, Vause CV, Ho TW, Bigal ME, Durham PL. Elevated saliva calcitonin gene-related peptide levels during acute migraine predict therapeutic response to rizatriptan. Headache 2009;49:1258-1266.
7. Goadsby PJ, Edvinsson L, Ekman R. Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. Ann Neurol 1990;28:183-187.
8. Marcus R, Goadsby PJ, Dodick D, Stock D, Manos G, Fischer TZ. BMS-927711 for the acute treatment of migraine: A double-blind, randomized, placebo controlled, dose-ranging trial. Cephalalgia 2013.
9. De Hoon J, Montieth D, Vermeersch S, et al. Safety, pharmacokinetics, and pharmacodynamics of LY2951742: a monoclonal antibody targeting CGRP. Cephalalgia 2013;33:247.
10. Sacco S, Ricci S, Degan D, Carolei A. Migraine in women: the role of hormones and their impact on vascular diseases. J Headache Pain 2012;13:177-189.
11. Buse DC, Loder EW, Gorman JA, et al. Sex differences in the prevalence, symptoms, and associated features of migraine, probable migraine and other severe headache: results of the American Migraine Prevalence and Prevention (AMPP) Study. Headache 2013;53:1278-1299.
12. Lipton RB, Stewart WF, Diamond S, Diamond ML, Reed M. Prevalence and burden of migraine in the United States: data from the American Migraine Study II. Headache 2001;41:646-657.
13. Martin VT. Menstrual migraine: a review of prophylactic therapies. Curr Pain Headache Rep 2004;8:229-237.
14. Lakso M, Pichel JG, Gorman JR, et al. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc Natl Acad Sci U S A 1996;93:5860-5865.
15. Yamada M, Soda M, Fujiwara S. Follicle-stimulating hormone and oestradiol levels during perimenopause in a cohort of Japanese women. Int J Clin Pract 2008;62:1623-1627.
16. Mattsson P. Hormonal factors in migraine: a population-based study of women aged 40 to 74 years. Headache 2003;43:27-35.
17. Wang SJ, Fuh JL, Lu SR, Juang KD, Wang PH. Migraine prevalence during menopausal transition. Headache 2003;43:470-478.
18. Cupini LM, Matteis M, Troisi E, Calabresi P, Bernardi G, Silvestrini M. Sex-hormone-related events in migrainous females. A clinical comparative study between migraine with aura and migraine without aura. Cephalalgia 1995;15:140-144.
19. Granella F, Sances G, Zanferrari C, Costa A, Martignoni E, Manzoni GC. Migraine without aura and reproductive life events: a clinical epidemiological study in 1300 women. Headache 1993;33:385-389.
20. Neri I, Granella F, Nappi R, Manzoni GC, Facchinetti F, Genazzani AR. Characteristics of headache at menopause: a clinico-epidemiologic study. Maturitas 1993;17:31-37.
21. Freeman EW, Sammel MD, Lin H, Gracia CR, Kapoor S. Symptoms in the menopausal transition: hormone and behavioral correlates. Obstet Gynecol 2008;111:127-136.
22. Martin V, Wernke S, Mandell K, et al. Medical oophorectomy with and without estrogen add-back therapy in the prevention of migraine headache. Headache 2003;43:309-321.
23. Jonsdottir GM, Herzog A, Istre O. Laparoscopic bilateral oophorectomy - feasible migraine management? Acta obstetricia et gynecologica Scandinavica 2012;91:271-272.
24. Panay N, Hamoda H, Arya R, Savvas M, British Menopause S, Women's Health C. The 2013 British Menopause Society & Women's Health Concern recommendations on hormone replacement therapy. Menopause Int 2013;19:59-68.
25. Facchinetti F, Nappi RE, Tirelli A, Polatti F, Nappi G, Sances G. Hormone supplementation differently affects migraine in postmenopausal women. Headache 2002;42:924-929.
26. MacGregor EA, Frith A, Ellis J, Aspinall L, Hackshaw A. Prevention of menstrual attacks of migraine: a double-blind placebo-controlled crossover study. Neurology 2006;67:2159-2163.
27. Kaiser HJ, Meienberg O. Deterioration or onset of migraine under oestrogen replacement therapy in the menopause. Journal of neurology 1993;240:195-196.
28. MacGregor A. Estrogen replacement and migraine aura. Headache 1999;39:674-678.

29. Negro A, Lionetto L, Simmaco M, Martelletti P. CGRP receptor antagonists: an expanding drug class for acute migraine? Expert opinion on investigational drugs 2012;21:807-818.
30. Spector JT, Kahn SR, Jones MR, Jayakumar M, Dalal D, Nazarian S. Migraine headache and ischemic stroke risk: an updated meta-analysis. Am J Med 2010;123:612-624.
31. Schurks M, Rist PM, Bigal ME, Buring JE, Lipton RB, Kurth T. Migraine and cardiovascular disease: systematic review and meta-analysis. BMJ 2009;339:b3914.
32. Gadducci A, Biglia N, Cosio S, Sismondi P, Genazzani AR. Progestagen component in combined hormone replacement therapy in postmenopausal women and breast cancer risk: a debated clinical issue. Gynecol Endocrinol 2009;25:807-815.
33. Schuurmans A, van Weel C. Pharmacologic treatment of migraine. Comparison of guidelines. Can Fam Physician 2005;51:838-843.
34. Steiner TJ, MacGregor EA, Davies PTG. Guidelines for All Healthcare Professionals in the Diagnosis and Management of Migraine, Tension-Type, Cluster and Medication-Overuse Headache (3rd edn). 2007;http://www.bash.org.uk/wp-content/uploads/2012/07/10102-BASH-Guidelines-update-2_v5-1-indd.pdf.
35. Gennari C, Nami R, Pecchi S, De Franco V, Panza F, Pavese G. Plethysmographic evaluation of the vascular effects of human calcitonin gene-related peptide in man. Angiology 1991;42:462-467.
36. Han TH, Blanchard RL, Palcza J, et al. Single- and multiple-dose pharmacokinetics and tolerability of telcagepant, an oral calcitonin gene-related peptide receptor antagonist, in adults. Journal of clinical pharmacology 2010;50:1367-1376.
37. Etminan M, Takkouche B, Isorna FC, Samii A. Risk of ischaemic stroke in people with migraine: systematic review and meta-analysis of observational studies. BMJ 2005;330:63.
38. Kurth T, Schurks M, Logroscino G, Buring JE. Migraine frequency and risk of cardiovascular disease in women. Neurology 2009;73:581-588.
39. Kurth T, Bubes V, Buring JE. Relative Contribution of Migraine with Aura to Cardiovascular Disease Occurrence in Women. . Neurology 2013;80:S40.001.
40. Kurth T, Mohamed S, Maillard P, et al. Headache, migraine, and structural brain lesions and function: population based Epidemiology of Vascular Ageing-MRI study. BMJ 2011;342:c7357.
41. Palm-Meinders IH, Koppen H, Terwindt GM, et al. Structural brain changes in migraine. JAMA 2012;308:1889-1897.
42. Kalaydjian A, Zandi PP, Swartz KL, Eaton WW, Lyketsos C. How migraines impact cognitive function: findings from the Baltimore ECA. Neurology 2007;68:1417-1424.
43. Li CI, Mathes RW, Bluhm EC, et al. Migraine history and breast cancer risk among postmenopausal women. J Clin Oncol 2010;28:1005-1010.

CHAPTER 10

Reduced trigeminovascular cyclicity in patients with menstrually related migraine

Based on: Neurology, 2015, 84:2, 125-131

K. Ibrahimi, W.P.J. van Oosterhout, W. van Dorp, A.H.J. Danser, I.M. Garrelds, S.A. Kushner, E.M.E.H. Lesaffre, G.M. Terwindt, M.D. Ferrari, A.H. van den Meiracker & A. MaassenVanDenBrink

Abstract

Objective: A case-control study to investigate the effect of the menstrual cycle on trigeminal nerve-induced vasodilation in healthy women and patients with menstrually-related migraine (MRM). **Methods:** Using a laser-Doppler imager, we compared the vasodilator effects of capsaicin application and electrical stimulation (ES) on the forehead skin, a trigeminal nerve-innervated dermatome, in premenopausal MRM patients (n=22), healthy controls (n=20), and postmenopausal women without migraine (n=22). Blood samples were collected for female sex hormones measurements. **Results:** Dermal blood flow (DBF) responses to capsaicin were higher in controls during days 1-2 than during days 19-21 of their menstruation cycle (Mean E_{max} ±SEM: 203±28 a.u. vs 156±27 a.u. (p=0.031) for 0.06 mg/ml capsaicin and 497±25 a.u. vs 456±24 a.u. (p=0.009) for 6.0 mg/ml capsaicin). In contrast, MRM patients demonstrated DBF responses without significant cycle-dependent variability (day 1-2 vs day 19-21: E_{max} 148±20 a.u. vs 154±20 a.u. (p=0.788) for 0.06 mg/ml capsaicin and 470±17 a.u. vs 465±20 a.u. (p=0.679) for 6.0 mg/ml capsaicin). DBF response to ES were not different between either MRM patients or controls, at either occasion. Estradiol levels on day 19-21 of the menstrual cycle were higher in healthy controls (Mean±SEM: 75±8 pg/ml) than in MRM patients (52±4 pg/ml, p=0.014). In postmenopausal women, DBF responses to capsaicin and ES, as well as estradiol levels at both visits, were all significantly reduced compared to MRM patients and controls (in all cases, $p<0.05$). **Conclusions:** Our study provides evidence for a reduced menstrual cyclicity of both estradiol levels and the trigeminovascular vasodilator system in MRM patients.

Introduction

Migraine is twice as prevalent in women than in men[1]. Migraine incidence in women changes because of estrogen variations around menarche and menopause, but also during the menstrual cycle.[2, 3] Estrogen withdrawal increases migraine attack incidence migraine attacks,[4] a process that may be postponed by estradiol injections.[5-7] Migraine attacks associated with menstruation are generally perceived as more severe than attacks outside this period.[8, 9]

The potent vasodilator calcitonin gene-related peptide (CGRP), a key mediator in migraine,[10] is released from primary afferents of the trigeminal ganglion, exerting its effects via the trigeminovascular system.[11, 12] Given the relationship between migraine attack incidence and hormonal fluctuations, an interaction between female sex hormones and CGRP seems likely.[13, 14]

We have developed a human model to study trigeminal nerve-mediated vasodilatation, by applying capsaicin and electrical stimulation (ES) to the forehead skin, a trigeminal nerve-innervated dermatome.[15] Capsaicin activates the transient receptor potential vanilloid type 1 (TRPV1) channel, thereby enhancing CGRP release from trigeminal nerve terminals.[15] In contrast, ES appears to act directly on trigeminal nerve terminals, without the need for TRPV1 activation to evoke release of CGRP.[15]

We investigated whether (1) varying levels of sex hormones during the menstrual cycle affect trigeminal nerve-mediated vasodilatation, and (2) this pattern differs between patients with menstrually-related migraine (MRM) and healthy controls. We hypothesized that (1) trigeminal nerve-mediated vasodilatory responses are increased preceding the menstruation, (2) this increase is more pronounced in patients with MRM than in controls, and (3) trigeminal nerve-mediated vasodilatory responses are consistently low in postmenopausal women.

Methods

Standard protocol approvals, registrations, and patient consents

The Independent Ethics Committee of Erasmus MC, Rotterdam, The Netherlands, reviewed and approved the study protocol. All participants gave written informed consent after explanation of the study, which was conducted in accordance with local laws, the ethical principles of the Declaration of Helsinki, as well as the principles of Good Clinical Practice.

Design and procedures

For our case-control study design we compared patients with MRM to age-matched healthy controls. We included postmenopausal women as a reference group (without menstrual cycle) for both the patients with MRM and their age-matched healthy controls. Patients with MRM were recruited via a Dutch Web site inviting migraineurs to participate in research as part of the LUMINA project.[16] On the website, patients completed validated questionnaires based on the *International Classification of Headache Disorders, third edition (beta version) (ICHD-3-b)* criteria to diagnose migraine and MRM.[17] MRM is classified as migraine without aura occurring on day 1±2 of the menstrual cycle with additional attacks of migraine at other times of the menstrual cycle.[17] Our MRM diagnosis was adapted from the *ICHD-3-b* with a record of 3 menstrual cycles, of which 2 were prospective, and an interview. We recruited women without migraine via advertisement in local (Rotterdam, Netherlands) newspapers and flyers distributed in the Erasmus MC.

Data on medical history, medication, and information about the menstrual cycle or menopausal status were collected via an additional questionnaire. Information about headache frequency and severity of the patients with MRM was acquired from the LUMINA database.[16] Only patients with MRM who were not using migraine prophylactic treatment and who consented to refrain from the use of acute migraine therapy 48 hours before visits participated to prevent bias. Patients with MRM and healthy age-matched controls with a regular menstrual cycle, not using hormonal contraceptives, were eligible for inclusion.

Recruitment started March 2011 and continued until August 2012. Research was executed in the Internal Medicine Department of Erasmus MC. Patients with MRM did not have any medical condition besides migraine. Healthy age-matched controls and postmenopausal women were screened with a thorough interview checking for

any (cardiovascular) disease or medication use. Nonsmoking healthy women were included. For premenopausal women, one study visit was 19-21 days after the first day of menstruation and the second visit on day 1-2 of the subsequent menstruation (Figure 1). For postmenopausal women, 2 visits were scheduled 7-10 days apart. Weight, height and (supine) blood pressure were measured.

The first research visit was in July 2011 and the last one in September 2012. Migraine attack incidence was recorded from 1 month before the first research visit until 2 months later. Follow-up ended December 2012.

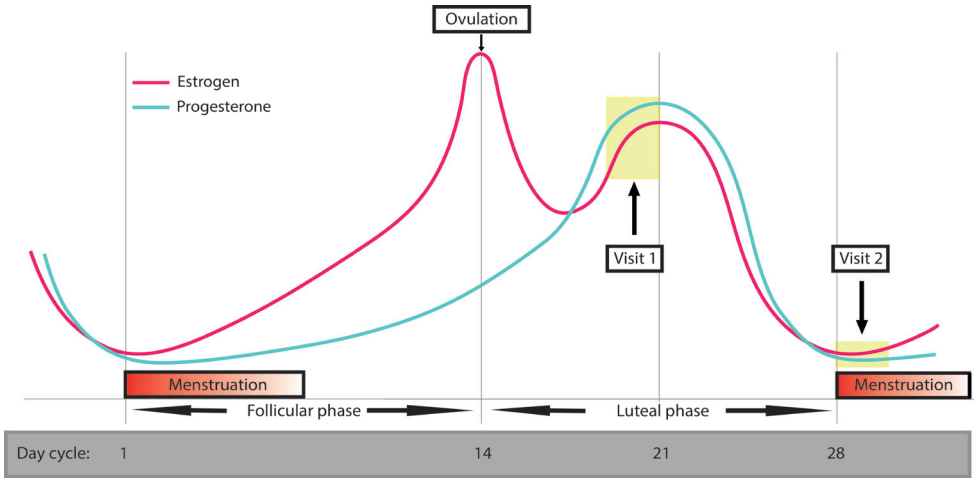


Figure 1. Estrogen and progesterone levels during menstrual cycle and time points of investigations. Yellow highlighted sections: time point of investigations for MRM patients and controls.

Forehead dermal blood flowstudies

Experiments were performed in a quiet, temperature-controlled room. Participants fasted for 3 hours before the measurements and both visits were during the same period of the day.

After 15 min acclimatization, 3 electrodes containing a 0.5-ml reservoir were placed on the forehead (for details, see our model validation article[15]). The electrodes were subsequently filled with three different types of solutions: normal saline, 0.06 mg/ml capsaicin, and 6.0 mg/ml capsaicin. A fourth electrode was placed in the neck region. An iontophoresis device (PeriIont 382b, Perimed, Sweden) was connected with the negative lead to the electrode containing saline and the positive lead to the electrode in the neck. Dermal blood flow (DBF) was measured with the PeriScan PIM-3 system (Perimed).

DBF at the site of the electrodes on the forehead was continuously measured for 40 min. After 2 min baseline measurement, a current (0.2 mA) was applied for 1 min to the electrode containing saline. DBF was subsequently measured during 6 min. This

process was repeated with increasing current intensities (0.4 mA, 0.6 mA, 0.8 mA), up to 1.0 mA.

Peripheral dermal blood flow response to ischemic stimulus

Post-occlusive reactive hyperemia (PORH) was measured at the volar site of the nondominant forearm (area 1x1 cm). A blood pressure cuff was placed around the upper arm. After a 2-min baseline DBF measurement, the pressure in the cuff was quickly increased to 200 mmHg, maintained for 5 min and subsequently released. PORH was continuously measured for 10 min.

CGRP measurements in saliva

Test subjects were given a cotton Salivette swab (Sarstedt AG & Co., Nümbrecht, Germany) to chew for 5 min. The swab was collected in the Salivette and stored at -80ºC. CGRP was determined by radioimmunoassay (Phoenix Pharmaceuticals, Inc, Burlingame, CA, USA), according to the manufacturer's instructions. Total protein content was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, Il, USA). Samples were measured in 2 blinded batches.

Estrogen and progesterone levels

Blood was collected via the cubital vein. Serum estradiol and progesterone levels were determined with the Coat-A-Count Estradiol and the Coat-A-Count Progesterone radioimmunoassay kits (Siemens Medical Solutions, Erlangen, Germany). Samples were measured in 2 blinded batches.

