Migraine as a cardiovascular risk factor for women

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CHAPTER 5

Decreased role of neuropeptides in the microvascular function in migraine patients with polycystic ovary syndrome

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ABSTRACT

Aims - To better understand the pathophysiological mechanisms underlying migraine as a cardiovascular risk factor, we studied neuropeptide action and endothelial function as measures of peripheral microvascular function in middle-aged women with or without migraine.

Methods and Results - We included women with the endocrine disorder Polycystic Ovary Syndrome (PCOS), a population with supposed elevated cardiovascular risk, with and without comorbid migraine. In 26 women without and 23 women with migraine in the interictal phase (mean age 50.8 years (SD=2.9)) Local Thermal Hyperemia (LTH) of the skin of the volar forearm was measured coss-sectionally under control conditions, after inhibition of neuronal axon reflex and neuropeptide release by 5% lidocaine/prilocaine (EMLA) cream application, and after inhibition of nitric oxide formation by iontophoresis of NG-Monomethyl-L-arginine (L-NMMA). Hereafter, changes in the natural logarithm of the Reactive Hyperemia Index (lnRHI) and Augmentation Index (AI) during reperfusion after occlusion-derived ischemia were measured, using the EndoPAT device. While mean values under control conditions and L-NMMA conditions were similar, women with migraine had a significantly higher mean Area of the Curve (AUC) of the total LTH response after EMLA application than those without (86.7% (SD=26.5) versus 67.9% (SD=24.2); p=0.014). This was also reflected by a higher median AUC of the plateau phase under similar conditions in women with migraine compared to those without (83.2% (IQR [73.2–109.5]) versus 73.2% (IQR [54.3–92.0]); p=0.039). Mean changes in lnRHI (0.73 (SD=0.25) versus 0.67 (SD = 0.29)) and AI (11.53 (SD=14.57) versus 8.68 (SD=15.17)) scores were similar in both groups.

Conclusion - In women with PCOS neuropeptide action was lower in those with comorbid migraine compared with those without migraine. These findings have consequences for the development of preventive measures against the elevated cardiovascular risk in migraine patients. Indeed, it provides a potential mechanism supporting previous findings that migraine is not associated with traditional risk factors, including atherosclerosis.

TRANSLATIONAL PERSPECTIVE

It is currently unknown which pathophysiological mechanisms are related to the increased cardiovascular risk in migraine patients. By comparing the microvascular status in women with PCOS, and with or without migraine, using a sophisticated technique, we identified a putative mechanism underlying the increased cardiovascular risk of migraine patients with comorbid PCOS. This association of migraine with an affected peripheral neuropeptide activity is not reflected by traditional cardiovascular risk scores and, therefore, challenges our current ideas on how to assess and mitigate this risk in migraine patients.

1. INTRODUCTION

Migraine is a chronic neurovascular headache disorder, typically characterised by attacks of debilitating unilateral headache, accompanied by photo- and phonophobia, nausea and emesis (1). About one-third of migraine patients experience migraine aura and have attacks that are preceded by spreading, reversible neurological disturbances, often consisting of visual symptoms (1). Migraine is classified as the top cause of disability in persons under fifty years (2, 3), and is two to three times more prevalent in women (global age-standardised prevalence 19%) than in men (4, 5). In addition to the disabling nature of its headache attacks, migraine – particularly with aura – has been associated with significant cardiovascular endpoints, including cardiac mortality, especially in women. More specifically, population-based cohort studies report increased risks of myocardial infarction, coronary heart disease, ischemic and haemorrhagic stroke, and venous thromboembolism in migraine patients (6-14). Recently, a cohort study by Kurth et al. confirmed the importance of migraine with aura as a cardiovascular risk factor, compared to other traditional risk factors, like obesity or unfavourable lipid levels (10).

Despite its high prevalence, the exact pathophysiological mechanism of migraine has not yet been unravelled. There is growing evidence that, besides the involvement of neuronal factors, this neurovascular disorder is driven by small artery disease or dysfunction of the microvasculature (15), which can be distinguished into (i) endothelial function (i.e. endothelium-dependent responses) and (ii) smooth muscle cell function as a response to neuropeptide release from sensory nerves mediated by an axon reflex (i.e. endothelium-independent responses). Endothelial dysfunction involves decreased bioavailability of (potent) vasodilatory factors that are released by the endothelium, including the gaseous signalling molecule nitric oxide (NO) – which is believed to be one of the mediators in migraine initiation and maintenance (16, 17). The second mechanism is thought to originate from brainstem neural activation and involves the simultaneous trigeminovascular release of neuropeptides, including calcitonin gene-related peptide (CGRP), substance P, neurokinin A and pituitary adenylate cyclase-activating peptide (PACAP-38) (18-23). This release causes dilation of cranial (dural and pial) blood vessels and pain sensation (24-27).

Both mechanisms are also involved in (peripheral) cardiovascular disease. Endothelial dysfunction has been described as an early indicator of cardiovascular risk and a reversible phase of atherosclerosis (28), while neuropeptides contribute to vasodilation in the peripheral cardiovascular system and are implicated in cardiovascular health and disease pathogenesis. For instance, neuropeptide Y is increasingly implicated in a variety of cardiovascular diseases, including atherosclerosis, myocardial ischemia and infarction, as well as hypertension, as reviewed by Tan et al. (29). In contrast, the potent vasodilator CGRP is considered to possess cardioprotective characteristics, including a protective role in vessel remodelling and atherosclerosis, which highlights the need for further research regarding the long-term effects of anti-CGRP monoclonal antibodies for migraine treatment (30-32). The contribution of the endothelium to vasodilatory responses induced by CGRP is, however, debated (31).

This is the first study using an innovative method to study the microvasculature patients with migraine. Therefore, we set out to study these techniques in a population with a possibly challenged microvascular system. Specifically, we studied middle-aged women with Polycystic Ovary Syndrome (PCOS). PCOS is a common endocrine disorder affecting 8-15% of women of reproductive age (33). Women with PCOS may present with ovulatory dysfunction, clinical or biochemical signs of hyperandrogenism and polycystic ovarian morphology (35). Contradictory results have been reported regarding the association of PCOS with cardiovascular disease. Some studies found an association of PCOS with an increased cardiovascular risk profile (36, 37), including a twofold increase in stroke prevalence compared to controls, and with cardiovascular risk factors, such as metabolic syndrome, multivessel coronary artery disease, obesity and hypertension (38, 39). This is in contrast to results from other cohorts, showing no evidence of increased all-cause mortality, cardiovascular disease (risk) or more severe atherosclerosis in women with PCOS (40, 41). Several studies describe increased endothelial dysfunction in women with PCOS; however, contrasting views also exist on whether this association is dependent on more general risk factors, like obesity (35, 42, 43).