Statistical analysis

The number of cases and controls was based on the previously published validation study of our model.[15] Based on this study, a sample size of 20 is sufficient to be able to detect a 25% shift in the DBF response during the cycle and between the groups to 6 mg/ml capsaicin with an 80% power and 5% significance.

For each subject the maximal DBF (E_{max}) responses to capsaicin, and the ischemic stimulus were calculated. Similarly, for each subject the maximal DBF responses to each ES current intensity (0.2 mA-1.0 mA) was calculated. Individual data were analyzed in a blinded manner. Group values are provided as mean values and SEM, except for the demographic data in the Table, where SDs and ranges are presented as indicated. Differences between groups were analyzed using analysis of variance (ANOVA) and unpaired Student *t* test. Differences within groups were examined by Student paired *t* test. Repeated-measures ANOVA with multiple comparison tests were computed. Linear regression analysis was performed and β values with 95% confidence intervals are presented. The difference in DBF responses to capsaicin (Δ DBF) between groups and across the menstrual cycle were tested with the Mann-Whitney U test. A p value <0.05 was considered to indicate statistical significance. No correction for multiple testing was applied.

	Patients with migraine	Controls	Postmenopausal women
Population, *n*	22	20	22
Age, years	37±7 (21-45)	33±9 (19-45)	60±5 (50-68)
BMI, kg/m^2	22.9±3.9	24.0±1.6	23.8±2.3
BP, mmHg			
Systolic	105±17	109±9	118±9
Diastolic	66±7	64±6	71±6
HR , bpm	61±8	63±9	62±8
Age at migraine onset, years	18±7 (4-36)		
Disease duration, years	19±9 (6-41)		
Attack frequency, attacks per year			
1–2	1		
3–6	2		
7–12	5		
13–54	14		
Migraine incidence on day 1±2 of the menstrual cycle, No. of attacks			
Day 27	1		
Day 28	1		
Day 1	7		
Day 2	1		
Day 3	2		

Table 1. Demographics of the study population. BMI, body mass index. BP, blood pressure. HR, heart rate. Mean ± SD (Range).

Results

Subjects

Flow diagrams providing details on the recruitment are provided in Supplemental Figure 1. BMI, blood pressure, and heart rate were similar between groups (Table). Migraine attack incidence of the patients with MRM was highest on day 1 of the menstrual cycle (Table).

Forehead DBF responses

In healthy controls, DBF responses to 0.06 mg/ml and 6.0 mg/ml capsaicin were significantly higher during day 1-2 of their menstrual cycle compared to day 19-21 (Figures 2A and 2B). In contrast, in patients with MRM, DBF responses to 0.06 mg/ml and 6.0 mg/ml capsaicin were similar throughout their menstrual cycle. The increase in DBF responses of healthy controls to 0.06 mg/ml capsaicin between visits (Δ: 47±20 a.u.) was significantly different from the slight decrease in DBF responses

of the MRM patients (Δ: 6±24 a.u., p=0.040), whereas the slightly smaller increase in response to 6.0 mg/ml capsaicin (Δ: 41±14 a.u.) in healthy controls was not significantly different from that in patients with MRM (Δ: 6 ±14 a.u., p=0.078). There was no association between DBF responses of patients with MRM and the time to/since their migraine attacks. DBF responses in postmenopausal women were significantly lower than those in women with a menstrual cycle (controls and patients with MRM) and were similar between visits. DBF responses to ES between visits did not differ for either of the groups (Figure 2C). Repeated-measures ANOVA with a Greenhouse-Geisser correction revealed that mean DBF responses to ES differed significantly between stimulation intensities (0.2 mA-1 mA ($F(2.017, 121.032)$: 414.878, $p<0.0001$). Repeated-measures ANOVA revealed a group effect, with the lowest responses to ES in the postmenopausal women.

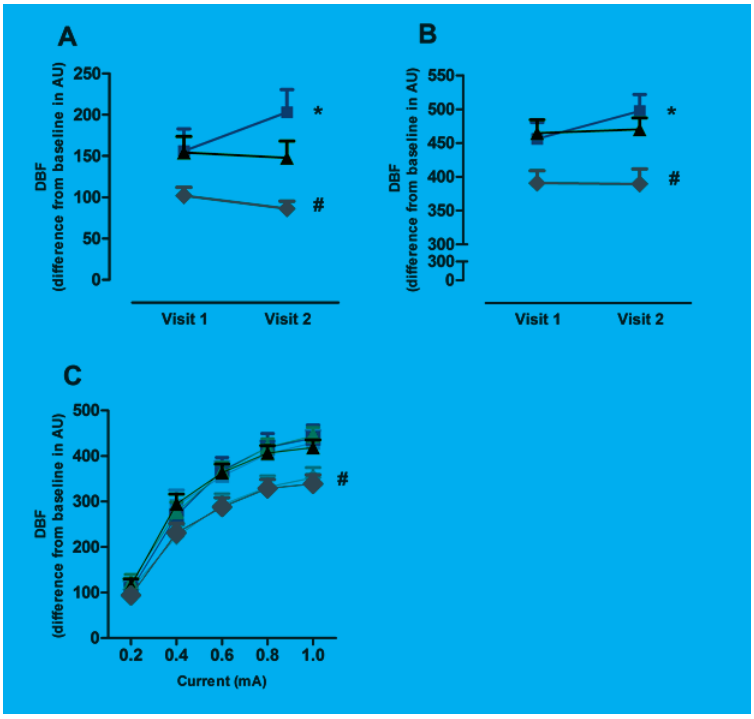


Figure 2. Maximal forehead dermal blood flow responses (DBF). DBF responses to 0.06 mg/ml capsaicin (2A), 6.0 mg/ml capsaicin (2B) and electrical stimulation (2C). For Controls (green ■) and patients with MRM (red ▲): Visit 1 = days 19-21 of the menstrual cycle and Visit 2 = days 1-2 of menstruation. For Postmenopausal women (blue ♦): Visit 1 and Visit 2 planned randomly with 7-10 days in-between. Figure 2C: visit 2 Controls (light green ■), visit 2 MRM patients (light red ▲) and visit 2 post-menopausal women (light blue ♦) *: Significant difference in Emax between Visit 1 and Visit 2. #: Significant difference in Emax during both visits compared to MRM patients and Controls.

Hormone levels

Serum estradiol levels on day 19-21 of patients with MRM were significantly lower than those of the healthy controls (52±4 pg/ml vs. 75±8 pg/ml respectively), while no differences in estradiol levels between MRM patients and healthy controls (25±5 pg/ml vs. 21±4 pg/ml respectively) were present on day 1-2 of the cycle (Figure 3A).

Serum progesterone levels in patients with MRM and healthy controls at the 2 occasions were similar (Figure 3B). Estradiol and progesterone levels during day 19-21 of the menstrual cycle were significantly higher than during day 1-2. Estradiol and progesterone levels of the postmenopausal women were low, consistent with their nonreproductive life stage.

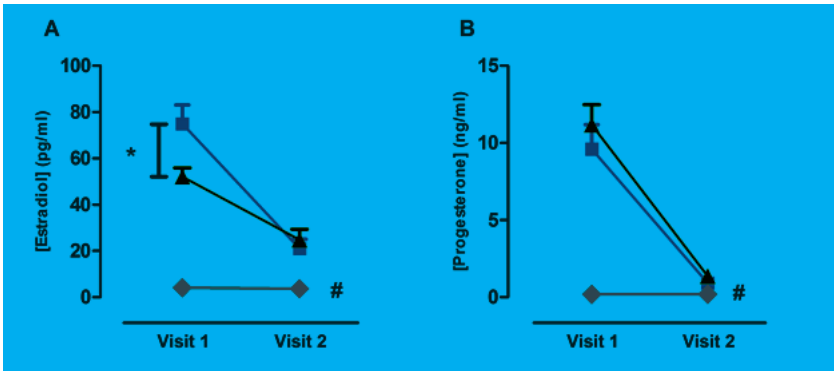


Figure 3. Serum estradiol levels (A) and progesterone levels (B). For Controls (green ■) and MRM patients (red ▲): Visit 1 = days 19-21 of the menstrual cycle and Visit 2 = days 1-2 of menstruation. For Postmenopausal women (blue ♦): Visit 1 and Visit 2 planned randomly with 7-10 days in-between. *: Significant difference in estradiol levels between MRM patients and controls. #: Significantly lower levels in Postmenopausal women during both visits compared to patients with MRM and controls.

Peripheral dermal blood flow response to ischemic stimulus

There were no significant differences in PORH responses between visits or between groups (Figure 4). Both age and the maximum PORH response were significant predictors for the E_{max} DBF response to 1 mA electrical stimulation (respectively: β:-2.455, 95% CI[-4.206; -0.705] and β: 0.813, 95% CI[0.165; 1.461]).

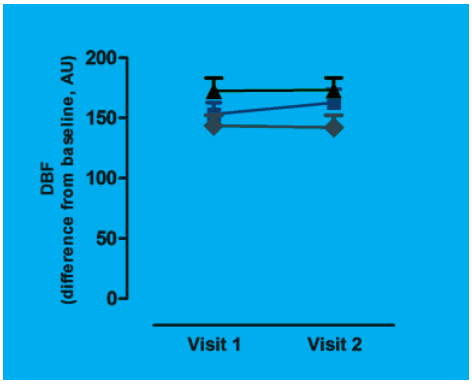


Figure 4. Maximal post occlusive reactive hyperemia (PORH) responses. For Controls (green ■) and patients with MRM (red ▲): Visit 1 = days 19-21 of the menstrual cycle and Visit 2 = days 1-2 of menstruation. For Postmenopausal women (blue♦): Visit 1 and Visit 2 planned randomly with 7-10 days in between.

CGRP in saliva

No differences in salivary CGRP levels between visits or between groups were observed (Supplemental 2). There was no correlation between saliva CGRP levels and the time to/since the last migraine attack of the MRM patients. The saliva volume collected from postmenopausal women was significantly lower than from healthy controls and patients with MRM on cycle days 19-21.

Discussion

We investigated the association between the menstrual cycling of female sex hormones on trigeminal nerve-mediated vasodilatation in healthy controls and MRM patients.

We clearly demonstrated a DBF response difference to capsaicin over the course of the menstrual cycle in healthy women, indicating that our model is suitable to detect cycle-dependent changes in trigeminovascular reactivity. In contrast, this cyclic DBF response difference was absent in patients with MRM. Notably, DBF responses to ES were similar throughout the menstrual cycle in both patients with MRM and healthy controls. As expected because of their stable low female sex hormone levels, postmenopausal women showed no cyclicity in their DBF responses to either capsaicin or ES between visits. Rather, when compared to the healthy controls and patients with MRM, they responded significantly lower to both stimuli. Another surprising finding of our study is the relatively low mean estradiol level detected during days 19-21 of the menstrual cycle of the patinets with MRM, which seems in agreement with their reduced cyclicity in DBF responses.

In accordance with our hypothesis, DBF responses to capsaicin during days 1-2 of the menstrual cycle were increased in healthy controls. The enhanced DBF response to capsaicin of healthy women around the time of their menstruation is consistent with previously published data, whereby, in healthy women, sensory and vasomotor responses to intradermal capsaicin injections of the forehead were increased during the menstruation compared to responses during the luteal phase.[18] The higher responses were attributed to either the direct effect of estrogen withdrawal on trigeminal nerve-innervated vasculature or to its effect on modulation of serum levels of ionized magnesium. Indeed, estrogen has been suggested to enhance neurogenic vasodilatation, primarily by CGRP, in rats.[19, 20] Because we did not detect cyclic responses to ES, the higher responses during menstruation in healthy controls may be attributed to differential activity of the TRPV1 channel. Alternatively, release of other neuropeptides in response to ES might mask the CGRP-specific component of the DBF response. Finally, the lack of cyclic responses to ES could be attributed to the stimulation time and intensity. With ES, we applied a brief (1-min) stimulus with a 6-min recovery time. Therefore, this recovery time might have been sufficient for the replenishment of the readily releasable pool of neuropeptide vesicles at the nerve terminals.[21] In contrast, with capsaicin application there is no recovery phase, but rather a constant stimulation during 40 min. This might lead to not only the exhaus-

tion of the readily releasable pool of vesicles, but also of the more slowly replenished neuropeptide vesicle reserve pool.

Contrary to our expectations, the DBF responses to capsaicin in patinets with MRM were unchanged throughout their menstrual cycle. Elevated CGRP levels in jugular blood during migraine attacks have been previously reported.[22] Consequently, we expected patients with MRM to have higher DBF responses to capsaicin during days 1-2 of their menstrual cycle. Our results may be explained by activity-dependent transport of neuropeptide in the trigeminal nerve. In particular, the higher release of CGRP from the dural fibres of the trigeminal nerve during the perimenstrual period might be attributable to enhanced anterograde transport from the cell soma, where CGRP is synthesized and packaged, to the nerve terminals from where it is synaptically released. Supporting such a potential mechanism is recent evidence from *Drosophila* that also suggests an activity-dependent mechanism of neuropeptide release.[23] Another possible explanation for the lack of cyclic responses in MRM patients could be an inhibition of TRPV1 channel function induced by the lower decline in estradiol levels in migraine patients as compared with healthy controls. Admittedly, the role of the TRPV1 channel in migraine is still ambiguous.[24, 25]

We included salivary measurements of CGRP levels as these reflect the activation state of the trigeminovascular system. Although previous studies have detected elevated CGRP levels in saliva in migraine patients during the premonitory and headache phase of a migraine attack,[26, 27] this finding was not replicated in a study with chronic migraine patients.[28] Notably, we observed similar levels of salivary CGRP in patinets with MRM and healthy controls. These data confirm our DBF measurements, because both the fibres of ophthalmic branch on the forehead (DBF response), as well as the fibres of the mandibular branch of the trigeminal nerve (saliva production) show similar responses in migraine patients independent from the timing in the menstrual cycle or the temporal relationship to a migraine attack. It is important to note that, contrary to the studies mentioned above, we did not collect saliva samples during the premonitory or headache phase of our patients with migraine, because we primarily intended to investigate the relation between CGRP and the menstrual phase of healthy controls and patients with migraine. Because this limits the conclusions that can be drawn from our salivary measurements, it would be interesting to study ictal salivary CGRP levels in patients with MRM in future.

As hypothesized, the DBF responses to capsaicin application and electrical stimulation in postmenopausal women were consistently lower than in menstruating women. To verify whether these findings were caused by general, age-dependent decreases of microcirculatory function, we performed the PORH test. Notably, the PORH response between groups was not significantly different. The relatively low age and good health of the included postmenopausal women may explain the normal PORH responses. Although not significant, the PORH responses of the postmenopausal women tended to be lower than the PORH responses of the premenopausal groups (Figure 4). However, this slight difference in PORH responses is not of such

magnitude to plausibly explain the significantly lower DBF responses of the postmenopausal women to capsaicin application and ES, which was confirmed by the regression analysis. Lower DBF responses to capsaicin application and ES thus seem to be related to the low estradiol levels after the menopause, which specifically affect the trigeminovascular system and do not induce generalized microcirculatory dysfunction.

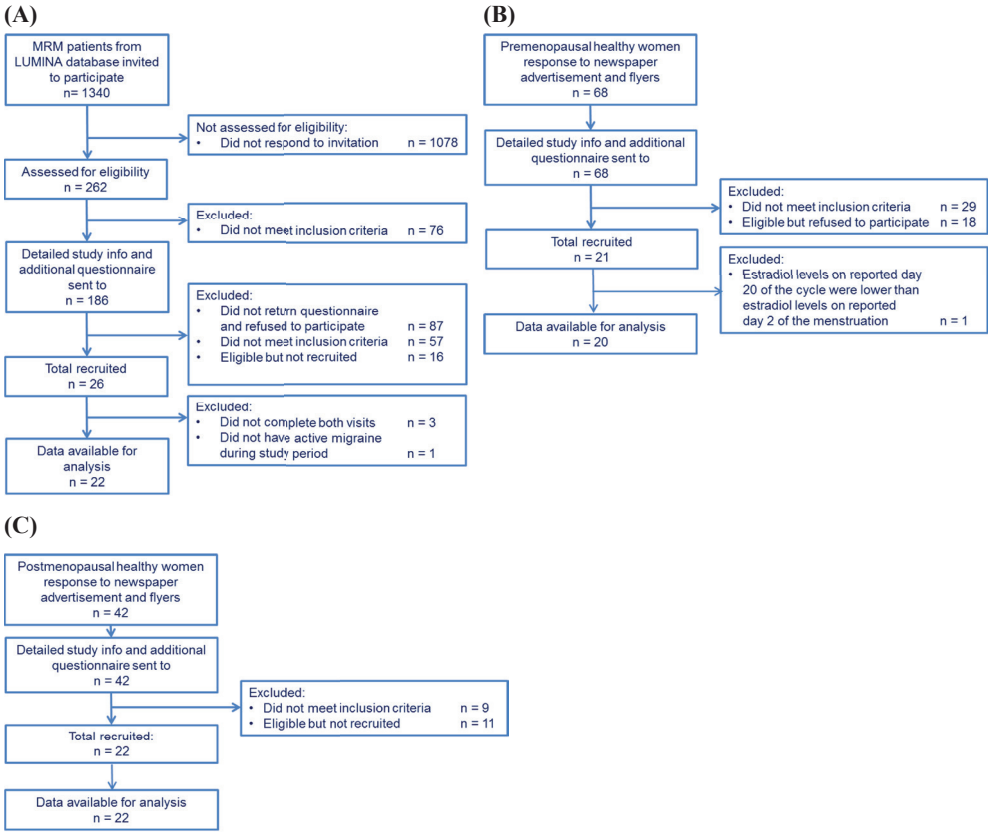
Our surprising finding of lower estradiol levels measured in patients with migraine during days 19-21 of the menstrual cycle is not attributable to the mean age of our study groups. In fact, previous studies have reported higher estradiol levels in perimenopausal women.[29] In our study, the patients with MRM had a slightly, but not significantly, higher mean age than the healthy controls (Table), despite having lower estradiol levels during the luteal phase (days 19-21 of the cycle). A relationship between low luteal estradiol levels and MRM has never previously been reported. High estradiol levels preceding ovulation have been linked to migraine with, but not to migraine without, aura.[30] Our observation is in accordance with previously published data,[4] in which the ratio between a urinary metabolite of estradiol measured during the luteal phase and during menses in menstrual migraine patients is the same as in our study (estradiol levels in patients with MRM: days 19-21:days 1-2 = 2:1). To replicate the data observed in our healthy controls, we used data from a previous study at our institution on healthy women with a regular menstrual cycle (n=9).[31] Indeed, the ratio between serum estradiol during the luteal phase and menses was the same as in our current study (estradiol levels healthy controls: days 19-21:days 1-2 = 4:1). We conceive that the number of subjects in the current study and the replication group is relatively small to make definite statements about the hormone levels in patients with MRM. Future studies should investigate these levels in a larger set of patients with MRM.