As ageing is one of the determinants of NO bioavailability in the human body, as well as cardiovascular health (45, 46), we incorporated the influence of aging by investigating middle-aged women.

In the current cross-sectional study, the primary objective was to determine and compare the microvascular status as well as the cardiovascular risk as traditionally determined by the Framingham Risk Score (FRS) in middle-aged women with PCOS with or without migraine in a non-invasive manner. To understand the role of microvascular dysfunction and the increased risk of cardiovascular disease in these patients, we differentiated between endothelium-dependent responses (the NO bioavailability or endothelial dysfunction) and endothelium-independent responses (the contribution of neuropeptide activity). Therefore, middle-aged women with or without migraine, all with PCOS, underwent two different measurements to determine their (micro)vascular health, including their endothelial function. The secondary objective was to compare these different measurement techniques of the microvasculature.

2. METHODS

Our study is part of CREW-MIST, an acronym for: “Cardiovascular RiskprofilE in Women: MIcrovascular Status”, which is a consortium aimed at elucidating the female-specific cardiovascular risk profile. The CREW study has been approved by the Medical Research Ethics Committee (institutional review board) of Leiden University Medical Center, Leiden, the Netherlands (P.15.384). Local approval was obtained from the Medical Research Ethics Committee of Erasmus Medical Center, Rotterdam, the Netherlands. Written informed consent was obtained from all participants after written and verbal explanation of the study. The study was conducted according to the principles of the declaration of Helsinki (Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, and amended by the 64th WMA General

Assembly, Fortaleza, Brazil, October 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO).

2.1 STUDY POPULATION

The study population consisted of women who were previously diagnosed with PCOS (approximately 3-5 years prior to inclusion for the current study), as defined by the (revised) Rotterdam consensus criteria (47). According to these criteria, women must fulfil at least two of the following criteria to be diagnosed with PCOS: (i) oligo- or anovulation, which includes irregular menses, (ii) characteristics of hyperandrogenism, including clinical symptoms and high plasma levels of testosterone, and (iii) polycystic ovaries, observed using on ultrasonography.

The original study population consisted of 200 women with PCOS who had been invited in an earlier stage of the CREW consortium for extensive endocrine and cardiovascular assessments; these outcome measures have been described in detail previously (41). Eligible subjects were selected and invited for the current study. The assessment of PCOS had taken place at an earlier stage in the context of a previous study (41). Women aged between 40 and 60 years who fulfilled the criteria for the diagnosis PCOS and who were capable and willing to provide informed consent were included. Patients with any of the following conditions were excluded: the presence of dermal diseases, scars or tattoos on the ventral sides of lower arm that could interfere with the measurements, current pregnancy, insufficient mastery of the Dutch language, any serious illness which obstructed study participation or an allergy for EMLA cream or the compound NG-monomethyl-L-arginine (L-NMMA).

2.2 MIGRAINE CLASSIFICATION

All participants were asked to fill in the Leiden University Migraine Neuro-Analysis (LUMINA) questionnaire to diagnose lifetime migraine. This validated web-based questionnaire aids in diagnosing migraine, including further subdivisions: migraine with aura (MA), migraine without aura (MO), aura without headache and subjects without migraine. Migraine auras are transient focal neurological symptoms, that manifest as visual, sensory, motor or verbal disturbances. The division was based on the third edition of the International Classification of Headache Disorders (ICHD) criteria (48). Both women with a history of migraine – i.e. women who had migraine in the past, but no migraine headache (attacks) and/or aura symptoms in the past 12 months – and women with active migraine were included. The investigator (LA-H) verified the migraine diagnosis during the visit by conducting a semi-structured interview. Moreover, a random sample of these diagnoses – including doubtful cases – has been judged independently by a specialized headache neurologist (GMT). We assured that none of the migraine patients experienced migraine on the day of the assessment, as our aim was to measure the microvascular status in the interictal period.

2.3 ADDITIONAL DATA

In addition, the questionnaire included baseline characteristics as displayed in Table 1 and the Supplementary Material, consisting of questions regarding health, lifestyle, intoxications, medication use, and gynaecological/obstetric as well as cardiovascular history. Self-reported amenorrhea (at least 12 months) was used to classify participants according to their reproductive stage.

Additional data for every participant were collected, including the weight and length (the latter was asked) for calculations of the Body Mass Index (BMI)), and the circumference of the waist and hip for the Waist-to-Hip ratio (WHR). The blood pressure was measured three times in total at different time points, in a sitting position after at least five minutes of rest with an automated oscillometric device. Information regarding intoxications, use of medications as well as menstruation or menopausal status was obtained. Lastly, we collected blood samples for the assessment of hormone levels and lipid profiles. Total cholesterol levels, HDL-cholesterol levels, sex, age, current smoking (yes/no), use of antihypertensive medication(s), systolic blood pressure, and diabetes (yes/no) were used as variables to calculate the FRS that assess the 10-year risk of developing overall cardiovascular disease (49). Former smokers were considered as non-smokers when calculating this score.

2.4 MEASUREMENT TECHNIQUES

Participants were restricted from food and drinks (except for water and necessary medications due to ethical reasons) three hours before the start of the experiments. In addition, consumption of caffeine and heavy meals was not allowed after midnight on the day before the experiments. All measurements were performed consistently by the same investigator in a silent and temperature-controlled room (21.6°C ± 1.3). Subjects were comfortably seated with the right arm in supine position supported by the armrest and with uncrossed legs. During the measurements, subjects were not allowed to talk or sleep.