Taken together, our data confirms the pre-existing theory that the premenstrual withdrawal of estradiol influences the trigeminovascular system. Moreover, our study provides evidence for a disturbed systemic as well as trigeminovascular cyclicity in patients with MRM, which may augment their susceptibility to migraine around the time of menstruation.

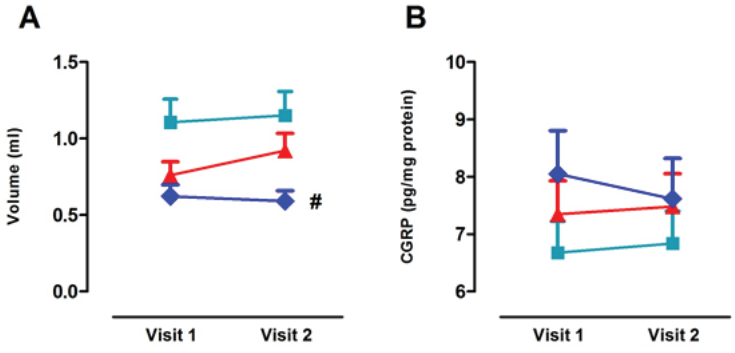
References

1. Buse DC, Loder EW, Gorman JA, et al. Sex Differences in the Prevalence, Symptoms, and Associated Features of Migraine, Probable Migraine and Other Severe Headache: Results of the American Migraine Prevalence and Prevention (AMPP) Study. Headache 2013.
2. Bille B. A 40-year follow-up of school children with migraine. Cephalalgia 1997;17:488-491; discussion 487.
3. Lipton RB, Stewart WF, Diamond S, Diamond ML, Reed M. Prevalence and burden of migraine in the United States: data from the American Migraine Study II. Headache 2001;41:646-657.
4. MacGregor EA, Frith A, Ellis J, Aspinall L, Hackshaw A. Incidence of migraine relative to menstrual cycle phases of rising and falling estrogen. Neurology 2006;67:2154-2158.
5. Somerville BW. The role of estradiol withdrawal in the etiology of menstrual migraine. Neurology 1972;22:355-365.
6. Somerville BW. Estrogen-withdrawal migraine. I. Duration of exposure required and attempted prophylaxis by premenstrual estrogen administration. Neurology 1975;25:239-244.
7. Somerville BW. Estrogen-withdrawal migraine. II. Attempted prophylaxis by continuous estradiol administration. Neurology 1975;25:245-250.
8. Couturier EG, Bomhof MA, Neven AK, van Duijn NP. Menstrual migraine in a representative Dutch population sample: prevalence, disability and treatment. Cephalalgia 2003;23:302-308.
9. MacGregor EA, Hackshaw A. Prevalence of migraine on each day of the natural menstrual cycle. Neurology 2004;63:351-353.
10. Edvinsson L, Villalón CM, MaassenVanDenBrink A. Basic mechanisms of migraine and its acute treatment. Pharmacology & therapeutics 2012;136:319-333.
11. Eftekhari S, Salvatore CA, Calamari A, Kane SA, Tajti J, Edvinsson L. Differential distribution of calcitonin gene-related peptide and its receptor components in the human trigeminal ganglion. Neuroscience 2010;169:683-696.
12. Edvinsson L, Elsas T, Suzuki N, Shimizu T, Lee TJ. Origin and Co-localization of nitric oxide synthase, CGRP, PACAP, and VIP in the cerebral circulation of the rat. Microscopy research and technique 2001;53:221-228.
13. Sarajari S, Oblinger MM. Estrogen effects on pain sensitivity and neuropeptide expression in rat sensory neurons. Exp Neurol 2010;224:163-169.
14. Multon S, Pardutz A, Mosen J, et al. Lack of estrogen increases pain in the trigeminal formalin model: a behavioural and immunocytochemical study of transgenic ArKO mice. Pain 2005;114:257-265.
15. Ibrahimi K, Vermeersch S, Danser AH,, et al. Development of an experimental model to study trigeminal nerve-mediated vasodilation on the human forehead. Cephalalgia 2014;34: 514-522.
16. van Oosterhout WPJ, Weller CM, Stam AH, et al. Validation of the web-based LUMINA questionnaire for recruiting large cohorts of migraineurs. Cephalalgia 2011;31:1359-1367.
17. Headache Classification Committee of the International Headache S. The International Classification of Headache Disorders, 3rd edition (beta version). Cephalalgia 2013;33:629-808.
18. Gazerani P, Andersen OK, Arendt-Nielsen L. A human experimental capsaicin model for trigeminal sensitization. Gender-specific differences. Pain 2005;118:155-163.
19. Gupta S, Mehrotra S, Villalón CM, Perusquia M, Saxena PR, MaassenVanDenBrink A. Potential role of female sex hormones in the pathophysiology of migraine. Pharmacology & Therapeutics 2007;113:321-340.
20. Gupta S, Villalon CM, Mehrotra S, et al. Female sex hormones and rat dural vasodilatation to CGRP, periarterial electrical stimulation and capsaicin. Headache 2007;47:225-235.
21. Rizzoli SO, Betz WJ. Synaptic vesicle pools. Nature reviews Neuroscience 2005;6:57-69.
22. Goadsby PJ, Edvinsson L. The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Annals of neurology 1993;33:48-56.
23. Shakiryanova D, Tully A, Levitan ES. Activity-dependent synaptic capture of transiting peptidergic vesicles. Nat Neurosci 2006;9:896-900.
24. Meents JE, Neeb L, Reuter U. TRPV1 in migraine pathophysiology. Trends Mol Med 2010;16:153-159.
25. Shimizu T, Shibata M, Toriumi H, et al. Reduction of TRPV1 expression in the trigeminal system by botulinum neurotoxin type-A. Neurobiol Dis 2012;48:367-378.
26. Cady RK, Vause CV, Ho TW, Bigal ME, Durham PL. Elevated saliva calcitonin gene-related peptide levels during acute migraine predict therapeutic response to rizatriptan. Headache 2009;49:1258-1266.
27. Bellamy JL, Cady RK, Durham PL. Salivary levels of CGRP and VIP in rhinosinusitis and migraine patients. Headache 2006;46:24-33.
28. Cady R, Turner I, Dexter K, Beach ME, Cady R, Durham P. An Exploratory Study of Salivary Calcitonin Gene-Related Peptide Levels Relative to Acute Interventions and Preventative Treatment With OnabotulinumtoxinA in Chronic Migraine. Headache 2014;54:269-277.

29. Santoro N, Brown JR, Adel T, Skurnick JH. Characterization of reproductive hormonal dynamics in the perimenopause. J Clin Endocrinol Metab 1996;81:1495-1501.
30. Nagel-Leiby S, Welch KM, Grunfeld S, D'Andrea G. Ovarian steroid levels in migraine with and without aura. Cephalalgia 1990;10:147-152.
31. van Santbrink EJ, Hop WC, van Dessel TJ, de Jong FH, Fauser BC. Decremental follicle-stimulating hormone and dominant follicle development during the normal menstrual cycle. Fertility and sterility 1995;64:37-43.



Supplemental Figure 1. Recruitment flow diagram. MRM patients (A), premenopausal healthy controls (B) and postmenopausal healthy controls (C).



Supplemental Figure 2. Collected saliva and measured CGRP levels. Collected saliva volume (5A) and CGRP levels (5B). For Controls (green ■) and patients with MRM (red ▲): Visit 1 = days 19-21 of the menstrual cycle and Visit 2 = days 1-2 of menstruation. For Postmenopausal women (blue ♦): Visit 1 and Visit 2 planned randomly with 7-10 days in-between. #: Significant difference in saliva levels during both visits.

CHAPTER 11

The influence of migraine and the menstrual cycle on capsaicin-induced dermal blood flow

Cephalalgia, under revision

K. Ibrahimi, S. Vermeersch, P. Frederiks, V. Geldof, C. Draulans,
L. Buntinx, E.M.E.H. Lesaffre, A. MaassenVanDenBrink & J. de Hoon

Abstract

Background: Migraine is much more common in females than in males and occurrence is associated with changes in female sex hormones. Calcitonin gene-related peptide (CGRP) plays a key role in migraine and variations in female sex hormones may affect CGRP sensitivity and or production. **Objectives**: Investigate repeatability, gender differences, influence of the menstrual cycle and of migraine on CGRP-dependent changes in dermal blood flow (DBF). **Methods**: CGRP-dependent increases in DBF were assessed using laser Doppler perfusion imaging after topical application of 300 or 1000 μg capsaicin on the forearm of healthy subjects and migraine patients. **Results**: In healthy males, DBF response did not vary over time and was comparable with DBF in male migraineurs. In healthy females, capsaicin-induced DBF responses to both doses of capsaicin were higher during menstruation compared to the late-secretory phase (p= 0.004-0.049). Compared to healthy subjects, female migraineurs displayed a higher DBF during menstruation and the late-secretory phase, which was not significantly different during the 2 phases of the cycle. **Conclusions**: An increased capsaicin-induced, CGRP-mediated DBF response was observed during menstruation in healthy women, but in female migraine patients this increased response was not affected by the menstrual cycle.

Introduction

Migraine headache is a neurovascular disorder in which the trigeminovascular system is primarily involved. Migraine is much more prevalent in women than in men[1, 2] and women with migraine also have higher rates of most migraine symptoms, more severe associated impairment, and higher healthcare resource utilization than males.[2]

Migraine attacks seem to be associated with changes of female sex hormone levels[3] as peaks of migraine frequency occur when estrogen levels drop;[4] i.e. around the menstruation period,[5, 6] at the end of pregnancy (postpartum) and during the transition to menopause. In pre-adolescent boys and girls, migraine prevalence is similar. However after menarche, migraine prevalence becomes approximately 3 times higher in females than males during the reproductive years of life.[7] Furthermore, in female migraine patients, a higher occurrence of attacks without aura is observed 2 days before onset of menses and on the first days of menses.[8] The exact diagnostic criteria for pure menstrual migraine without aura and menstrually related migraine without aura are defined by The International Classification of Headache Disorders.[9] As mentioned above, a relationship between migraine and estrogen withdrawal has been suggested, while rising levels of estrogen appear to be protective against migraine.[4] This theory is confirmed by studies reporting improvement of migraine during pregnancy,[10, 11] particularly during the second and third trimester, when serum levels of estradiol and progesterone are much higher than peak levels during native menstrual cycles. Postpartum, migraine reoccurs, but less in lactating women. It has even been suggested that the improvement observed during pregnancy is maintained by breast-feeding, a period of anovulation.[12] During the transition period to menopause, when estrogen levels vary, a higher migraine prevalence is

observed.[13] After the menopause, when female hormone levels are low, migraine in many patients disappears.[14]

Sex steroids can exert effects on neurotransmitter systems and pain processing networks relevant to migraine headache[15] and at the level of downstream signaling.[16] Estrogen affects growth factors and influences the function of neurons, glia cells and the vasculature. Therefore, estrogens may contribute to neurological disorders including migraine.[17] Mechanisms by which estrogens exert vascular functional effects, are nicely reviewed by Miller and Duckles.[18]

Findings over the last two decades also clearly indicate a pivotal role for calcitonin-gene related peptide (CGRP) in the pathophysiology of migraine. CGRP is a very potent vasodilator and a neuropeptide involved in pain transmission in the trigeminovascular system. Blocking CGRP is efficacious in migraine treatment.[19-21] To test receptor engagement of CGRP receptor antagonists in early clinical trials, a human exploratory biomarker model was developed.[22] This capsaicin-induced dermal blood flow (DBF) model was validated[23] as a reproducible human neurogenic inflammation model to elicit endogenous CGRP release via transient receptor potential vanilloid 1 (TRPV1) activation at peripheral nerve endings.[24] The resulting vasodilatation, as measured with laser Doppler perfusion imaging, was clearly shown to depend on CGRP.[25]

After validating the long term repeatability of the DBF model in healthy male volunteers, this study investigated the influence of the female menstrual cycle and of migraine on changes in skin perfusion resulting from the endogenous release of CGRP induced by the local application of capsaicin. We hypothesized that the CGRP response is fortified by migraine and/or dependent on the female hormonal cycle.

Methods

Subjects

This study was approved by the ethics committee of the University Hospitals of Leuven (Leuven, Belgium). Subjects were recruited via public advertisements at the university campus and in student magazines. Additionally, a volunteer database was used for subject recruitment. During a telephone call and e-mail communication, a first eligibility check was performed. During a subsequent screening visit, all in- and exclusion criteria were further checked. Written informed consent was obtained from all subjects during the screening visit. All subjects were adult, white, nonsmoking, healthy subjects (apart for migraine) based on medical history. No hormonal contraceptive method was allowed. As part of the health and demographic information questionnaire, women reported the first day and length of their most recent menstruation and confirmed having a regular menstrual cycle. Migraine patients suffering from moderate to severe migraine headache with or without aura were included as diagnosed according to IHS criteria (International Committee on the Classification of Headache Disorders, 2004). Subjects using cardiovascular medication or medication affecting the nervous system were excluded. In case of

acute anti-migraine medication use, a time window of 5 half-lives between medication intake and the experiment was taken into account. Subjects taking prophylactic migraine medication within 1 month of the screening visit or taking medication for acute headache on more than 10 days per month were excluded.

Study design

During a screening visit, medical history, inclusion and exclusion criteria were checked and basic demographic data collected. During each study visit, supine systolic blood pressure, diastolic blood pressure, and heart rate were measured at the dominant arm with a semi-automated oscillometric device (Omron HEM-705CP; Omron Healthcare, Hamburg, Germany) after an acclimatization period of at least 20 min. Subsequently, baseline DBF was determined as described previously,[22] using a laser Doppler perfusion imager (HR-LDPI system, Periscan PIMII). Only responders, defined as subjects with a capsaicin-induced increase in DBF of ≥100% in both proximal sites of both forearms were included after the screening visit. All measurements were performed while the subjects rested in a supine position on a comfortable bed in a quiet, temperature-controlled room (ambient temperature 23 ± 1°C). Subjects were instructed to abstain from chocolate-, alcohol-, and caffeine-containing beverages and food during the 12 h period preceding each study visit. General fasting for 3 hours before the experiment was demanded. Blood samples were taken from participating women to assess female hormone levels (i.e. estrogen and progesterone).

Repeatability of capsaicin-induced DBF over time

Male healthy subjects were included in a 4-period, single-blind study. Study periods with capsaicin-induced DBF assessments were separated by a wash-out period of 7(±1) days.

Menstrual cycle effects in healthy females

For the study part assessing menstrual cycle effects in healthy females, capsaicin-induced DBF was evaluated weekly up to 2 menstrual cycles. Women were included at random periods of their menstrual cycle to avoid confounding effects over time. Measurements from women with an irregular menstrual cycle were excluded from the analysis.

Migraine patients

In the study part with migraine patients, male subjects were assessed at 2 study visits apart from the screening visit. Average capsaicin-induced DBF was calculated from these 2 visits. After screening, female migraineurs underwent the DBF experiment one week before expected menstruation, and once during their menstruation period. All experiments in migraine patients were performed during the interictal period.

Capsaicin-induced DBF response

In each subject, 3 equally spaced (minimum distance of 4 cm) 10-mm rubber O-rings

(8 mm inner diameter) were placed on the volar surface of each forearm. In the 2 most proximal rings, subjects received a topical dose of 300 or 1000 μg/20 μL capsaicin (single-blind, randomized). In the distal ring of both arms, placebo (i.e. vehicle) was applied. The application of capsaicin/placebo started on the dominant arm (t=0) with a 1-min interval between each ring. The applications at the non-dominant arm started 5 min after the first application at the dominant arm. DBF was assessed using laser Doppler perfusion imaging just before and at 10, 20, 30 and 40 min after capsaicin/vehicle application.