We performed measurements of the peripheral microvascular function by using the following techniques: (i) measurement of changes in dermal blood flow (DBF) after Local Thermal Hyperemia (LTH) by using the Laser Doppler Perfusion Imager (LDPI, PeriScan Pim 3 system, Perimed, Järfälla, Sweden) and (ii) measurement of the post-occlusive reaction of the forearm by using the EndoPAT device (Endo-PAT2000, Itamar Medical, Caesarea, Israel).

2.4.1 LTH measurements

The first method is based on a model described by Roustit and Cracowski (50). We aimed to study the LTH response of the skin at the volar site of the right forearm. DBF was measured in the superficial dermal layers by using the LDPI. Heating probes (Moor Instruments Ltd, Devon, UK) filled with water were used for the induction of local heating. The skin was heated up to 40°C after a five-minute baseline measurement at 33°C to standardize the temperature of the skin. Within ten minutes after the start of the skin heating generally an initial peak in DBF appears, caused by a neuronal axon reflex, which is largely mediated by neuropeptides (51, 52).

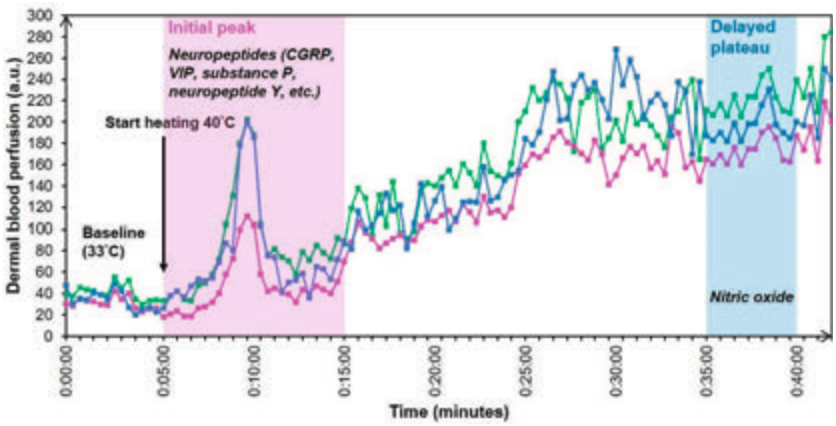
The peak phase is followed by the plateau phase 30-35 minutes after heating. NO is the key contributor to this plateau phase, responsible for approximately 60% of the response (50, 52-54).

To unravel the influence and attribution of endothelium-independent and endothelium-dependent pathways responsible for the reactive vasodilation after heating, we blocked these selectively. Therefore, we applied 5 grams lidocaine/prilocaine cream (both 25 mg/g, EMLA®, Aspen Netherlands b.v., Gorinchem, The Netherlands) at least one hour prior to the experiment to inhibit neuropeptide release, similar to the protocol applied by Minson et al. (52). Additionally, iontophoresis at 60 µA during 7 minutes as previously validated in pilot experiments (data not shown, Perilont LI611, Järfälla, Perimed Sweden (55)) of the NO-synthase inhibitor L-NMMA (Bachem, Clinalfa®, Bubendorf, Switzerland) was used to deliver L-NMMA to the upper dermal layers to inhibit NO formation, allowing a study of smooth muscle cell dysfunction. We used a 2% solution of L-NMMA, which was dissolved in hypotonic water (Fresenius Kabi).

A so-called "iontophoresis artefact", or current-induced vasodilation, was present in several participants. This artefact presents itself as an increased DBF during baseline (thus, before heating) only on the site where iontophoresis of L-NMMA was applied. Therefore, we extended the baseline period of 5 minutes during the LTH measurements, in case an artefact was visually observed to obtain a stable baseline and to avoid possible biased outcomes before heating the skin to 40°C as much as possible.

Figure 1 provides a chronological illustration of a single LTH experiment including the different phases of the vasodilatory response under three conditions (control, EMLA and L-NMMA).

Figure 1. An example of a LTH response



Induction of LTH on the skin of the forearm under control conditions (green line), application of EMLA (lidocaine/prilocaine) (pink line) and L-NMMA (NO synthase inhibitor) (blue line). Abbreviations: a.u.: arbitrary units; CGRP: calcitonin gene-related peptide; VIP: vasoactive intestinal polypeptide.

To answer our primary objective, we calculated and compared the Area Under the Curve (AUC) (expressed in ms*a.u.) for the entire curve, including the five minute baseline period, as the main outcome measure for all three conditions (control conditions, after application of EMLA cream, and after iontophoresis of L-NMMA) by using the trapezoidal rule. DBF responses after EMLA application and iontophoresis of L-NMMA were expressed as a percentage relative to DBF responses under control conditions for each participant. DBF responses under control conditions only have been expressed as such. Additional outcome measures included the AUC of the LTH peak phase (defined as the ten-minute phase after heating) and plateau phase (defined as the five-minute phase starting 30 minutes after heating) separately, for all three conditions (control conditions, after application of EMLA cream, and after iontophoresis of L-NMMA).

Anomalies in individual measurements caused by movement and motion of the subject were visually detected and reported by the investigator for later adjustment. Additionally, possible outliers were detected in case the standard deviation (SD) of a single image deviated 1.5 times from the mean SD of all images of the entire LDPI measurement.

We only corrected for outliers, if consensus was reached with an independent investigator. Both the independent investigator and principal investigator were blinded for the migraine diagnosis. We handled these outliers by imputation of the concerning value, using the average of one image before and after, as calculated by the LDPI software.

In case of an "iontophoresis artefact", we systematically corrected for the increased baseline. If the LTH response inhibited by L-NMMA (blue line, Figure 1) had a 1.5-fold higher baseline of 5 minutes before heating to 40°C compared to the LTH response without pretreatment (green line, Figure 1), we took the average of the DBF measurements of approximately 1.5 minutes before until 1.5 minutes after start heating.