Estradiol and progesterone plasma levels

Serum estradiol and progesterone levels were determined with the Coat-A-Count Estradiol and the Coat-A-Count Progesterone kits (Siemens Medical Solutions, Netherlands). These are no-extraction, solid-phase ^{125}I radioimmunoassay kits designed for measurement of estradiol and progesterone in serum.

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 of 20%.[22] To assess repeatability of DBF after capsaicin/vehicle application, percentage change in DBF from baseline to 40 min after capsaicin/vehicle application was calculated by ANOVA including dose, time point and week in the model. For comparison between healthy and migraine subjects, percentage change in DBF (corrected for the vehicle response) was calculated at each time point (pre-capsaicin, 10, 20, 30 and 40 min post-capsaicin). The area under the curve of DBF response from 0 up to 40 min after capsaicin application ($AUC_{0\text{-}40}$, % · min) was calculated as a summary measure using DBF ratios. Unless stated otherwise, mean and standard error of the mean (SEM) are presented.

SPSS version 21 (SPSS inc, IBM,Chicago, IL, USA) was used for statistical analysis. Repeated-measures analysis of variance (ANOVA) with a Bonferroni post-hoc test was used to assess reproducibility in healthy males, menstrual cycle effects in healthy females, and comparison of differences between healthy subjects and migraine patients. To compare the effects of female hormones on capsaicin-induced DBF with those recently reported in literature,[26] we performed paired t-tests comparing the response during menses and the late-secretory phase of the menstrual cycle. Kruskal-Wallis ANOVA was used to assess hormonal changes during the 4-week menstrual cycle of healthy subjects and migraine patients. With a Dunn's post hoc test we compared the hormone levels within and between groups. A two-tailed p-value <0.05 was considered statistically significant.

Results

Subjects

Five healthy subjects and two migraineurs were excluded at screening being non-

responder to capsaicin. Data of 5 subjects were excluded from analysis because of medication use; 4 others were excluded because of irregular menstrual cycles. Due to technical breakdown of the LDI scanner, data from 7 out of 26 healthy females were exclusively used for analysis of menstrual cycle effects within the group of healthy females and excluded from comparison with migraine women as they were scanned with a differently calibrated scanner. Likewise, data from 2 out of 16 healthy men were only used for weekly repeatability analysis within healthy males, and not for inter-group comparison.

Repeatability in males

Figure 1 describes DBF response 10, 20, 30 and 40 min after application of vehicle, 300 or 1000 µg capsaicin solution, assessed at 4 consecutive weeks in 16 healthy men (average age 23.6 years, range: 20-28). Significant differences were found between doses (placebo, 300 µg and 1000 µg capsaicin; $p<0.001$) and between time points (10, 20, 30 and 40 min post-capsaicin; $p<0.001$). Over a period of 4 weeks the capsaicin-induced increase in DBF did not change ($p=0.06$).

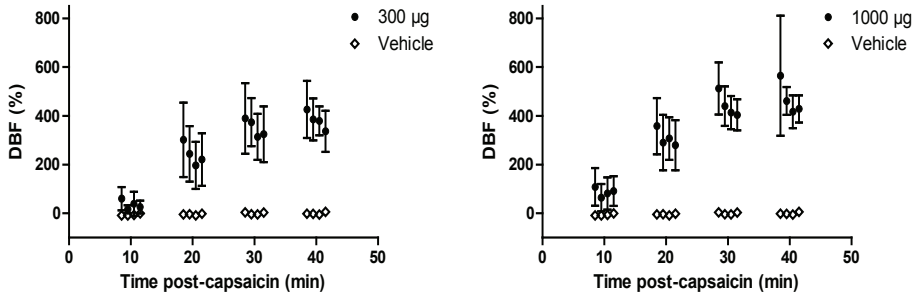


Figure 1: Repeatability of dermal blood flow over 4 consecutive weeks in healthy men (n=16). DBF is presented as percentage change from baseline. Mean and 95% CI. DBF: dermal blood flow.

Menstrual cycle effects in healthy females

Twenty-six healthy females, not taking hormonal contraceptives were enrolled (average age 21.6 years, range 18-26). DBF was expressed as (i) percentage increase in DBF 40 min post-capsaicin application compared to placebo, as well as (ii) area-under-the-curve of percentage change in DBF response, expressed as percentage increase compared to placebo, from 0 up to 40 min (AUC_{0-40}). Repeated measures ANOVA did not reveal a significant effect of the menstrual cycle on the 40 min DBF response to 300 µg capsaicin (figure 2A), neither on the 40 min DBF response (figure 2B) and nor on the AUC of the DBF response to 1000 µg (figure 2D). We did however find a trend for significance of the menstrual cycle effect on the AUC of the DBF response to 300 µg capsaicin (figure 2C, $P=0.053$). The 40 min DBF response to 300 µg capsaicin. When specifically comparing the menses with the late secretory phase, we in most comparisons observed increased DBF responses to both doses of capsaicin during the menstruation (figure 2A: $p=0.049$, figure 2B: $p=0.004$, figure 2C: $p=0.023$, figure 2D: $p=0.065$).

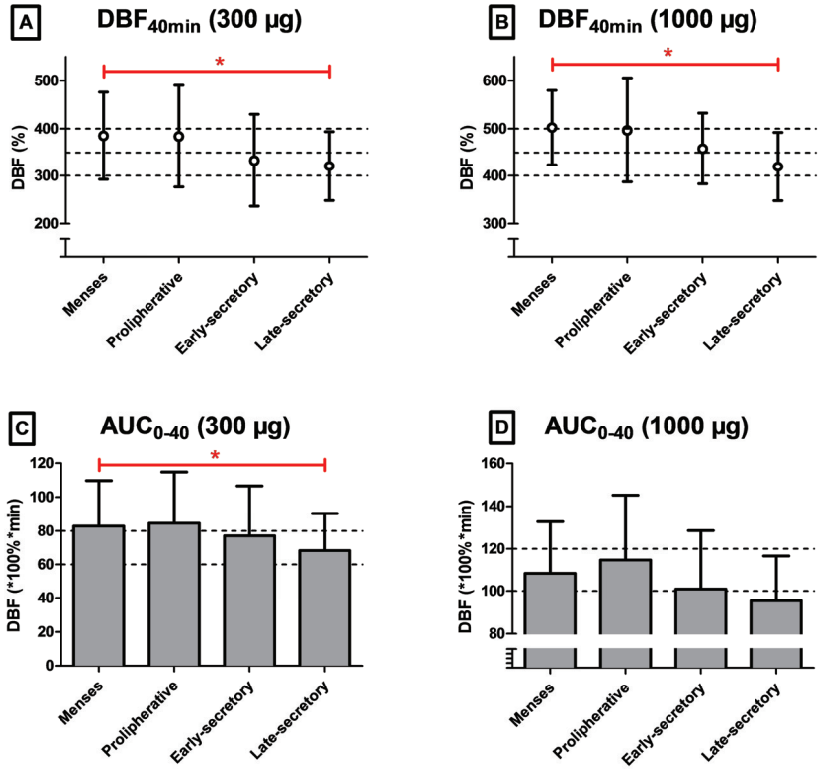


Figure 2: DBF during each week of the menstrual cycle in healthy females (n=26). DBF was expressed as percentage change in perfusion after 300 µg (panel A) and 1000 µg capsaicin (panel B) compared to vehicle. DBF_{40min}: capsaicin-induced dermal blood flow 40 min after capsaicin application; AUC_{0-40}: area under the curve of the DBF response from 0 till 40 min after 300 µg (panel C) and 1000 µg (panel D) capsaicin application; * Student's paired t-test comparing menses with late secretory phase, $p<0.05$. Mean and 95% CI.

Migraine patients

Demographics for inter-group comparison of healthy subjects and migraine patients are displayed in table 1. Overall, migraine patients were well matched with healthy control groups for age, body mass index and blood pressure parameters. However, the female migraine patient group was slightly older than the female control group (25.1 versus 21.5 years; $p<0.001$). On average, migraine patients suffered from migraine for 11 years (± 5.8 years), with a frequency of 5.6 attacks (± 6.9 attacks) over the last 3 months preceding participation to this study. Sixteen (=53%) of participating migraine patients had aura attacks preceding their headache: 7 out of 14 (50%) females and 9 out of 16 males (56%). Fifteen patients (50%) reported dietary causes of migraine attacks, 15 patients (50%) reported a sleep pattern relationship and 17 patients (57%) indicated stress-related migraine attacks. From the 14 female migraineurs, 4 (29%) reported migraine attacks were associated with menstruation. For acute treatment of their migraine attacks, participants used paracetamol (21 patients, i.e. 70%), non-steroidal anti-inflammatory drugs (14 patients, i.e. 47%), opioids (1 subject, i.e. 3%), anti-emetics (12 subjects, i.e. 40%), and triptans (2 subjects, i.e. 7%).

	♀			♂		
	Healthy (N=19)	**Migraine (N=14)**	***p-value***	**Healthy (N=14)**	**Migraine (N=16)**	***p-value***
Age (years)	21.5 ± 1.8	25.1 ± 3.5	*0.0006*	22.8 ± 2.0	21.6 ± 3.4	*0.21*
BMI (kg/m^2)	22.5 ± 2.0	22.5 ± 3.9	*0.97*	23.2 ± 2.8	22.2 ± 3.1	*0.42*
BP, (mm Hg)						
Systolic	114.6 ± 9.6	114.7 ± 9.8	*0.97*	125.2 ± 10.7	122.4 ± 7.7	*0.40*
Diastolic	66.6 ± 6.3	69.8 ± 7.4	*0.20*	65.9 ± 6.6	65.1 ± 5.5	*0.70*
Heart rate, bpm	65.6 ± 10.8	67.4 ± 9.6	*0.63*	60.6 ± 6.3	60.3 ± 8.2	*0.92*

Table 1: Inter-group comparison of demographics of healthy subjects and migraine patients. Data presented as mean ± SD.

Repeated measures ANOVA did not reveal a significant effect of the menstrual cycle phase within the migraine patients on the 40 min response or on the AUC of the capsaicin induced DBF neither for 300 nor for 1000 µg capsaicin (figure 3A-figure 3D). When comparing the DBF response at 40 min between migraine patients and healthy women together in one analysis, effect of disease state (healthy vs. migraine, p=0.052) appeared to influence the response to the low dose of capsaicin (300 µg), but not (p=0.07) when studying the high dose (1000 µg) of capsaicin. In contrast, the AUC was significantly affected by disease state for both doses of capsaicin. These differences in AUC values are in line with the observation that migraine patients tended to reach their maximal response to capsaicin at an earlier time point than the healthy women (supplementary figure 1). No difference in DBF was found between healthy men and men suffering from migraine after both 300 and 1000 µg capsaicin (figure 3).

Hormone levels

During the menstrual cycle within the healthy women (73 ± 12 pg/ml to 25 ± 3 pg/ml, $p<0.05$) and within migraine patients (75 ± 12 pg/ml to 28 ± 3 pg/ml, $p<0.001$), a significant drop in estrogen before menstruation was observed. No differences in estrogen levels were found between both groups during menstruation and late-secretory week.

Similarly, within both the healthy women (10 ± 2 ng/ml to 0.6 ± 0.1 ng/ml, $p<0.001$) as well as migraine patients (5 ± 1 ng/ml to 0.7 ± 0.2 ng/ml, $p<0.05$), a significant drop in progesterone before menstruation was observed. No differences in progesterone levels were found between groups during either the late-secretory week or menstruation.

Discussion

The present study confirms the repeatability of DBF changes induced by weekly applications of capsaicin in healthy males without evidence for major desensitization. Unlike men, females displayed fluctuations in the CGRP-dependent dermal blood

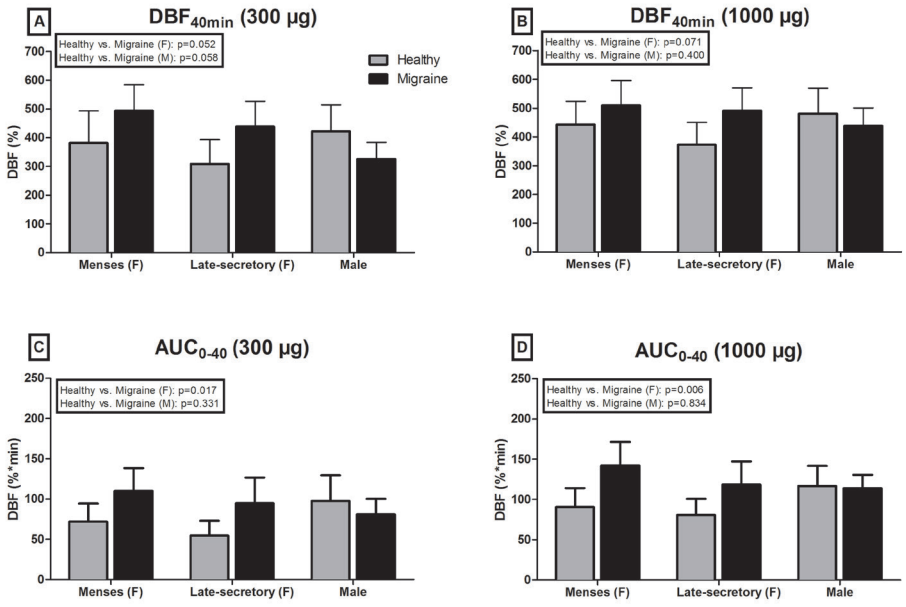


Figure 3: Increased DBF in migraine women (n=14) compared to healthy women (n=19). No difference between healthy men (n=14) and migraine men (n=16) after 300 and 1000 µg capsaicin. DBF was expressed as percentage change in perfusion after 300 µg or 1000 µg capsaicin compared to vehicle. DBF_{40min}.: capsaicin-induced dermal blood flow 40 min after capsaicin 300 µg (panel A) and 1000 µg (panel B) capsaicin application; AUC_{0-40}: area under the curve of the DBF response from 0 till 40 min after capsaicin after 300 µg (panel C) and 1000 µg (panel D) capsaicin application. Mean and 95% CI.

flow response, which was increased during the menstruation period. DBF responses were elevated in female migraine patients compared to healthy subjects.

Since the capsaicin-induced DBF response is repeatable over time in healthy male volunteers , the "capsaicin-model" can also be used as target-engagement biomarker for the prospective follow-up of long-acting biologicals including monoclonal antibodies targeting CGRP,[27] next to short acting small molecule CGRP-receptor antagonists in early clinical drug development.[25]

As there was no significant desensitization to capsaicin observed in healthy males and, moreover, because women were included at random weeks during their menstrual cycle, the changing DBF response in healthy women during the menstrual cycle can most likely be attributed to hormonal changes related to the menstrual cycle. This was previously suggested by findings of Gazerani *et al.*[26, 28] After intradermal capsaicin injection to the forehead, they found an increased flare area in women during the menstrual phase compared to the luteal phase. Accordingly, after topical application of capsaicin on the forearm, we found an increased DBF in healthy females during their menstrual phase compared to the luteal phase. In contrast, we did not find an effect of the menstrual cycle on the capsaicin-induced DBF in

migraine patients. Interestingly, upon comparison of corresponding phases of the menstrual cycle, female migraine patients responded with higher increases in DBF to capsaicin application than healthy women did. This effect may have been augmented by an earlier response to capsaicin in patients (Supplemental Figure 1) and suggests an increased availability of CGRP or an increased sensitivity to capsaicin and/or CGRP. The lack of menstrual cycle-dependent variation in migraine has previously been reported in our study where we assessed the influence of the menstrual cycle on capsaicin-induced forehead DBF, thus comparing trigeminovascular activation in healthy women to that in women with menstrually related migraine.[26]

The importance of CGRP in the pathophysiology of migraine and a correlation between migraine incidence, menstrual cycle and decreasing estrogens, as described in literature, are in line with our findings. A substantial period with high estrogen exposure seems to be necessary for migraine attacks to result from estrogen withdrawal.[29-31] Many studies have described an association between the occurrence of migraine attacks and menstrual cycle,[4] pregnancy,[32, 33] partus[34] and menopausal transition.[13] However, effective hormonal treatment of migraine associated with menstruation and the menopause is lacking.[3, 35-37] Also, association studies focusing on the role of sex hormone receptor gene polymorphisms in migraine remain inconclusive.[38] Nevertheless, changes in levels of female sex hormones like estrogen and progesterone, clearly modulate neurotransmitter systems,[17] influence brain function[15] and vascular function[18] in diverse ways.

In contrast to females, comparison of healthy men with male migraine patients showed no difference in DBF response. Gender differences (due to hormonal fluctuations) are suggested to have implications on both migraine susceptibility and migraine mechanism between males and females.[39] Gender differences further illustrate the complexity of migraine and may support the hypothesis that clinically observed migraine features are modulated by different mechanistic pathways that trigger migraine.

Migraine subjects included in this study met the criteria for episodic migraine as classified in the International Classification for Headache (ICHD-II). No distinction was made between menstrual, menstrually-related or non-menstrual migraine subjects due to small subgroup under powering. As there are subtle differences between these groups, the hormonal effects observed in our study should represent a more general migraine population, but might be more pronounced in purely menstrual migraine without aura patients. Hormone levels between healthy female subjects and migraine patients in our study did not differ, suggesting that in this group of migraine patients higher sensitivity to female hormones is more important than absolute plasma levels.