2.4.2. EndoPAT measurements

Approximately five minutes after completion of the LTH measurements, EndoPAT measurements were performed using the index fingers of both arms. The EndoPAT device measures plethysmographic changes during reperfusion after a five-minute period of occlusion-derived ischemia of the brachial artery. This occlusion, which we applied on the left arm (in contrast to the LTH method), and which was obtained by rapidly inflating a blood pressure cuff to a pressure of 200 mmHg, reflects a downstream hyperemic response and is a measure of peripheral arterial endothelial function (56, 57). The ratio of the post-to-pre occlusion peripheral arterial tone (PAT) amplitude of the tested finger (on the left arm), divided by the post-to-pre occlusion ratio of the control finger (on the right arm) was automatically calculated by the device to obtain the Reactive Hyperemia Index (RHI). The lnRHI is a monotonic natural log transformation of this same index in arbitrary units (a.u.), which provides a better double-sided (Gaussian) distribution than the RHI, and which has been described to be a measure of the peripheral vascular endothelial function (57, 58). A normal lnRHI (or EndoScore) is defined as an outcome above 0.51 – i.e. approximately a 1.665-fold

higher ratio of the occluded arm compared to the control arm – whereas an abnormal score is an outcome of less than or equal to 0.51 and is associated with endothelial dysfunction (59). In addition, we used the augmentation index (AI), normalized to heart rate of 75 bpm (AI@75), as a measure for arterial stiffness (56).

Both measures were calculated automatically by the EndoPAT software (version 3.6.2.) that uses computerized algorithms.

A previous study of the Framingham Cohort found a significant inverse relation between cross-sectional measurements of the endothelial function (LnRHI) and multiple traditional cardiovascular risk factors (including male sex, BMI, total/HDL cholesterol, diabetes, (current) smoking, and use of lipid-lowering treatment) (60). Therefore, we performed secondary analyses to investigate the external validity of the relationship between the EndoScore and FRS as a measure of cardiovascular risk in women with PCOS. To investigate potential effect modification by migraine, we stratified the results according to the migraine status.

2.5 STATISTICAL ANALYSIS

The distribution of values, whether normally distributed or not, was determined with the Shapiro-Wilk test. Various statistical tests have been applied to compare the gynaecological/obstetric, endocrine and cardiovascular outcomes as well as the LTH and EndoPAT measurement outcomes between patients with and without migraine: Student T-tests for continuous variables with a normal distribution, Mann–Whitney U tests for non-normally distributed continuous data, and Fisher's exact tests for categorical variables.

Secondary analyses were performed in all participants to investigate the association between both techniques, the EndoPAT results (including the lnRHI and AI@75) and LTH results. In addition, to study the relationship between traditionally determined cardiovascular risk and both techniques, we investigated the association between the EndoPAT results and FRS and between the LTH results under control conditions and FRS. The Pearson correlation coefficient (r) was used to assess the strength of these associations. We performed a natural log transformation of the FRS to ensure a normal distribution of these data.

Two-tailed P-values of ≤ 0.05 were considered to be statistically significant. No corrections for multiple testing of LTH results of the primary objective have been made, as we considered each individual condition (control, EMLA, and L-NMMA) as independent variables. All data were handled and analysed blinded for migraine diagnosis using GraphPad Prism software version 8.0.1 and SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. RESULTS

Forty-nine women with PCOS of the invited 200 PCOS patients fulfilled the inclusion criteria and were willing to participate, comprising 23 individuals with migraine (16 with MA and 7 with MO) and 26 individuals without migraine. In total, 6 women experienced migraine in the past and 17 women experienced a migraine headache and/or aura symptoms recently (no longer than 12 months prior to their visit).

Table 1 and the Supplementary Material present the baseline characteristics, various cardiometabolic, endocrine and gynaecological/obstetric parameters for all participants and for both women with or without migraine separately. No differences were observed between women with or without migraine. Blood collection and the assessment of lipid levels were unsuccessful in one woman without migraine.

On average, migraine patients experienced their first migraine headache attack at the age of 24 years (SD=11). Triptans were used by 7 participants, namely sumatriptan (5 patients) and rizatriptan (2 patients). None of the migraine patients used triptans on the day of the assessments. Moreover, no use of ergotamine was reported.

Table 1. Study population characteristics

	All subjects (N = 49)	**Women with migraine (N = 23)**	**Women without migraine (N = 26)**
General parameters and intoxications[1]			
Age (years)	50.4 (48.7–52.9)	50.2 (49.4–53.5)	50.7 (48.3–52.7)
Ethnicity (race)			
Black	2 (4.1%)	0 (0%)	2 (7.7%)
Mediterranean	3 (6.1%)	2 (8.7%)	1 (3.8%)
Multi-ethnic	2 (4.1%)	2 (8.7%)	0 (0%)
White	42 (85.7%)	19 (82.6%)	23 (88.5%)
Alcohol consumers[2]	32 (65.3%)	16 (69.6%)	16 (61.5%)
Caffeine intake (units/week)	38.5 (23.3–52.5)	38.5 (28.0–52.5)	36.9 (14.0–55.1)
Illicit drugs use	1 (2.0%)	0 (0%)	1 (3.8%)
Current smoker	4 (8.2%)	1 (4.3%)	3 (11.5%)
Pack years of previous and current smokers[3]	7.5 (3.2–16.2)	8.7 (2.8–18.1)	7.5 (3.2–21.0)
Anthropometrics			
BMI (kg/m^2)	30.3 (6.7)	30.1 (7.7)	30.4 (5.9)
Waist/Hip ratio[3,4]	0.9 (0.8–0.9)	0.9 (0.8–0.9)	0.9 (0.8–0.9)
Cardiometabolic parameters			
Systolic BP (mm Hg)	128.7 (16.6)	130.5 (17.9)	127.1 (15.5)
Diastolic BP (mm Hg)	80.4 (10.4)	80.8 (10.0)	80.0 (10.9)
(Previous) myocardial infarction[1,4]	2 (4.2%)	1 (4.5%)	1 (3.8%)

Table 1. Continued

	All subjects (N = 49)	Women with migraine (N = 23)	Women without migraine (N = 26)
Diabetes Mellitus[1,5]	6 (12.2%)	3 (13.0%)	3 (11.5%)
Framingham Risk Score (10 year Cardiovascular Disease Risk %)[4,5]	4.9% (3.7–8.5)	4.8% (3.4–8.8)	5.6% (3.7–7.8)
Framingham Risk Score categories[4,5]			
Low risk (<10%)	41 (85.4%)	19 (82.6%)	22 (88.0%)
Intermediate risk (10–20%)	5 (10.4%)	3 (13.0%)	2 (8.0%)
High risk (>20%)	2 (4.2%)	1 (4.3%)	1 (4.0%)
Antihypertensive drug use	14 (28.6%)	8 (34.8%)	6 (23.1%)
Gynaecological/obstetric parameters			
Current use of hormones[1,6]	15 (30.6%)	7 (30.4%)	8 (30.8%)
Reproductive stage			
Pre-/perimenopausal stage	23 (46.9%)	11 (47.8%)	12 (46.2%)
Postmenopausal stage	6 (12.2%)	2 (8.7%)	4 (15.2%)
Unclear[7]	20 (40.8%)	10 (43.5%)	10 (38.5%)
Endocrine parameters[3]			
Estradiol (pmol/L)[8]	58.4 (55.1–168.8)	70.1 (55.1–232.1)	55.1 (55.1–130.9)
SHBG (nmol/L)	50.0 (37.0–79.8)	48.3 (39.3–86.2)	50.4 (32.4–73.6)
Testosterone (nmol/L)	0.7 (0.5–1.0)	0.9 (0.6–1.1)	0.6 (0.4–0.9)
Dihydrotestosterone (nmol/L)[9]	0.2 (0.2–0.2)	0.2 (0.2–0.2)	0.2 (0.2–0.2)
FAI	1.3 (0.9–2.2)	1.5 (0.8–2.4)	1.3 (1.0–1.9)

Abbreviations: BMI: Body Mass Index; BP: blood pressure; FAI: Free Androgen Index; SHBG: sex hormone-binding globulin

Values of all subjects are displayed as means (standard deviation) or medians (interquartile range, $Q_3 - Q_1$), or as numbers (percentage). Differences between participants with or without migraine were tested with Student's T-test for variables with a normal distribution, while the Mann-Whitney U test was used for variables with a skewed distribution and for variables with a non-normal distribution in one group. The Fisher's exact test was used for categorical data.

[1] Self-reported; [2] This includes both frequent and (very) infrequent alcohol consumers; [3] Data are missing in one migraine patient, that has been excluded from the analyses; [4] Data are missing in one patient without migraine, that has been excluded from the analyses; [5] Four participants did not know whether they are diabetic. These patients have been considered as non-diabetics, based on their medication use; [6] This includes OCP use, having a hormone spiral, but also use of hormone therapy during menopause or hormonal treatments for breast cancer; [7] Unclear, for example due to hormone use, which masks their natural menstrual cycle, or due to a hysterectomy; [8] Hormone levels below the detection limit have been considered to be at the detection limit, which is 55.1. This was the case in 10 migraine patients and 14 patients without migraine; [9] Hormone levels below the detection limit have been considered to be at the detection limit, which is 0.2. This was the case in 15 migraine patients and 20 patients without migraine.

3.1 LTH MEASUREMENTS

LTH measurements were performed in all 49 participants. The measurement of one participant was shorter than 40 minutes. Therefore, only data on the AUC of the peak phase have been included for this particular participant, while the AUC of the plateau phase as well as the total AUC have not been taken into account. Furthermore, the "iontophoresis artefact" was observed in 12 subjects in total and was not related to the migraine diagnosis ($p = 0.748$), as this subgroup consisted of 5 women with migraine and 7 women without migraine. As we observed no significant differences in the LTH baseline between individuals with or without migraine under all three conditions (control conditions (95% CI of the difference [-5.70–2.62]; $p = 0.460$), iontophoresis of L-NMMA ($p = 0.444$), and application of EMLA cream ($p = 0.866$)), results are presented without baseline corrections. Overall and stratified LTH results of the three conditions are presented in Table 2.

Firstly, we analysed differences under control conditions between participants with or without migraine, as expressed by the AUC. We did not detect any significant difference in the AUC of the total LTH response under control conditions between both groups, which was also reflected by similar responses between subjects with or without migraine in the AUC of the ten-minute peak phase or in the mean AUC of the plateau phase (Table 2).

LTH RESPONSES UNDER EMLA CREAM CONDITIONS

Secondly, we analysed LTH responses of the total response, peak phase, and plateau phase after EMLA application relative to control conditions in the total study population. Overall, application of EMLA cream led a total LTH response of 76.53% (SD=26.70) compared to control conditions, indicating an inhibition of 23.47%. More specifically, we observed a median AUC of the peak phase of 55.35% (IQR [41.45–77.31]) compared to control conditions in all participants, which implies an inhibition of 44.65%. Also, under similar circumstances, it reduced the median AUC of the plateau phase to 78.07% (IQR [62.25–97.77]), implying an inhibition of 21.93%.

We further studied differences of LTH responses between women with or without migraine after EMLA application. We observed a higher AUC of the total LTH response in participants with migraine after inhibition by EMLA cream relative to control conditions expressed as a percentage, see Figure 2A. Although inhibition of neuropeptides by EMLA cream did not lead to significant differences in the AUC of the peak phase compared to control conditions, we found a consistent difference in the AUC after inhibition in the plateau phase, see Figure 2B.

Figure 2A. Box plot showing the significant difference of the AUC of the total LTH response between subjects without migraine and with migraine after inhibition by EMLA cream (pink area). This is expressed as a percentage relative to the responses under control conditions (green area) (error bars: 95% CI). The right-sided graph represents an example of a single LTH response under EMLA conditions.

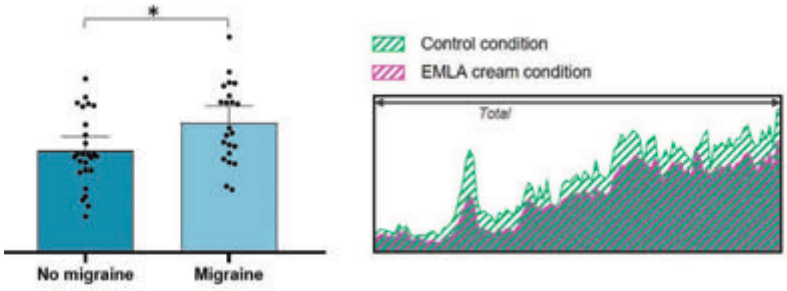


Figure 2B. Box plot that depicts the significant difference of the AUC in the plateau phase of the LTH response between subjects without migraine and with migraine after inhibition by EMLA cream (pink area). This is expressed as a percentage relative to the responses under control conditions (green area) (error bars: 25th and 75th percentiles). The right-sided graph represents an example of a single LTH response under EMLA conditions.