The exclusion of non-responders to capsaicin could be a limitation of this study as this may bias our results. However, we excluded 5 healthy subjects and only 2 migraineurs because of this criterion, implicating that inclusion of non-responders would even fortify our findings. The reason why some subjects do not respond to topical applied capsaicin is unknown, but has been observed in other studies as

well.[23, 40] Genetic analysis of non-responders to detect TRPV1 polymorphisms or other mutations could provide an explanation. However, it has been suggested that the absence of a DBF response may be purely related to insufficient skin penetration rather than to the absence of (functional) TRPV1 channels. This was supported by the observation that subjects with an apparently absent response to capsaicin applied on the forearm do respond after application on the forehead skin.[41]

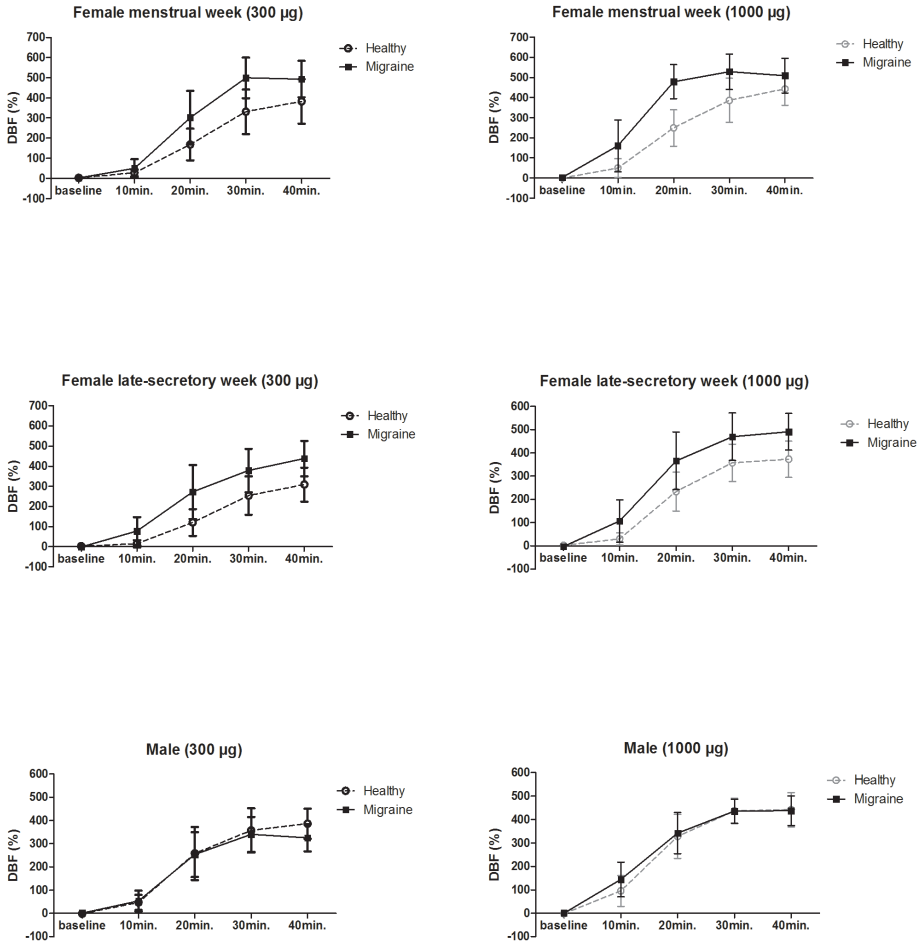
Molecular and physiological mechanisms for increased DBF response after capsaicin application could be the result of several mechanisms including increased neuronal sensitivity to capsaicin, perhaps due to increased TRPV1 expression, increased release of CGRP, or increased sensitivity to CGRP. Female hormones influence neurotransmitter systems,[17] vasculature[18] and thus migraine,[15] but further research is needed to clarify which mechanisms are involved.[42]

In summary, we first validated long-term repeatability of the CGRP-dependent DBF response in men. Secondly, we showed increased DBF response during the menstruation period in healthy women. Finally, a higher and seemingly faster CGRP-dependent DBF response that was not influenced by the menstrual cycle, was confirmed in women suffering from migraine. Our data demonstrate the influence of female menstrual cycle and migraine on CGRP-mediated vascular responsiveness, which further supports hormonal effects and increased female susceptibility to migraine.

References

1. Vos T, Flaxman AD, Naghavi M, et al. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 2012;380:2163-2196.
2. Buse DC, Loder EW, Gorman JA, et al. Sex differences in the prevalence, symptoms, and associated features of migraine, probable migraine and other severe headache: results of the American Migraine Prevalence and Prevention (AMPP) Study. Headache 2013;53:1278-1299.
3. Brandes JL. The influence of estrogen on migraine: a systematic review. Jama 2006;295:1824-1830.
4. MacGregor EA, Frith A, Ellis J, Aspinall L, Hackshaw A. Incidence of migraine relative to menstrual cycle phases of rising and falling estrogen. Neurology 2006;67:2154-2158.
5. MacGregor EA, Hackshaw A. Prevalence of migraine on each day of the natural menstrual cycle. Neurology 2004;63:351-353.
6. Couturier EG, Bomhof MA, Neven AK, van Duijn NP. Menstrual migraine in a representative Dutch population sample: prevalence, disability and treatment. Cephalalgia 2003;23:302-308.
7. Stewart WF, Lipton RB, Celentano DD, Reed ML. Prevalence of migraine headache in the United States. Relation to age, income, race, and other sociodemographic factors. Jama 1992;267:64-69.
8. Stewart WF, Lipton RB, Chee E, Sawyer J, Silberstein SD. Menstrual cycle and headache in a population sample of migraineurs. Neurology 2000;55:1517-1523.
9. (IHS) HCCotIHS. The International Classification of Headache Disorders, 3rd edition (beta version). Cephalalgia 2013;33:629-808.
10. Sances G, Granella F, Nappi RE, et al. Course of migraine during pregnancy and postpartum: a prospective study. Cephalalgia 2003;23:197-205.
11. Somerville BW. A study of migraine in pregnancy. Neurology 1972;22:824-828.
12. Marcus DA, Scharff L, Turk D. Longitudinal prospective study of headache during pregnancy and postpartum. Headache 1999;39:625-632.
13. Wang SJ, Fuh JL, Lu SR, Juang KD, Wang PH. Migraine prevalence during menopausal transition. Headache 2003;43:470-478.
14. Neri I, Granella F, Nappi R, Manzoni GC, Facchinetti F, Genazzani AR. Characteristics of headache at menopause: a clinico-epidemiologic study. Maturitas 1993;17:31-37.
15. Martin VT, Behbehani M. Ovarian hormones and migraine headache: understanding mechanisms and pathogenesis--part I. Headache 2006;46:3-23.
16. Gupta S, McCarson KE, Welch KM, Berman NE. Mechanisms of pain modulation by sex hormones in migraine. Headache 2011;51:905-922.
17. Scharfman HE, MacLusky NJ. Estrogen-growth factor interactions and their contributions to neurological disorders. Headache 2008;48 Suppl 2:S77-89.
18. Miller VM, Duckles SP. Vascular actions of estrogens: functional implications. Pharmacol Rev 2008;60:210-241.
19. Olesen J, Diener HC, Husstedt IW, et al. Calcitonin gene-related peptide receptor antagonist BIBN 4096 BS for the acute treatment of migraine. N Engl J Med 2004;350:1104-1110.
20. Hewitt DJ, Aurora SK, Dodick DW, et al. Randomized controlled trial of the CGRP receptor antagonist MK-3207 in the acute treatment of migraine. Cephalalgia 2011;31:712-722.
21. Ho TW, Ferrari MD, Dodick DW, et al. Efficacy and tolerability of MK-0974 (telcagepant), a new oral antagonist of calcitonin gene-related peptide receptor, compared with zolmitriptan for acute migraine: a randomised, placebo-controlled, parallel-treatment trial. Lancet 2008;372:2115-2123.
22. Van der Schueren BJ, Rogiers A, Vanmolkot FH, et al. Calcitonin gene-related peptide8-37 antagonizes capsaicin-induced vasodilation in the skin: evaluation of a human in vivo pharmacodynamic model. J Pharmacol Exp Ther 2008;325:248-255.
23. Van der Schueren BJ, de Hoon JN, Vanmolkot FH, et al. Reproducibility of the capsaicin-induced dermal blood flow response as assessed by laser Doppler perfusion imaging. Br J Clin Pharmacol 2007;64:580-590.
24. Brain SD, Grant AD. Vascular actions of calcitonin gene-related peptide and adrenomedullin. Physiol Rev 2004;84:903-934.
25. Sinclair SR, Kane SA, Van der Schueren BJ, et al. Inhibition of capsaicin-induced increase in dermal blood flow by the oral CGRP receptor antagonist, telcagepant (MK-0974). Br J Clin Pharmacol 2010;69:15-22.
26. Ibrahimi K, van Oosterhout WPJ, van Dorp W, et al. Reduced trigeminovascular cyclicity in patients with menstrually related migraine. Neurology 2015;84:125-131.
27. Vermeersch S, Van Hecken A, Abu-Raddad E, et al. Translational Pharmacodynamics of CGRP Monoclonal Antibody LY2951742 in Capsaicin-Induced Dermal Blood Flow Model. Cephalagia 2013;33:249-250.
28. Gazerani P, Andersen OK, Arendt-Nielsen L. A human experimental capsaicin model for trigeminal

sensitization. Gender-specific differences. Pain 2005;118:155-163.
29. MacGregor EA. Oestrogen and attacks of migraine with and without aura. Lancet Neurol 2004;3:354-361.
30. Somerville BW. The role of estradiol withdrawal in the etiology of menstrual migraine. Neurology 1972;22:355-365.
31. Epstein MT, Hockaday JM, Hockaday TD. Migraine and reporoductive hormones throughout the menstrual cycle. Lancet 1975;1:543-548.
32. Loder E. Migraine in pregnancy. Semin Neurol 2007;27:425-433.
33. Granella F, Sances G, Pucci E, Nappi RE, Ghiotto N, Napp G. Migraine with aura and reproductive life events: a case control study. Cephalalgia 2000;20:701-707.
34. Klein AM, Loder E. Postpartum headache. Int J Obstet Anesth 2010;19:422-430.
35. Loder E, Rizzoli P, Golub J. Hormonal management of migraine associated with menses and the menopause: a clinical review. Headache 2007;47:329-340.
36. Calhoun AH. Current topics and controversies in menstrual migraine. Headache 2012;52 Suppl 1:8-11.
37. Silberstein SD. Sex hormones and headache. Rev Neurol (Paris) 2000;156 Suppl 4:4S30-41.
38. Schurks M, Rist PM, Kurth T. Sex hormone receptor gene polymorphisms and migraine: a systematic review and meta-analysis. Cephalalgia 2010;30:1306-1328.
39. Guidetti V, Lucchese F, Bellini B. Is the migrainous female brain different? Some new evidence. Brain 2012;135:2311-2313.
40. Ferrell WR, Wong BB, Lockhart JC, Ramsay JE. Gender differences in regional cutaneous microcirculatory responses to capsaicin. Fundam Clin Pharmacol 2004;18:195-200.
41. Ibrahimi K, Vermeersch S, Danser A, et al. Development of an experimental model to study trigeminal nerve-mediated vasodilation on the human forehead. Cephalalgia 2014;34:514-522.
42. Gupta S, Mehrotra S, Villalón CM, Perusquia M, Saxena PR, MaassenVanDenBrink A. Potential role of female sex hormones in the pathophysiology of migraine. Pharmacol Ther 2007;113:321-340.



Supplemental Figure 1. DBF in migraine women (n=14) compared to healthy women (n=19) and healthy men (n=14) and migraine men (n=16) after 300 µg and 1000 µg capsaicin. DBF was expressed as percentage change in perfusion after 300 µg or 1000 µg capsaicin compared to vehicle.

CHAPTER 12

Summarizing discussion and future perspectives

Part I: Experimental models of migraine

Since the advent of triptans, no other family of drugs has been as successful in the treatment of migraine. Despite being widely used, triptans are not effective in all patients. Due to their vascular effects they are contraindicated in patients with cardiovascular disease, and 14-30 % of patients are non-responders.[1, 2] Thus, the search for new anti-migraine therapy, preferably prophylactic therapy that could prevent attacks altogether, is still ongoing. The potent vasodilator calcitonin gene-related peptide (CGRP) is the key peptide in the pathophysiology of migraine and together with its receptor(s) the most promising target for new drug development.[3] In ***Chapter 3*** we reviewed several *in vitro* and *in vivo* experimental models that can be applied for the discovery and characterization of new CGRP receptor antagonists. These models are also well suitable in researching the recently developed antibodies directed against CGRP and its receptor, that are now undergoing Phase II and Phase III clinical trials. Migraine models not only are essential in investigating new anti-migraine therapy, they also provide an opportunity to study migraine pathophysiology.

We do have to keep in mind that experimental models not always accurately predict or mimic disease. For example, in ***Chapter 4*** we have commented on a study investigating vasodilatation of intra- and extra-cranial arteries in relation to attacks of migraine without aura (MO) and treatment with sumatriptan. In comparison with earlier publications where migraine attacks in migraine patients were provoked by infusion of CGRP, the results of this study with spontaneous migraine were contradictory.[4] The results of the migraine provocation study indicated that MO was associated with dilation of cranial arteries and that the headache location was associated with the location of vasodilatation.[5] In contrast, the results of the study with spontaneous migraine showed no significant dilatation of the extracranial arteries on the migraine headache pain side and only minor dilatation of the cerebral arteries.[4] This contrast could be due to differences between mechanisms leading to a provoked or a spontaneous migraine attack, but might also be due to technical limitations of the detection method that was used. In both the above-mentioned studies, vasodilatation was assessed with magnetic resonance imaging (MRI). A limitation of MRI is that the intracranial part of the middle meningeal artery cannot be measured, while this intracranial portion of the middle meningeal artery is most likely crucial in the pathophysiology of migraine. As with most experimental migraine models, MRI does not integrate the neuronal and vascular components of migraine.

Considering this lack, we set up a non-invasive human model where we could investigate the trigeminovascular activation of the forehead skin in humans. In ***Chapter 5,*** we have described the development and validation of this model. It is known that during migraine, CGRP is released from the trigeminal sensory nerves.[6] Within our model we induce the release of endogenous CGRP by applying capsaicin, the pungent component of chili peppers, to the forehead skin. Capsaicin activates the transient receptor potential vanilloid type 1 (TRPV1) cation channel and subsequently causes the release of CGRP.[7, 8] Similar to the meninges, the dermatome of the forehead is innervated by the trigeminal nerve, making this anatomical location

suitable to investigate trigeminovascular mechanisms of migraine. The capsaicin-induced CGRP release causes increases in dermal blood flow (DBF) that we can detect and quantify with laser Doppler perfusion imaging. Beside capsaicin we induce increases in DBF with electrical stimulation. The involvement of CGRP in the capsaicin-induced increases in DBF has previously been demonstrated with a CGRP receptor antagonist.[8] Unfortunately, CGRP receptor antagonists were not available at the time of our study to assess to what extent CGRP was involved in the increases in DBF caused by electrical stimulation. With a within subject coefficient of variation (WCV) of 6% for capsaicin (6 mg/ml) and of 11% for electrical stimulation (1.0 mA), this trigeminal nerve-mediated vasodilatation model was well reproducible in healthy volunteers. In ***Chapter 6,*** we validated the trigeminal nerve-mediated vasodilatation model as a biomarker for the trigeminovascular effects of anti-migraine drugs in a double blind placebo-controlled crossover study with sumatriptan. For the validation in this study we investigated the effect of sumatriptan, the most established and the first marketed out of 7 triptans now available,[9] on the CGRP-mediated rise of capsaicin-induced DBF in healthy volunteers. With this study we demonstrated that sumatriptan inhibited the capsaicin-induced vasodilatory DBF response, but not the vasodilatory DBF response to electrical stimulation. These results confirmed that sumatriptan is indeed able to inhibit the release of CGRP within our trigeminal nerve-mediated vasodilatation model. Thus, we established that with our experimental human model the trigeminovascular effects of future anti-migraine drugs could be investigated.

Migraine is comorbid with Raynaud's phenomenon and other small vessel diseases, such as RVCL (autosomal dominant Retinal Vasculopathy with Cerebral Leukodystrophy) and CADASIL (Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy).[10, 11] Considering that migraine is a major risk factor for cardiovascular disease in women,[12] there seems to be ample evidence for an association between migraine and generalized vascular dysfunction. However, as mentioned in the introduction of this thesis, results of cross-sectional studies investigating vascular dysfunction in migraine patients are conflicting. One of the reasons for these conflicting results might be the use of unreliable methods in these studies to investigate vascular function, or methods that are reliable but measure different aspects of vascular function. Therefore, in ***Chapter 7*** we investigated the reproducibility of and correlation between different measures of vascular function. To exclude variations in our results due to variant hormone levels, we performed this study in healthy non-smoking and as well as in smoking men; this latter group was included to also involve men with presumed endothelial dysfunction. We assessed three different measures of vascular function: flow-mediated dilatation (FMD), local thermal hypaeremia (LTH) and post-occlusive reactive hyperaemia (PORH). We also investigated the nitric oxide (NO)-dependency of LTH.