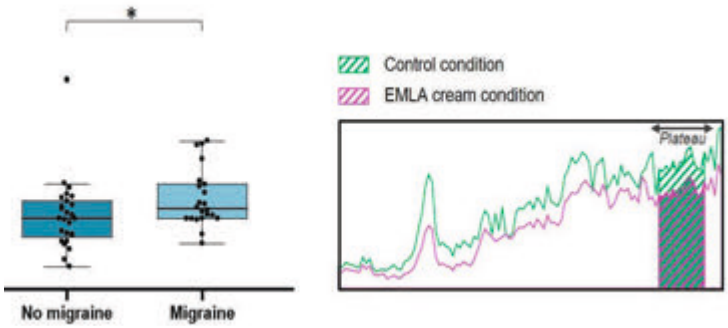


Table 2. LTH responses under different conditions

	All subjects	Women with migraine	Women without migraine	*P-value*
CONTROL CONDITIONS				
Total LTH response in ms*a.u. [IQR]	$2.00*10^8$ [$1.47*10^8$– $2.48*10^8$]	$1.80*10^8$ [$1.37*10^8$– $2.47*10^8$]	$2.10*10^8$ [$1.59*10^8$– $2.55*10^8$]	0.280
Peak phase in ms*a.u. [IQR]	$3.78*10^7$ [$3.01*10^7$–$4.89*10^7$]	$3.64*10^7$ [$2.78*10^7$–$4.46*10^7$]	$4.08*10^7$ [$3.15*10^7$–$5.06*10^7$]	0.421
Plateau phase in ms*a.u. (SD)	$3.71*10^7$ ($1.29*10^7$)	$3.41*10^7$ ($1.09*10^7$)	$3.97*10^7$ ($1.42*10^7$)	0.141
EMLA CREAM CONDITIONS				
Total LTH response in % (SD)	76.53 (26.70)	86.69 (26.48)	67.94 (24.15)	0.014*
Peak phase in % [IQR]	55.35 [41.45–77.31]	61.16 [40.48–79.44]	54.07 [44.13–72.24]	0.655
Plateau phase in % [IQR]	78.07 [62.25–97.77]	83.16 [73.17–109.51]	73.17 [54.31–92.01]	0.039*
L-NMMA CONDITIONS				
Total LTH response in % [IQR]	98.31 [72.97–117.20]	91.50 [66.39–128.30]	98.31 [78.54–110.48]	0.720
Peak phase in % [IQR]	107.66 [81.02–128.68]	107.66 [74.42–131.48])	105.70 [81.18–116.53]	0.613
Plateau phase in % [IQR]	88.46 [76.47–109.75]	87.63 [63.04–125.33]	88.46 [79.48–101.90]	0.927

Median AUC (with the corresponding IQR) or mean AUC (with the corresponding SD) of LTH responses of all subjects and stratified for women with or without migraine under control conditions, under EMLA conditions compared to control conditions (expressed as a percentage, %), and under L-NMMA conditions compared to control conditions (expressed as a percentage, %). LTH responses of women with or without migraine have been analyzed, as displayed by the P-values.

LTH RESPONSES UNDER L-NMMA CONDITIONS

Thirdly, we analysed LTH responses of the total graph, peak phase, and plateau phase after iontophoresis of L-NMMA relative to control conditions. Overall, iontophoresis of L-NMMA led to a median AUC of the peak phase of 107.66% (IQR [81.02–128.68]) and a median AUC of the plateau phase of 88.46% (IQR [76.47–109.75]) compared to control conditions in all participants.

We also further studied differences of LTH responses between women with or without migraine after iontophoresis of L-NMMA. No statistically significant differences between both groups were found after inhibition of NO by application of L-NMMA in the AUC of the total LTH response, peak phase or plateau phase during 35 minutes after start heating relative to control conditions (Table 2).

3.2 ENDOPAT MEASUREMENTS

EndoPAT measurements were performed in 43 participants in total: 21 women with migraine and 22 women without migraine. The occlusion period was shorter than 5 minutes in one participant (approximately 2:15 minutes), due to uncomfortable pain sensations. In another participant, the blood pressure cuff was inflated to a pressure of 180 mmHg, instead of 200 mmHg, due to pain sensations.

The EndoPAT measurements showed no statistically significant difference in the mean lnRHI scores between individuals with or without migraine ($p = 0.475$). Women with migraine had a mean lnRHI of 0.73 (SD=0.25), while women without migraine had a mean lnRHI of 0.67 (SD=0.29). In addition, no differences were observed in the AI@75 ($p = 0.534$) between both groups. Women with migraine had a mean AI@75 of 11.53 (SD=14.57), while women without migraine had a mean AI@75 of 8.68 (SD=15.17).

3.3 ASSOCIATIONS BETWEEN LTH AND ENDOPAT MEASUREMENTS

Based on the aforementioned hypothesis that endothelial dysfunction is characterized by decreased NO bioavailability, secondary analyses were performed in 42 participants to investigate whether the EndoPAT score shows an agreement with the (NO-mediated part of the) LTH measurements. The AUC of the plateau phase under control conditions, corrected for their baselines, did not show any positive correlations with the lnRHI score (Pearson's $r = 0.07$ (95% CI [-0.24–0.37]; $p = 0.641$). In addition, the AUC of the plateau phase under L-NMMA conditions relative to control conditions (square root transformed in order to obtain normally distributed data), also corrected for their baselines, did not show any positive correlations with the lnRHI score either (Pearson's $r = 0.03$ (95% CI [-0.28–0.33]; $p = 0.855$).

The FRS was used as a traditional measure of cardiovascular risk and did not show statistically significant differences between participants with or without migraine (Table 1). The supplementary materials contain additional analyses on cardiovascular risk score in relation to LTH measurements and Endopat measurements (see Supplementary Material).

4 DISCUSSION

In this cross-sectional study, in a population at supposed elevated CVD risk, namely patients with PCOS, we investigated the microvascular status in women with and without migraine.

A variety of studies have reported measurements of the endothelial function as a measure of cardiovascular risk by using and validating the EndoPAT method in different populations (61, 62). This device measures the flow-mediated vasodilation after brachial occlusion and does not specifically target the microvasculature.

This is in contrast with our novel method that involves measurements of the skin perfusion during local heating after applying EMLA cream and L-NMMA. Blockade of the two main components involved in response to heating, neuropeptides and NO, allows a more detailed evaluation of their influences in our study population. This method has not been described before.

4.1. SUMMARY OF LTH MEASUREMENTS

We did not find differences in LTH responses under control conditions. Also, we did not detect differences in the NO bioavailability, as assessed by iontophoresis of L-NMMA, between patients with or without migraine. However, EMLA inhibited neuropeptide action in women without migraine to a larger extent than in women with migraine. This was reflected by the AUC of both the total response and the plateau phase after application of EMLA cream.

These results indicate a significantly decreased neuropeptide action in migraine patients in the interictal period, which may either be due to a reduced release or due to a diminished postsynaptic response in comparison with women without migraine. Our finding contributes to the hypothesis that an altered action of neuropeptides in the periphery is associated with the pathophysiology of migraine. This finding is in accordance with a previous study conducted by Tuka et al., who found decreased levels of PACAP-38 in peripheral blood plasma in the interictal period in migraine patients as compared to healthy subjects (64). Considering other studies that have found elevated CGRP levels in the peripheral vascular system outside migraine attacks, our results might also contribute to the hypothesis of receptor desensitization in patients with migraine (65). Hypothetically, this receptor desensitization could be caused by a higher release in female migraine patients at a younger age, as suggested by results of a human in vivo model by Ibrahimi et al. (66).

The absence of differences in LTH responses under control conditions might indicate that certain mechanisms are “masked” under control conditions, with the result that the detection of subtle differences is only possible when applying a more sophisticated model. Also, the presence of other compensatory mechanisms – systems beyond the neuropeptide release induced by neuronal axon reflexes and NO-dependent vasodilation – might mask the presence of LTH differences under control conditions. These compensatory mechanisms are more likely to be present in the overall LTH response, and might be reflected by percentages above 100% in some individuals after blockade by L-NMMA or EMLA cream. One of these possible

mechanisms might be related to a class of factors called endothelium-derived hyperpolarizing factors (EDHFs), whose effects cannot be investigated using the current method and protocol. EDHFs cause vasodilation mainly through calcium-activated potassium channels present on both smooth muscle cells and the endothelial cell membrane, and are shown to be upregulated when endothelium derived NO is suppressed (54, 67). EDHFs have been described to compensate the loss of NO synthesis in humans (68).

It has been generally accepted that NO accounts for approximately 60% of the plateau phase of LTH (52-54), and in our previous study, in which we applied a similar technique using the LDPI device in healthy men (mean age 40 years (SD=12)), we also confirmed a robust decrease of the LTH peak and plateau by 31% (SD=21) and 65% (SD=14), respectively, after iontophoresis of the NO-synthase inhibitor L-NMMA. However, in the current study, we found no profound decrease of the AUC of the plateau phase after iontophoresis of L-NMMA. Compared to our previous study, we modified the iontophoresis protocol and validated it in pilot studies. Indeed, the protocol that we applied in our previous study mentioned above, in which we included healthy men, increased the occurrence of an iontophoresis artefact in the current study population of middle-aged women. Therefore, the presence of PCOS (and related comorbidities) and/or age and sex differences are most likely to be responsible for the absence of effects after NO synthase blockade. Although we assume that sufficient L-NMMA was properly delivered in the upper dermal layers, we cannot entirely exclude influences of our enhanced iontophoresis protocol that was applied in this specific study population for the first time, that further hampered proper analyses (55).

We also observed no differences of NO bioavailability between individuals with and without migraine. This is in line with a previous study, which showed no differences in endothelium-dependent vasodilatation, basal endothelial NO production and stimulated release of tissue plasminogen activator in forearm resistance vessels between migraine patients and healthy controls (70). In addition, this observation could again support the predominant effects of EDHF in the LTH response. A study performed by Brunt and Minson in a younger study population (18–30 years of age) showed that NO and EDHF have an equally important role in the plateau phase, which leads to the additional hypothesis that the effects of EDHF are larger than NO in middle-aged women with PCOS (54). Another explanation for the low NO bioavailability in these women with PCOS might be related to their low estrogen levels (71), masking potential differences between women with or without migraine, that might be present in women with a normal hormone profile.

The decreased action of neuropeptides, observed in the current study, may be part of the explanation for the increased cardiovascular risk in female migraine patients (6, 9), as certain neuropeptides, for example CGRP, are proposed to exhibit cardioprotective effects in cardiovascular disease (72).

4.2. SUMMARY OF ENDOPAT MEASUREMENTS AND FRAMINGHAM RISK SCORES

Outcomes of the EndoPAT measurements of patients with or without migraine and comorbid PCOS did not differ and fell within the normal range (56, 59). This value indicates a normal endothelial function or arterial stiffness for this population, which is in line with previous research in these study participants, that indicated a similar (low) cardiovascular risk profile compared to age-matched controls (41). This was also reflected by the relatively low FRS in the participants, similar to our current study. We did not detect any significant differences of the FRS between women with or without migraine and comorbid PCOS. The possibility exists that these women with PCOS already received proper preventive treatments and were counselled at an early age to avoid developing diabetes and cardiovascular problems in relation to their disease. Importantly, the FRS is mostly based on traditional risk factors. Despite the increased cardiovascular risk in migraine patients, previous studies found no relationship between migraine and coronary artery calcification scores or atherosclerosis – which are generally viewed as traditional markers for cardiovascular disease (73, 74). In combination with our findings, the increased cardiovascular risk in migraine patients might be explained through non-traditional, non-atherosclerotic pathways, including a subtle difference in the peripheral activity of neuropeptides. Our finding concerning the decreased action of neuropeptides in migraine patients might, therefore, be a possible non-traditional risk factor for the increased cardiovascular risk in these individuals. This hypothesis that alternative pathways might be implicated in migraine is in line with findings of a recent population based study, where migraine patients, compared to individuals without migraine, appear to have less arterial calcification in the intracranial carotid artery (75). In addition, our findings could be relevant in view of the cardiovascular safety of novel anti-migraine medications that may interfere with neuropeptide function (31).