Our results show that the coefficient of variation of the LTH peak and plateau response was 16% and 21%, of the PORH peak and AUC 11% and 10%, and of the FMD response 12%. The LTH peak response tended to be lower, while the LTH-

plateau response was significantly lower in smokers than in non-smokers, whereas the FMD and PORH responses in smokers and non-smokers were identical. Both in non-smokers and smokers, the LTH peak and LTH plateau responses were inhibited by the NO synthase inhibitor N^G-monomethyl-l-arginine (L-NMMA), indicating that both the LTH peak and plateau response are in part mediated by increased NO release. The lower LTH plateau phase in smokers indicates less NO release, and consequently points to an endothelial dysfunction in the smokers. Additionally, we showed that the LTH plateau phase correlated with the PORH peak response ($r = 0.56$, $P = 0.008$), whereas FMD and PORH AUC were closely correlated ($r = 0.64$, $P = 0.002$). In both the LTH and the PORH response, endothelium-derived hyperpolarizing factor (EDHF) pathway activation is involved,[13, 14] likely explaining the correlation between PORH peak response and LTH plateau phase. As both PORH and FMD are caused by post-ischemic reactive hyperaemia, both measures are possibly dependent upon the release of similar vasodilatory mediators, resulting in a correlation between FMD and PORH. Neither the LTH peak nor the LTH plateau response correlated with the FMD response. Given that both responses are largely NO-dependent, this absent association, although previously reported in studies with similar group size,[15] is surprising. However, it should be kept in mind that the dynamic range of LTH and FMD is vastly different; while with LTH the DBF response can increase by 900% from baseline DBF, with FMD only diameter changes of up to 12% can be achieved. This difference in magnitude may underlie the lack of correlation between LTH and FMD. FMD is known to be a predictor of cardiovascular risk[16] and our results show that the LTH plateau response is highly endothelium-dependent. With LTH, but not FMD or PORH, we were able to show differences in endothelial function between non-smoking and smoking subjects. Therefore, in future studies assessing vascular function LTH, FMD and PORH should be complementary measures and are not interchangeable. PORH proved a reproducible and easily applicable measure of microvascular function. The PORH peak response is known to be mostly dependent on the release of neuropeptides and the time course of the PORH response is predominantly dependent upon activation of the endothelium-derived hyperpolarizing factor (EDHF) pathway causing the resulting vasodilation.[13]

In ***Chapter 8*** we applied PORH measurements in combination with laser Doppler flowmetery to perform *in vivo* microvascular characterization of the novel *TREX1* knock-in mouse model for RVCL. We specifically opted for PORH measurements, because we had not characterized the LTH response yet and PORH enabled us to study both the neurovascular and endothelium-dependent components of the microvasculature. Besides *in vivo* microvascular characterization, we performed detailed pharmacological *in vitro* macrovascular (aorta) characterization using Mulvany myographs. The PORH AUC responses were significantly attenuated in the 13-week to 100-week old homozygote V235fs KI animals. In the *in vitro* studies, acetylcholine induced significantly smaller relaxations in the aortae of the 100-week old transgenic compared to wild type mice. Our data are in line with preliminary patient results showing endothelial dysfunction in in RVCL patients.[17] This transgenic

mouse model for RVCL can be applied to obtain a further understanding of *TREX1* mutation in RVCL as well as migraine.

Part II: Migraine and female sex hormones

With a prevalence of 17% in women and 6% in men, migraine affects women much more than men.[18] Major hormonal changes during a woman's lifetime affect migraine and migraine frequency. One of those periods of hormonal change is the perimenopausal period. In ***Chapter 9*** we reviewed the effect of the perimenopausal period on migraine. During the perimenopausal period, the period of 2–8 years prior to the menopause as well as the year after the end of menses, estradiol levels start to fluctuate leading to an eventual drop towards the end of the perimenopausal period. During this period women report new onset migraine, as well as improvement and worsening of existing migraine. Improvement of migraine seems to occur most in women with MO.[19] Migraine incidence not only changes during hormonal milestones, during the reproductive years migraine incidence is also under the influence of the menstrual cycle. Approximately 50% of women with migraine have menstrually related migraine (MRM).[20] Despite the importance of female sex hormones in influencing migraine onset, incidence and frequency in women, there are not many studies investigating this link. Therefore, as described in ***Chapter 10,*** we assessed whether varying levels of sex hormones during the menstrual cycle affect trigeminal nerve–mediated vasodilatation. For this purpose we included MRM patients, their healthy age-matched controls, and postmenopausal women as a reference group with stable low estradiol levels, and investigated them within the our trigeminal nerve-mediated vasodilatation model described in ***Chapter 5***. We demonstrated a menstrual cycle-dependent response to capsaicin in healthy women. In contrast, responses to capsaicin were not dependent on the menstrual cycle in MRM patients. Postmenopausal women showed similar responses during the 2 research time-points. However, their overall DBF responses were significantly lower compared to the premenopausal women. During day 19-21 of the menstrual cycle, MRM patients had significantly lower estradiol levels compared to their healthy age-matched controls, confirming a possibly reduced cyclicity, which was also observed in the DBF responses to capsaicin. The lower estradiol levels in MRM patients, although seemingly in line with previous published data on MRM patients, should be confirmed in a study with a larger number of subjects.[21] We performed a similar study, as described in ***Chapter 11***, investigating the influence of migraine and the menstrual cycle on the capsaicin-induced DBF on the forearm. In this study, besides including healthy women and women with migraine, we also compared healthy male subjects with men with migraine. Measuring the capsaicin-induced DBF on the forearm allowed us to compare peripheral responses to that of the trigeminal nerve-mediated responses measured in ***Chapter 10***. Confirming our results as described in ***Chapter 10***, healthy women responded more strongly to capsaicin application during their menstruation, while there was no influence of the menstrual cycle on the DBF responses to capsaicin in women with migraine. In contrast to our results on the forehead, women with migraine responded more strongly to capsaicin

application on the forearm during all research time-points. These conflicting findings may be attributed to differences in the migraine patients that were included in both studies (MRM patients in ***Chapter 10*** and general migraine patients in ***Chapter 11***). Alternatively, the different finding in ***Chapter 10*** and ***Chapter 11*** could also be due to differences between trigeminovascular responses and peripheral responses to capsaicin. There may be a higher demand for CGRP in the meningeal region in migraine patients, leading to lower levels that can be released upon activation of the trigeminal afferents on the forehead. This difference in peripheral and trigeminal finding is an interesting contrast to study in the future.

Future Perspectives

Migraine and female sex hormones

Our studies have provided further insight into the effect of female sex hormones on the pathophysiology of migraine (Chapters 10 and 11), suggesting that the reactivity of the trigeminovascular system in migraine patients in response to varying levels of female sex hormones differs from that in healthy women without migraine. In addition, we obtained preliminary evidence (Chapter 10) that not only the *response to* varying levels of female sex hormones, but also the *levels* of these hormones is different in MRM patients compared to healthy women. Future studies should demonstrate whether our findings could indeed be confirmed in a larger study sample. Such studies should also focus on female sex hormones beyond estradiol and progesterone. Further, we have up to now assumed that the increased DBF responses during the menses in women are mediated by CGRP, since CGRP has been shown to almost exclusively mediate the DBF response to capsaicin in forearm studies.[8] However, since these studies[8] were performed in men, we cannot exclude that, during specific phases of the menstrual cycle, additional factors may come into play, like pituitary adenylate cyclase-activating peptide (PACAP) and vasoactive intestinal peptide (VIP), and substance P. Therefore, it is to be recommended that, when compounds interfering with CGRP (i.e., CGRP receptor antagonists or antibodies directed against CGRP or its receptor) are available for use in humans, the cycle-dependent changes in DBF to capsaicin are studied in their presence. This would allow more insight into the involvement of other vasodilatory factors, besides CGRP, in the DBF responses to capsaicin. In addition, since female sex hormones do influence many of the mechanisms involved in migraine, they should be taken into account when studying the link between migraine and cardiovascular disease, as well as the effects of drugs interfering with the CGRP-ergic system of nerve stimulation (see below).

Migraine and cardiovascular disease

As mentioned above, migraine is a major cardiovascular disease risk factor in women. Not only is there an association between stroke and migraine [22], but migraine patients are now also known to have a higher chance of cardiac events.[23] A recent study indicates that migraine may be an even larger cardiovascular risk factor than the classical risk factors diabetes, obesity and smoking.[12] The increased cardiovascular

risk due to migraine is not explained by a single vascular risk factor and in fact seems to be independent from diabetes mellitus and atherosclerosis.[24] Thus, it is essential to characterize the vascular system of migraine patients, using a method that can discriminate between different components involved in vasorelaxation. Such studies could be performed using LTH, as this microvascular measure is suitable to pharmacologically assess the role of specific vasodilator components, like NO (see ***Chapter 7***), neuropeptides, and EDHF (pilot studies currently ongoing in our laboratory). In addition, the LTH response may be related to the DBF increase induced by an endothelium-independent vasodilator, such as glyceryl trinitrate. The knowledge on the pathophysiological mechanisms involved in the vascular profile of migraine patients thus obtained will allow a better prevention in these patients. Unfortunately, mechanistic studies that focus on vascular function show inconsistent results. A particular limitation of these studies, seems to be the inclusion criteria for patients.[25] Regrettably, current knowledge obtained from epidemiological studies cannot be directly applied for the selection of patients in mechanistic studies. Uniform criteria for identification of migraine patients at risk of cardiovascular do not exist. If methods for correct sampling would be available to bridge these two scientific fields, pathophysiological studies may more consistently elucidate the underlying vascular dysfunction in migraine patients.

Antibodies directed against CGRP and its receptor

Due to the central role of CGRP in the pathophysiology of migraine, this neuropeptide has been a target for drug development for several years. Up to several years ago, research focused on the development of CGRP receptor antagonists (gepants). CGRP receptor antagonists were promising, as they were as effective as triptans, apparently without the vascular side effects. Studies even indicated that triptan non-responders could benefit from gepants.[26] However, the development of olcegepant was terminated because of pharmacokinetic limitations of this compound.[27] Subsequently, increased liver transaminases were detected in a phase 3 trial of telcagepant, testing its capacity as a prophylactic drug, leading to the immediate termination of further development of this compound. These unfortunate events have delayed the development of this class of drugs.[28] Monoclonal antibodies against CGRP and its receptor bypass the liver metabolism and as such became the new focus of anti-migraine drug development.[29] Preliminary data show promising results of treatment with CGRP antibodies.[30, 31] Although blocking CGRP does not have direct vascular effects, like the vasoconstrictor effect with triptans, the cardiovascular safety of antibodies directed against CGRP or its receptor has to be evaluated extensively, because CGRP is involved in ischemia and reperfusion events,[32] and blocking the function of CGRP for a longer period of time could have detrimental effects. Studies with CGRP knockout mice in an angiotensin II-induced hypertension model have demonstrated that the CGRP knockout mice display enhanced hypertension and aortic hypertrophy compared to wild type mice, reinforcing the importance of the protective role of CGRP in the cardiovascular system.[33]

Extracranial peripheral nerve stimulation

In recent years, there has been a focus on device-driven management of migraine. Based on the premise that extracranial peripheral nerves can influence migraine, several nerve stimulators have been developed. Occipital nerve stimulation (ONS), an invasive method of nerve stimulation, has been investigated in clinical trials, but yielded variable results. The Occipital Nerve Stimulation for the Treatment of Chronic Migraine Headache (ONSTIM) study reported a reduction of at least 50% in headache frequency in migraine, while no improvement was found in the sham-stimulated or medically treated groups.[34] In the largest randomized clinical trial in patients with chronic migraine however, no difference in primary outcome (50% reduction of mean daily headache intensity) between active and sham treatment was found.[35] However, a sub-analysis indicated a 30% reduction in mean headache days ($p<0.05$) and a decrease in migraine-related disability score (MIDAS) ($p<0.01$).[35] Non-invasive peripheral nerve-stimulating methods include transcutaneous supraorbital nerve stimulation (tSNS) and transcutaneous vagus nerve stimulation (tVNS).[36, 37] In a randomized, double-blind sham-controlled trial, tSNS significantly decreased the number of mean monthly migraine days.[36] In an open label study in 30 patients, tVNS in the neck was effective in aborting migraine attacks in 21% of patients (painfree at two hours).[37] The studies on peripheral extracranial nerve stimulation seem promising, however a major limitation of clinical trials with nerve stimulation is the sham treatment. Due to the sensation of nerve stimulation correct blinding is very difficult to achieve. Studies that validate sub-threshold stimulation are necessary. For example, our trigeminal nerve-mediated vasodilatation model is well suitable to assess the effect of tSNS on CGRP release by measuring CGRP-induced increases in DBF. In addition, similar methods should be developed to assess the effects of vagal nerve stimulation. In this way, these novel modes of nerve stimulation might be validated and the role of CGRP and other neuropeptides, such as PACAP and VIP in their effects may be studied, leading into more insight into the pathophysiology of migraine.

References

1. Plosker GL, McTavish D. Sumatriptan. A reappraisal of its pharmacology and therapeutic efficacy in the acute treatment of migraine and cluster headache. Drugs 1994;47:622-651.
2. MaassenVanDenBrink A, Reekers M, Bax WA, Ferrari MD, Saxena PR. Coronary side-effect potential of current and prospective antimigraine drugs. Circulation 1998;98:25-30.
3. Wrobel Goldberg S, Silberstein SD. Targeting CGRP: A New Era for Migraine Treatment. CNS drugs 2015.
4. Amin FM, Asghar MS, Hougaard A, et al. Magnetic resonance angiography of intracranial and extracranial arteries in patients with spontaneous migraine without aura: a cross-sectional study. The Lancet Neurology 2013;12:454-461.
5. Asghar MS, Hansen AE, Amin FM, et al. Evidence for a vascular factor in migraine. Annals of neurology 2011;69:635-645.
6. Goadsby PJ, Edvinsson L. The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Annals of neurology 1993;33:48-56.
7. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 1997;389:816-824.
8. Sinclair SR, Kane SA, Van der Schueren BJ, et al. Inhibition of capsaicin-induced increase in dermal blood flow by the oral CGRP receptor antagonist, telcagepant (MK-0974). British journal of clinical pharmacology 2010;69:15-22.
9. Chan KY, Vermeersch S, de Hoon J, Villalón CM, MaassenVanDenBrink A. Potential mechanisms of prospective antimigraine drugs: a focus on vascular (side) effects. Pharmacology & therapeutics 2011;129:332-351.
10. Brand FN, Larson MG, Kannel WB, McGuirk JM. The occurrence of Raynaud's phenomenon in a general population: the Framingham Study. Vascular medicine 1997;2:296-301.
11. Stam AH, Haan J, van den Maagdenberg AM, Ferrari MD, Terwindt GM. Migraine and genetic and acquired vasculopathies. Cephalalgia : an international journal of headache 2009;29:1006-1017.
12. Kurth T, Bubes V, Buring JE. Relative Contribution of Migraine with Aura to Cardiovascular Disease Occurrence in Women. . Neurology 2013;80:S40.001.
13. Cracowski JL, Gaillard-Bigot F, Cracowski C, Sors C, Roustit M, Millet C. Involvement of cytochrome epoxygenase metabolites in cutaneous postocclusive hyperemia in humans. J Appl Physiol (1985) 2013;114:245-251.
14. Brunt VE, Minson CT. KCa channels and epoxyeicosatrienoic acids: major contributors to thermal hyperaemia in human skin. J Physiol 2012;590:3523-3534.
15. Hansell J, Henareh L, Agewall S, Norman M. Non-invasive assessment of endothelial function - relation between vasodilatory responses in skin microcirculation and brachial artery. Clin Physiol Funct Imaging 2004;24:317-322.
16. Ras RT, Streppel MT, Draijer R, Zock PL. Flow-mediated dilation and cardiovascular risk prediction: a systematic review with meta-analysis. International journal of cardiology 2013;168:344-351.
17. Vermeersch S, Stam AH, Zielman R, et al. Trex1-mutation associated with endothelial dysfunction in RVCL patients. Cephalalgia : an international journal of headache 2011;31:13.
18. Buse DC, Loder EW, Gorman JA, et al. Sex differences in the prevalence, symptoms, and associated features of migraine, probable migraine and other severe headache: results of the American Migraine Prevalence and Prevention (AMPP) Study. Headache 2013;53:1278-1299.
19. Mattsson P. Hormonal factors in migraine: a population-based study of women aged 40 to 74 years. Headache 2003;43:27-35.
20. Martin VT. Menstrual migraine: a review of prophylactic therapies. Current pain and headache reports 2004;8:229-237.
21. MacGregor EA, Frith A, Ellis J, Aspinall L, Hackshaw A. Incidence of migraine relative to menstrual cycle phases of rising and falling estrogen. Neurology 2006;67:2154-2158.
22. Etminan M, Takkouche B, Isorna FC, Samii A. Risk of ischaemic stroke in people with migraine: systematic review and meta-analysis of observational studies. Bmj 2005;330:63.
23. Sacco S, Ornello R, Ripa P, et al. Migraine and risk of ischaemic heart disease: a systematic review and meta-analysis of observational studies. European journal of neurology : the official journal of the European Federation of Neurological Societies 2015;22:1001-1011.
24. Sacco S, Pistoia F, Degan D, Carolei A. Conventional vascular risk factors: their role in the association between migraine and cardiovascular diseases. Cephalalgia : an international journal of headache 2015;35:146-164.
25. Sacco S, Ripa P, Grassi D, et al. Peripheral vascular dysfunction in migraine: a review. The journal of headache and pain 2013;14:80.
26. Ho TW, Olesen J, Dodick DW, Kost J, Lines C, Ferrari MD. Antimigraine efficacy of telcagepant based

on patient's historical triptan response. Headache 2011;51:64-72.
27. Farinelli I, De Filippis S, Coloprisco G, Missori S, Martelletti P. Future drugs for migraine. Intern Emerg Med 2009;4:367-373.
28. Ho TW, Connor KM, Zhang Y, et al. Randomized controlled trial of the CGRP receptor antagonist telcagepant for migraine prevention. Neurology 2014;83:958-966.
29. Bigal ME, Walter S. Monoclonal antibodies for migraine: preventing calcitonin gene-related peptide activity. CNS drugs 2014;28:389-399.
30. Dodick DW, Goadsby PJ, Spierings EL, Scherer JC, Sweeney SP, Grayzel DS. Safety and efficacy of LY2951742, a monoclonal antibody to calcitonin gene-related peptide, for the prevention of migraine: a phase 2, randomised, double-blind, placebo-controlled study. The Lancet Neurology 2014;13:885-892.
31. Dodick DW, Goadsby PJ, Silberstein SD, et al. Safety and efficacy of ALD403, an antibody to calcitonin gene-related peptide, for the prevention of frequent episodic migraine: a randomised, double-blind, placebo-controlled, exploratory phase 2 trial. The Lancet Neurology 2014;13:1100-1107.
32. Russell FA, King R, Smillie SJ, Kodji X, Brain SD. Calcitonin gene-related peptide: physiology and pathophysiology. Physiological reviews 2014;94:1099-1142.
33. Smillie SJ, King R, Kodji X, et al. An ongoing role of alpha-calcitonin gene-related peptide as part of a protective network against hypertension, vascular hypertrophy, and oxidative stress. Hypertension 2014;63:1056-1062.
34. Saper JR, Dodick DW, Silberstein SD, et al. Occipital nerve stimulation for the treatment of intractable chronic migraine headache: ONSTIM feasibility study. Cephalalgia : an international journal of headache 2011;31:271-285.
35. Silberstein SD, Dodick DW, Saper J, et al. Safety and efficacy of peripheral nerve stimulation of the occipital nerves for the management of chronic migraine: results from a randomized, multicenter, double-blinded, controlled study. Cephalalgia : an international journal of headache 2012;32:1165-1179.
36. Schoenen J, Vandersmissen B, Jeangette S, et al. Migraine prevention with a supraorbital transcutaneous stimulator: a randomized controlled trial. Neurology 2013;80:697-704.
37. Goadsby PJ, Grosberg BM, Mauskop A, Cady R, Simmons KA. Effect of noninvasive vagus nerve stimulation on acute migraine: an open-label pilot study. Cephalalgia : an international journal of headache 2014;34:986-993.

Deel I: Experimentele migraine modellen

Geen andere medicijnklasse is meer succesvol geweest in de acute behandeling van migraine dan triptanen. Triptanen zijn evenwel niet in alle patiënten effectief; schattingen van non-responders variëren van 14-30%. Verder zijn triptanen wegens hun vasoconstricties effect gecontraïndiceerd bij patiënten met hart- en vaatziekten. Daarom gaat de zoektocht naar nieuwe antimigraine middelen nog steeds door, waarbij voorkeur uitgaat naar middelen die aanvallen kunnen voorkomen. In de pathofysiologie van migraine is de krachtige vaatverwijder "calcitonin gene-related peptide" (CGRP) het belangrijkste peptide en vormt samen met de CGRP-receptor het meest veelbelovend aanknopingspunt in de ontwikkeling van nieuwe antimigraine middelen.

In ***hoofdstuk 3*** worden de verschillende *in vitro* en *in vivo* experimentele modellen besproken die kunnen worden aangewend bij de ontwikkeling en karakterisering van CGRP-receptorantagonisten. Deze modellen zijn ook geschikt voor het onderzoeken van de onlangs ontwikkelde antilichamen, gericht tegen CGRP en de CGRP-receptor. Deze antilichamen worden momenteel in Fase II en Fase III klinische studies getest. Migraine modellen zijn niet alleen essentieel voor het onderzoek naar de werkzaamheid van nieuwe antimigraine middelen, maar bieden ook mogelijkheden om de pathofysiologie van migraine verder uit te zoeken, waarbij uiteraard rekening moet worden gehouden met het feit dat experimentele modellen beperkingen hebben en de klinische situatie niet altijd accuraat nabootsen. Bijvoorbeeld, in ***hoofdstuk 4*** bespreken wij een studie waarin de verwijding van intra- en extracraniële vaten in relatie tot spontane migraineaanvallen zonder aura (MO) en het effect van sumatriptan hierop is onderzocht. De resultaten van deze studie zijn tegenstrijdig met de resultaten van eerder gepubliceerde studies, waarin migraineaanvallen werden opgewekt door de infusie van CGRP. Verschillen in de mechanismen die leiden tot een opgewekte en een spontane migraineaanval, maar ook de technische beperkingen van de detectiemethode die gebruikt is zouden deze tegenstrijdigheid kunnen verklaren. In beide genoemde studies werd de vaatverwijding beoordeeld met behulp van magnetische resonantie beeldvorming (MRI). Een belangrijke beperking van MRI is dat het intracraniële deel van de arteria meningea media niet gemeten kan worden, terwijl dit intracraniële deel verondersteld wordt cruciaal te zijn in de pathofysiologie van migraine. Zoals dat ook geldt voor de meeste experimentele migrainemodellen, is het met MRI niet mogelijk om de neuronale en vasculaire componenten van migraine te integreren. Daarom hebben wij een non-invasief model ontwikkeld waarin de trigeminovasculaire activering van de huid van het voorhoofd in mensen onderzocht kan worden. ***Hoofdstuk 5*** beschrijft de ontwikkeling en validatie van dit model. Tijdens een migraineaanval komt CGRP vrij uit de sensorische zenuwuiteinden van de nervus trigeminus. In het door ons ontwikkeld model wordt het vrijkomen van endogeen CGRP geïnduceerd door het aanbrengen van capsaïcine op de huid van het voorhoofd. Capsaïcine is het pittige bestanddeel van chilipepers. Capsaïcine activeert het transiënte receptor potentiaal vanillaoid type 1 (TRPV1) ion-kanaal, hetgeen leidt tot de afgifte van CGRP. Zoals de hersenvliezen wordt de huid van het

voorhoofd geïnnerveerd door de nervus trigeminus, reden waarom dit dermatoom uitermate geschikt is voor het onderzoek van de trigeminovasculaire mechanismen in migraine. De capsaïcine-geïnduceerde CGRP-afgifte veroorzaakt een toename in de doorbloeding van de huid (DBF), die eenvoudig gekwantificeerd kan worden met behulp van laser Doppler perfusie imaging. Naast de capsaïcine-geïnduceerde toename werd gekeken naar de toename in DBF, geïnduceerd door elektrische stimulatie. De betrokkenheid van CGRP bij de capsaïcine-geïnduceerde toename in DBF is eerder aangetoond met een CGRP-receptorantagonist. Helaas konden we tijdens onze studies niet beschikken over een CGRP-receptorantagonist om vast te kunnen stellen of en in hoeverre CGRP betrokken is bij de toename van de DBF door elektrische stimulatie. Met een intra-individuele variatiecoëfficiënt (WCV) van 6% voor capsaïcine (6 mg/ml) en 11% voor elektrische stimulatie (1.0 mA), is dit trigeminale zenuw-gemedieerde vasodilatatiemodel in gezonde vrijwilligers goed reproduceerbaar. In ***hoofdstuk 6*** hebben wij het trigeminale zenuw-gemedieerde vasodilatatiemodel gevalideerd als een biomarker voor de trigeminovasculaire effecten van sumatriptan in een dubbelblinde, placebo-gecontroleerde cross-over studie. We onderzochten het effect van sumatriptan, omdat van de 7 triptanen die nu beschikbaar zijn dit middel het meest gebruikt wordt. In deze studie vonden we dat sumatriptan de door capsaïcine geïnduceerde DBF respons remde, maar niet de respons op elektrische stimulatie. Met ons trigeminale zenuw-gemedieerde vasodilatatiemodel konden we dus aantonen dat sumatriptan in staat is om de afgifte van CGRP te remmen. Onze conclusie is dan ook dat het ontwikkelde experimentele humane model de mogelijkheid biedt om trigeminovasculaire effecten van nieuwe anti-migraine middelen te onderzoeken.

Migraine is geassocieerd met het Raynaud-fenomeen en andere microvasculaire aandoeningen zoals RVCL (autosomaal dominante Retinale Vasculopatie met Cerebrale Leukodystrofie) en CADASIL (Cerebrale Autosomaal-Dominante Arteriopatie met Subcorticale Infarcten en Leukoencefalopathie). Omdat migraine het risico van cardiovasculaire ziekten in vrouwen verhoogt, wordt er een verband tussen migraine en gegeneraliseerde vasculaire disfunctie verondersteld. Zoals besproken in de inleiding van dit proefschrift, zijn de resultaten van de studies die vasculaire disfunctie in migraine patiënten hebben onderzocht niet eenduidig. Een mogelijke verklaring voor deze tegenstrijdige resultaten is het gebruik van minder valide methoden om de vasculaire functie te onderzoeken, of methoden die wel valide zijn maar andere aspecten van de vasculaire functies meten dan die zijn aangedaan bij migraine. Daarom hebben we in ***hoofdstuk 7*** de reproduceerbaarheid van en correlatie tussen verschillende meetmethoden van vasculaire functie onderzocht. Om variaties door variërende hormoonspiegels uit te sluiten, hebben we deze studie uitgevoerd bij gezonde niet-rokende en rokende mannen; deze laatste groep werd geïncludeerd omwille van de veronderstelde endotheeldisfunctie. Drie verschillende methoden om vasculaire functie te bepalen hebben we gebruikt: flow-gemedieerde dilatatie (FMD), lokale thermale hyperemie (LTH) en post-occlusieve reactieve hyperemie (PORH). Tevens hebben we de stikstofmonoxide (NO)-afhankelijkheid van LTH onderzocht. Onze resultaten tonen aan dat de variatiecoëfficiënt van de LTH-piek- en LTH-plateaure-

spons 16% en 21% bedraagt, van de PORH piek- en AUC-respons 11% en 10%, en van de FMD-respons 12%. De LTH-plateaurespons was minder bij rokers dan bij niet-rokers, terwijl de FMD en PORH-responsen bij rokers en niet-rokers identiek waren. Zowel bij rokers als niet-rokers werden de LTH-piek- en LTH-plateaurespons geremd door de NO-synthaseremmer N^G-monomethyl-l-arginine (L-NMMA), hetgeen erop wijst dat zowel de LTH piek- als plateaurespons, althans ten dele, gemedieerd wordt door een toegenomen NO-afgifte. De lagere LTH-plateaurespons in rokers impliceert een verminderde NO-afgifte, hetgeen in overeenstemming is met de veronderstelde endotheeldisfunctie bij rokers. De LTH-plateaurespons correleerde met de PORH-piek respons ($r = 0.56$, $P = 0.008$), terwijl FMD en PORH AUC sterk gecorreleerd waren ($r = 0.64$, $P = 0.002$). Bij zowel de LTH- als de PORH-respons is activering van de endotheel-afhankelijke hyperpolariserende factor (EDHF) pathway betrokken, hetgeen de correlatie tussen de PORH maximale respons en de LTH plateaurepons waarschijnlijk verklaart. Omdat de PORH-respons en FMD-respons veroorzaakt worden door post-ischemische reactieve hyperemie, zijn beide metingen afhankelijk van de afgifte van vergelijkbare vaatverwijdende stoffen, hetgeen de correlatie tussen FMD en PORH verklaart. Noch de LTH-piek, noch de LTH-plateaurespons correleerden met de FMD-respons. Aangezien beide responsen grotendeels NO-afhankelijk zijn, is het ontbreken van deze associatie verrassend, zij het eerder gerapporteerd in studies met vergelijkbare groepsgrootte. Hierbij kan worden aangetekend dat de dynamische range van LTH en FMD respons sterk verschillen; terwijl bij LTH de DBF met 900% kan toenemen, worden bij FMD veranderingen in diameter tot maximaal 12% bereikt. Dit verschil in dynamische range kan de afwezige correlatie tussen LTH en FMD wellicht verklaren. Het is bekend dat een verminderde FMD een voorspeller is van het risico van cardiovasculaire aandoeningen. Onze resultaten tonen aan dat de LTH-plateaurespons voor een groot deel endotheel-afhankelijk is. Met LTH, maar niet met FMD of PORH, waren we in staat om verschillen in endotheelfunctie aan te tonen tussen niet rokende en rokende proefpersonen. In toekomstige studies naar vasculaire (dis)functie zouden de LTH, FMD en PORH als aanvullende, maar niet als uitwisselbare metingen kunnen worden toegepast. PORH is een goed reproduceerbare en gemakkelijk uit te voeren meting om de microvasculaire functie te evalueren. De PORH-piekrespons is voornamelijk afhankelijk van de afgifte van neuropeptiden en het tijdsverloop van de PORH-respons is in grote mate afhankelijk van de activering van de EDHF-pathway. In ***hoofdstuk 8*** pasten we PORH metingen toe in combinatie met laser Doppler flowmetingen om *in vivo* de microvasculaire functie van het nieuwe *TREX1* knock-in muismodel (V235fs KI) voor RVCL te karakteriseren. We hebben voor PORH metingen gekozen, omdat we de LTH-respons nog niet geëvalueerd hadden en we met de PORH- respons zowel de neurovasculaire als de endotheelafhankelijke componenten van de microcirculatie konden bestuderen. Naast de *in vivo* karakterisering van de microcirculatie hebben we *ex vivo* de vasculaire functie van aorta onderzocht, gebruikmakend van de Mulvany myograaf. De PORH AUC-responsen waren significant verlaagd in de 13 tot 100 weken oude homozygote V235fs KI dieren. In de *ex vivo* studies veroorzaakte acetylcholine minder relaxatie in de 100 weken oude transgene muizen dan in

de controle muizen. Deze experimentele gegevens zijn in overeenstemming met de eerste resultaten verkregen in patiënten met RVLC. Het transgene muismodel voor RVCL kan worden gebruikt om enerzijds meer inzicht te krijgen in de rol van de *TREX1*-mutatie in RVCL en anderzijds in de pathofysiologie van migraine.

Deel II: Migraine en vrouwelijke geslachtshormonen

Met prevalenties van 17% in vrouwen en 6% in mannen, komt migraine veel vaker voor bij vrouwen dan bij mannen. De grote hormonale veranderingen tijdens een vrouwenleven zijn van invloed op de incidentie en de frequentie van migraine. Een periode van hormonale veranderingen is de perimenopauze. In ***hoofdstuk 9*** geven we een literatuuroverzicht van het effect van de perimenopauze op migraine. Tijdens de perimenopauze, de periode van 2–8 jaren voor de menopauze en het eerste jaar na de laatste menses, begint de oestradiolspiegel te fluctueren, uiteindelijke leidend tot een dalende spiegel rond het einde van de perimenopauze. Rond deze periode kunnen vrouwen voor het eerst migraineaanvallen krijgen, maar een verbetering of verergering van bestaande migraine kan eveneens optreden. Verbetering van bestaande migraine lijkt het meest voor te komen bij vrouwen met MO. De incidentie en frequentie van migraine aanvallen veranderen niet alleen tijdens hormonale mijlpalen. Ook tijdens de vruchtbare jaren wordt migraine beïnvloed door hormonale veranderingen samenhangend met de menstruele cyclus. Ongeveer 50% van de vrouwen met migraine hebben menstruatie-gerelateerde migraine (MRM). Ondanks de belangrijke rol van vrouwelijke geslachtshormonen op het ontstaan en de incidentie en frequentie van migraine bij vrouwen, is dit verband weinig onderzocht. Daarom hebben wij, zoals beschreven in ***hoofdstuk 10,*** middels een case-control studie geëvalueerd of veranderingen die optreden in de vrouwelijke geslachtshormonen gedurende de cyclus van invloed zijn op de nervus trigeminus-gemedieerde vasodilatatie. Gebruikmakende van ons trigeminale zenuw-gemedieerde vasodilatatie model zoals beschreven in ***hoofdstuk 5*** hebben we MRM patiënten, gezonde controles van dezelfde leeftijd en postmenopauzale vrouwen (een referentiegroep met een stabiele lage oestradiolspiegel) bestudeerd. In onze studie konden we aantonen dat de DBF-respons op capsaïcine in gezonde vrouwen afhankelijk is van de cyclus. Daarentegen waren de DBF-responsen in MRM patiënten onafhankelijk van de menstruele cyclus. De DBF-responsen van postmenopauzale vrouwen op capsaïcine waren identiek gedurende de 2 meetmomenten van het onderzoek, maar significant lager dan die in premenopauzale vrouwen. Gedurende dag 19-21 van de cyclus hadden MRM-patiënten lagere estradiolspiegels in vergelijking met de controles, hetgeen mogelijk de verminderde cycliciteit van de capsaïcine-respons verklaart. De lagere estradiolspiegels in MRM-patiënten, alhoewel in overeenstemming met eerder gepubliceerde studies, zouden in een grotere studie bevestigd moeten worden. In ***hoofdstuk 11*** hebben wij de invloed van migraine en menstruatiecyclus op de capsaïcine-geïnduceerde DBF in de onderarm onderzocht. In deze studie hebben we naast gezonde vrouwen en vrouwen met migraine ook gezonde mannen en mannen met migraine met elkaar vergeleken. Het meten van de DBF in de onderarm stelde ons in staat om de responsen in de onderarm te vergelijken met de trigeminale ze-

nuw-gemedieerde responsen zoals beschreven in ***hoofdstuk 10***. Gezonde vrouwen reageerden sterker op de toediening van capsaïcine tijdens hun menstruatie, terwijl de capsaïcine-geïnduceerde DBF-responsen niet werden beïnvloed door de cyclus bij vrouwen met migraine, hetgeen in overeenstemming is met de resultaten beschreven in ***hoofdstuk 10***. Echter, in tegenstelling tot de verminderde capsaïcine-respons op het voorhoofd, reageerden vrouwen met migraine sterker op de toediening van capsaïcine op de onderarm tijdens alle tijdspunten van het onderzoek. Deze tegenstrijdige resultaten kunnen wellicht worden toegeschreven aan verschillende migrainepatiënten die in beide studies geïncludeerd zijn: MRM patiënten in ***hoofdstuk 10*** en algemene migrainepatiënten in ***hoofdstuk 11***. Ook zou het verschil verklaard kunnen worden door eventuele verschillen tussen trigeminovasculaire en perifere responsen op capsaïcine. Er is mogelijk een grotere vraag naar CGRP in de meningeale vasculatuur bij migraine patiënten, hetgeen kan leiden tot lagere hoeveelheden CGRP die afgegeven kunnen worden bij de activering van de trigeminale afferenten in het voorhoofd. Het contrast in de perifere en trigeminale DBF resultaten is een interessante bevinding die verder onderzoek vereist.