As may be expected with our relatively small and homogenous study population, we did not find a correlation between the FRS and the outcome measures of microvascular function as assessed by the different techniques. The absence of a correlation between the FRS and EndoPAT outcomes in accordance with a previous study which found that the predictive value of the reactive hyperemia signal on late cardiovascular adverse events went beyond the traditional FRS in a similar age group (76). Besides, we found no correlations between outcome measures of microvascular function assessed by the EndoPAT and the LTH method. This might be related to the evident differences between both techniques, as the LTH technique assesses the microvasculature locally on the forearm, while the EndoPAT provides a composite outcome of the entire forearm.

4.3 LIMITATIONS AND STRENGTHS

There are several limitations to our study. Firstly, while the cross-sectional design of the study allows a direct comparison of the results, a disadvantage of this design is that both methods were performed only once and that no inferences about causality can be made. Also, restricted by our sample size, we could not assess the influence of all confounding variables in the association between the increased cardiovascular risk in PCOS and migraine patients.

Secondly, our measurements of endothelial dysfunction could be biased by the iontophoresis artefact we found, a phenomenon and possible confounding factor which deserves further attention as (the combination of) an electric current and NO might be involved. This current-induced vasodilation has been demonstrated to be triggered by C-fiber stimulation, and downstream pathways mainly involve cyclooxygenase-1 (COX-1) in healthy rats (77). Axon reflex responses via activation of mechano-insensitive C-nociceptors have also been hypothesized to be responsible, as EMLA cream results in elimination of this artefact (78). Yet, a clear-cut explanation of the occurrence of this artefact in only a selection of human participants is still lacking, but influences of the pH value of the skin, the skin thickness and vehicle, among others, have been described in detail elsewhere (78, 79).

We did not exclude participants exhibiting this artefact, as this could have led to potential selection bias related to the microvascular function. Thirdly, as we had no complete data on the exact timing of the previous and next migraine attack in migraine patients, we could not investigate the temporal association between our microvascular measurements and the prodromal or postdromal phases of a migraine attack. Lastly, we found a migraine prevalence of 47% in our study population, which approaches previous reports on the lifetime migraine prevalence of approximately 32% in these women (80, 81). This high prevalence is a surprising finding, as a previous case-control study by Pourabolghasem et al. (82) showed no significant increase in the migraine prevalence in women with PCOS compared to a control group. In contrast, a study in a nationwide Danish population of patients with PCOS described a significantly increased and two-fold higher risk of migraine (headache) in patients with PCOS compared to controls (83). While we did not include participants actively based on their migraine diagnosis, (self-) selection bias might have played a role in our study. Yet, the observed relatively high prevalence deserves further attention for additional investigation. Limited by our small sample size, we were unable to distinguish microvascular responses between MO and MA patients in this study, whereas it is the migraine type with aura that is especially associated with an increased cardiovascular risk (10, 84). Interestingly, the prevalence of MA in our population of women with PCOS far exceeded that of the general migraine population (70% versus 33%). One of the strengths of this study is that, to the best of our knowledge, this is the first study which assesses and compares endothelial dysfunction and neuropeptide activity using the LTH method in women suffering from PCOS with or without migraine. Blockade of the two main components involved in response to heating, being neuropeptides and NO, allows a more detailed evaluation of their influences in our study population. Using Laser Doppler Imaging to assess dermal blood flow allowed for a focus on the microvascular function. Secondly, we were able to detect subtle differences in the microvascular function using a more sophisticated and high-tech LTH technique, which were underexposed when using the already established, but more simple EndoPAT method. Therefore, the EndoPAT device provides a composite measure of the functioning of the inner lining of the blood vessels, but does not allow a closer study of the relative contribution of the various mediators involved in the vasodilatory response, in contrast to the LTH method.

Thirdly, migraine diagnoses were established through our well-validated questionnaire and subsequent confirmation by a headache specialist.

4.4 FUTURE WORK

Our study points to a difference in the blockade of neuropeptide action between women with or without migraine. However, our methods do not allow us to distinguish which neuropeptides are responsible for this difference, highlighting the need for basic and translational studies to disentangle their relative contribution and effects. Besides, research on the role of additional mechanisms involved in the vasodilatory response, including EDHFs, is warranted to better understand the mechanisms underlying the increased cardiovascular risk of PCOS and migraine patients. External validation of our findings in endocrinologically healthy and/or younger female migraine patients is needed to further investigate the effects of age and disease (PCOS in this case) on the microvasculature. It is yet also unknown to what extent these results are generalizable to other (middle-aged) women with migraine. Also, further studies to replicate our findings in larger settings are warranted.

Further investigation of the prevalence of migraine (with aura) in women with PCOS could be interesting. The different hormone profile in these women may be associated with an altered susceptibility to migraine and their cardiovascular risk profile may pose an ultimately supra-additive risk for cardiovascular disease in women with migraine. Therefore, incorporating such common pathways between migraine and PCOS in further (follow-up) studies might allow a more detailed measurement of the microvascular function to disentangle the contribution of different components and to understand the heterogeneity of LTH responses after EMLA and L-NMMA application between individuals. These longitudinal studies might also take the relationship between the microvascular function and the timing of migraine attacks into account.

As the endothelium and peripheral neuropeptides play a pivotal role in the development of cardiovascular disease, more knowledge on its functioning is needed to understand the underlying pathophysiology and to develop more targeted (and cardiovascularly safe) therapies in patients with migraine.

CONCLUSION

In conclusion, we determined the contribution of neuropeptide activity and NO bioavailability to peripheral microvascular function in women with or without migraine and comorbid PCOS using a novel set-up, namely the LTH technique. Despite no differences in the FRS – which is a traditional measure for the cardiovascular risk – we found a significantly decreased neuropeptide action in women with migraine and comorbid PCOS in the interictal period, compared to women with PCOS and without migraine. Although further studies are warranted to clarify the role of neuropeptides in younger as well as endocrinologically healthy female and male individuals with migraine, this decreased neuropeptide activity might serve as an explanation for the increased cardiovascular risk in migraine patients, which might be related to non-traditional cardiovascular risk factors. Lastly, it indicates that yet underexposed peripheral systems could serve as compensatory mechanisms for the decreased peripheral neuropeptide activity in patients with migraine.

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