Dankwoord

Na een bijzonder plezierige promotieperiode rest mij nu alleen om de mensen te danken die hebben bijgedragen aan de totstandkoming van dit proefschrift.

Op de eerste plaats wil ik mijn promotor prof.dr. A.H.J. Danser bedanken. Beste Jan, dank voor de gelegenheid die jij mij hebt gegeven om onderzoek te mogen doen bij de afdeling Farmacologie. Ik heb enorme bewondering voor de wijze waarop jij altijd tot de kern van zaken weet te komen en feilloos de zwakke punten van de naar jou toegestuurde manuscripten weet te vinden. Dank voor je snelle feedback op alle vragen en stukken die jij toegestuurd kreeg. Jij draagt met recht de titel "Promotor van het jaar".

Mijn copromotoren dr. A.H. van den Meiracker en dr. A. Maassen van den Brink wil ik in het bijzonder bedanken. Beste Ton, ik kwam bij jou als student om keuzeonderzoek te doen aan de afdeling Interne Geneeskunde. Jij stelde mij voor aan Antoinette en zo begonnen wij aan een nu vijf jaar durende samenwerking. Onze studies hebben veel profijt gehad van jouw jarenlange ervaring met klinisch onderzoek. Daarnaast was het ook erg fijn dat ik op vrijdagmiddagen altijd even langs kon komen om mijn studieresultaten te bespreken en we over wetenschappelijk onderzoek in het algemeen konden discussiëren. Dr. Maassen van den Brink, beste Antoinette, je was een geweldige copromotor, mentor en coach de afgelopen vier jaren. Dank voor alle discussies over studieresultaten, presentaties en "data is" of "data zijn". Ik heb erg veel van jou geleerd. Door jouw intelligentie, doorzettingsvermogen en talent voor het opzetten van samenwerkingsverbanden ben jij een geweldige bron van inspiratie.

Prof.dr. C.M. Villalón, dear Carlos, thank you for the collaboration on my first publication and for being a member of my Thesis committee. Ik wil ook graag prof. dr. J.S.E. Laven en prof.dr.ir. A.P.N. Themmen danken voor het zitting nemen in de kleine promotiecommissie.

Kayi, dank voor alle gezelligheid de afgelopen 4 jaar. Als onderdeel van het migraine clubje hebben we veel tijd met elkaar kunnen doorbrengen tijdens werk en ook daarbuiten. Toen ik begon heb je mij meteen welkom laten voelen en daarna zijn we goede vrienden geworden. Ik weet zeker dat we hierna contact blijven houden. Dank dat je mijn paranimf wilde zijn. Ik ben erg vereerd dat jij straks naast mij staat bij mijn verdediging.

Stephanie, dank dat jij mijn paranimf wilde zijn. Ondanks dat je het zelf erg druk hebt met het schrijven en het samenstellen van je eigen proefschrift heb je toch veel tijd weten vrij te maken om voor mijn promotie van alles te regelen. Verder dank voor alle gezamenlijke sportsessies. We kunnen nu in ieder geval op de maat ons mannetje staan. Ik wens je heel veel succes met het afronden van jouw eigen promotie.

Sieneke, je bent weliswaar niet meer werkzaam bij het Erasmus MC, maar we hebben toch 3 jaar lang erg leuk samengewerkt en daar wil ik je voor danken. Ook tijdens congressen, vooral die in Boston, hebben we het erg gezellig gehad. Net als

Kayi, maakte jij ook deel uit van het migraine clubje en daardoor hebben we erg leuke momenten gedeeld. Katie, ook jou wil ik bedanken voor de samenwerking en de leuke tijd in Valencia. Ik wens jou heel veel succes met het opzetten en uitvoeren van jouw eigen onderzoek.

Lieve Evelien, bedankt dat je me zo goed hebt opgevangen toen ik als student begon bij het onderzoekslab. Ik zal onze lunches samen missen.

Richard, René en Ingrid wil ik bedanken voor alle hulp bij het verkrijgen van een groot gedeelte van de data in dit proefschrift. Richard ook dank voor alle gezelligheid tijdens de tripjes naar Leiden voor het uitvoeren van experimenten.

Ik wil graag ook rest van mijn collega's bedanken: Anton, dank ook voor je gastvrijheid tijdens de etentjes bij jou thuis. Koen, Lodi, Paula, Langeza, Luuk, Joep, David, Arthur, Mahdi, Bruno, Usha, Dominique, Birgitte, Marcel, Estrellita, Bibi, Wendy, Frank, Jeanette, Edith Friesema, Gardi, Adrie, Edith Padberg, Sven, dank voor de uitzonderlijk prettige sfeer op de afdeling. Ook dank voor al jullie hulp bij mijn pilot experimenten. Ik heb dat altijd enorm gewaardeerd.

Voor de samenwerking met het Leids Universitair Medisch Centrum (LUMC) wil ik graag prof.dr. M.D. Ferrari, dr. G.M. Terwindt, prof.dr. A.M.J.M. van den Maagdenberg, drs. W.P.J. van Oosterhout, R.R. Klever MSc., dr. E.A. Tolner, I.A. Mulder MSc., en M. Schenk danken.

Prof.dr. J. de Hoon, S. Vermeersch MSc., en L. Buntinx MSc.: beste Jan, Steve en Linde, hartelijk dank voor de fijne samenwerking de afgelopen jaren. Linde, door jouw geweldige inzet tijdens de data analyse van het hormonen stuk hebben wij alles op tijd af gekregen.

Prof. dr. L. Edvinsson, dr. M.L. Edvinsson and dr. S. Eftekhari: dear Lars and Marie-Louise thank you for the great collaboration and the kind welcome in Lund. Sajedeh, thank you for the wonderful time during conferences and sharing your room with me during iHead.

Drs. E.G. Couturier wil ik graag danken voor de samenwerking en bijdrage aan de review beschreven in hoofdstuk 9 van dit proefschrift.

Dr. R. Draijer en Y. de Graaf van Unilever R&D Vlaardingen wil ik graag danken voor de erg leuke samenwerking. Young, door jou leken de meetdagen veel korter; dank voor het verbreden van mijn algemene kennis.

Dr. J.G. Langendonk, beste Janneke, dank dat je als onafhankelijke arts onze klinische studies hebt ondersteund.

Lieve vrienden: Eefje, kan niet wachten tot je weer terug bent in Nederland en we eindelijk al dat we gepland hebben kunnen uitvoeren. Mirka, dank voor je jarenlange steun en vriendschap. Ik weet dat ik altijd op je kan rekenen en ben blij dat we elkaar vaker kunnen zien nu je in Rotterdam woont. Nargis en Arezo, ook al hebben we

het druk gehad de laatste tijd, we weten altijd er voor elkaar te zijn wanneer het telt. Dank daarvoor. Lindsey, ook al kunnen we niet meer zo vaak samen sporten, we blijven elkaar zien onder het genot van een theetje.

Mijn lieve schoonouders, Romina, Alina en Shair; dank voor jullie begrip voor alle drukke periodes waardoor mijn tripjes naar Middelburg erg schaars waren de afgelopen tijd.

Mijn lieve ouders, Majid en Faouzia, aan jullie dank ik alles. Jullie hebben ervoor gezorgd dat, Ahmad, Atia en ik een toekomst hebben. Jullie hebben zonder aan jullie eigen toekomst en carrière te denken alles achtergelaten en een nieuw leven voor ons hier in Nederland opgebouwd. Jullie hebben ons aangemoedigd om nooit op te geven en om al onze mogelijkheden te benutten. Mijn dankbaarheid daarvoor is niet in woorden uit te drukken. Ahmad, Roya en Atia, dank voor alle liefde en steun de afgelopen 4 jaar. Zelfs bij het rekruteren van proefpersonen zijn jullie me te hulp geschoten.

Obayd, lieverd, als laatste wil ik jou bedanken. In mijn ogen zijn wij een ideaal team. Jij steunt mij niet alleen in alles wat ik doe, maar moedigt mij aan om nieuwe wegen in te slaan en gelooft ook nog eens onvoorwaardelijk in mij. Door jouw eigen ongeëvenaarde zelfdiscipline, integriteit en werkethiek stel je ook nog eens een voorbeeld voor mij waarmee jij mij vooruit helpt in moeilijke tijden.

Curriculum Vitae

Khatera Ibrahimi was born on November 25, 1984 in Kabul, Afghanistan. In 1993, she and her family moved to the Netherlands. She attended secondary school at the "Nehalennia Stedelijke Scholengemeenschap" in Middelburg. In 2003 she started to study medicine at the Erasmus University in Rotterdam. She started working at the Netherlands Institute for Innovative Ocular Surgery as a Technical analyst in 2004. For her Master's thesis she investigated the trigeminovascular activation on the forehead at the Erasmus MC, supervised by dr. Maassen van den Brink and dr. van den Meiracker. This resulted in the 5th chapter of this thesis. In 2011, she started her PhD training at the Department of Internal Medicine, Division of Vascular Medicine and Pharmacology.

List of publications

Ibrahimi K, Couturier EG, MaassenVanDenBrink A. Migraine and perimenopause. Maturitas. 2014;78(4):277-80.

Ibrahimi K, van Oosterhout WPJ, van Dorp W, Danser AH, Garrelds IM, Kushner SA, et al. Reduced trigeminovascular cyclicity in patients with menstrually related migraine. Neurology. 2015;84(2):125-31.

Ibrahimi K, Vermeersch S, Danser AHJ, Villalón CM, van den Meiracker AH, de Hoon J, et al. Development of an experimental model to study trigeminal nerve-mediated vasodilation on the human forehead. Cephalalgia. 2014;34(7):514-22.

Labruijere S, **Ibrahimi K**, Chan KY, MaassenVanDenBrink A. Discovery techniques for calcitonin gene-related peptide receptor antagonists for potential antimigraine therapies. Expert Opin Drug Discov. 2013;8(11):1309-23.

MaassenVanDenBrink A, **Ibrahimi K**, Edvinsson L. Intracranial and extracranial arteries in migraine. Lancet Neurol. 2013;12(9):847-8.

Pessoa BS, Slump DE, **Ibrahimi K**, Grefhorst A, van Veghel R, Garrelds IM, et al. Angiotensin II type 2 receptor- and acetylcholine-mediated relaxation: essential contribution of female sex hormones and chromosomes. Hypertension. 2015;66(2):396-402.

PhD Portfolio

Name PhD student:	Khatera Ibrahimi
PhD period:	2011-2015
Erasmus MC Department:	Department of Internal Medicine, Division of Vascular Medicine and Pharmacology
Promotor:	Prof.dr. A.H.J. Danser
Supervisors:	Dr. A. Maassen van den Brink Dr. A.H. van den Meiracker
Research School:	Cardiovascular Research School Erasmus University Rotterdam (COEUR)

General academic and research skills ***(3 ECTS)***

2012	Laboratory animal science, Erasmus MC, Rotterdam, The Netherlands

In-depth Courses ***(10.7 ECTS)***

2015	iHead Meeting, The International Headache Society, LUMC, Leiden, The Netherlands
2012-2013	COEUR Courses and Research Seminars, Erasmus MC, Rotterdam, The Netherlands
2012	Basis cursus klinisch onderzoekers (Brok), Erasmus MC, Rotterdam, The Netherlands

Teaching activities ***(5.3 ECTS)***

2011-2015	Pharmacology practical courses, scientific internship of Junior Med school students and (co)supervising Master's thesis of a Biomedical Sciences student.

Presentations *(5.7 ECTS)*

2015

17th International Headache Congress, Valencia. *In vivo and in vitro vascular characterization of a novel transgenic mouse model of migraine. (Poster presentation)*

25th ADMA Annual Meeting, Valencia. *In vivo and in vitro vascular characterization of a novel transgenic mouse model of migraine* (Oral presentation)

NVF Spring Meeting, Nijmegen. *Sumatriptan non-responders: assessment of a possible Biomarker. (Poster presentation)*

Wetenschapsdagen Interne Geneeskunde, Antwerpen. *In vivo and in vitro vascular characterization of a novel transgenic mouse model of migraine (Poster presentation)*

2014

2nd Benelux Congress On Physiology and Pharmacology (PHYSPHAR 2014), Maastricht. *Sumatriptan non-responders: assessment of a possible biomarker. (Oral presentation)*

4th European Headache and Migraine Trust International Congress (EHMTIC), 18-21 September 2014, Copenhagen. *Efficacy of sumatriptan: assessment of a possible biomarker. (Oral presentation)*

The 8th International Symposium on the CGRP family of peptides (CGRP, adrenomedullin,adrenomedullin 2, amylin and calcitonin), Ascona. *Efficacy of sumatriptan: assessment of a possible biomarker. (Poster presentation)*

16th FIGON Dutch Medicine Days, 6 - 8 October 2014, Ede. *Pathophysiological and pharmacological studies in a human model on trigeminovascular activation. (Oral presentation)*

Wetenschapsdagen Interne Geneeskunde, Antwerpen. *Efficacy of sumatriptan: assessment of a possible biomarker. (Poster presentation)*

2013

11th Dutch Endo-Neuro-Psycho Meeting (ENP 2012), Lunteren. *Patients with menstrually-related migraine lack the trigeminovascular menstrual cyclicity of healthy women. (Oral presentation)*

16th International Headache Congress, Boston. *Patients with menstrually-related migraine lack the trigeminovascular menstrual cyclicity of healthy women. (Poster presentation)*

16th Annual Scientific Meeting of the Dutch Headache Society (DHS), Leiden. *Inhibition of trigeminovascular CGRP release by sumatriptan studied in healthy volunteers. (Oral presentation)*

Wetenschapsdagen Interne Geneeskunde, Antwerpen. *Influence of varying estrogen levels on trigeminal CGRP release in healthy women. (Oral presentation)*

2012 3rd European Headache and Migraine Trust International Congress (EHMTIC), London. *Influence of varying estrogen levels on trigeminal CGRP release in healthy women. (Poster presentation)*

FIGON Dutch Medicine Days 2012, Lunteren. *Trigeminal CGRP release: influence of migraine and estrogen. (Oral presentation)*

Wetenschapsdagen Interne Geneeskunde, Antwerpen. *Development of a model to study trigeminal CGRP release in human subjects. (Poster presentation)*

2011 21st Anglo Dutch Migraine Association Annual Meeting, Maastricht. *Trigeminal CGRP release: development of a human research model. (Oral presentation)*

15th International Headache Congress, Berlin. *Development of a model to study trigeminal CGRP release in human subjects. (Poster presentation)*

FIGON Dutch Medicine Days 2011, Lunteren. *Development of a model to study trigeminal CGRP release in human subjects. (Oral presentation)*

International Conferences *(8.1 ECTS)*

2015 17th International Headache Congress, Valencia.

25th ADMA Annual Meeting, Valencia.

2014 4th European Headache and Migraine Trust International Congress (EHMTIC), 18-21 September 2014, Copenhagen.

The 8th International Symposium on the CGRP family of peptides (CGRP, adrenomedullin,adrenomedullin 2, amylin and calcitonin), Ascona.

2013 16th International Headache Congress, Boston.

23rd ADMA Annual Meeting, Haarlem

2012 3rd European Headache and Migraine Trust International Congress (EHMTIC), London.

22nd ADMA Annual Meeting, Brighton

2011 21st Anglo Dutch Migraine Association Annual Meeting, Maastricht.

15th International Headache Congress, Berlin.

Grants & Awards

IHS travel grant to attend the IHC 2015 (Valencia, 14-17 May)

2nd prize PhD Student Competition with an oral presentation titled: Pathophysiological and pharmacological studies in a human model on trigeminovascular activation". FIGON Dutch Medicine Days 2014 (Ede, 6-8 October)

Award for best abstract in the poster discussion session "Migraine Therapy" with the abstract: "Efficacy of sumatriptan: assessment of a possible biomarker". EHMTIC 2014 (Copenhagen, 18-21 September)

AHS travel award to attend IHC 2013 (Boston, 27-30 June)

IHS travel grant to attend EHMTIC 2012 (London, 20-23 September)

IHS travel grant to attend IHC 2011 (Berlin, 23-26 June)