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

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ABSTRACT

Background and aims: To understand 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: 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 ± 2.9 years) local thermal hyperemia (LTH) of the skin of the volar forearm was measured cross-sectionally under control conditions, after inhibition of neuro-peptide release by 5% lidocaine/prilocaine (EMLA) cream application, and after inhibition of nitric oxide for-mation by iontophoresis of NG-monomethyl-ʟ-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.
Results: While mean values under control conditions and L-NMMA conditions were similar, migraine patients had a significantly higher mean area of the curve (AUC) of the total LTH response after EMLA application than those without ($86.7 \pm 26.5\%$ *versus* $67.9 \pm 24.2\%$; $p = 0.014$). 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 and AI scores were similar in both groups.
Conclusions: In PCOS patients with migraine, neuropeptide action was lower compared with those without migraine. While larger studies are warranted, these findings provide a potential mechanism supporting previous findings that migraine may be independent from traditional risk factors, including atherosclerosis.

1. Introduction

Migraine is a chronic neurovascular headache disorder, typically characterized by attacks of debilitating unilateral headache, accompanied by photo- and phonophobia, nausea and emesis [1]. About one-third of migraine patients experience reversible neurological disturbances, i.e. aura symptoms, before the start of the migraine headache [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 attacks, migraine – particularly with aura – is a cardiovascular risk factor that has been associated with

significant cardiovascular endpoints, including myocardial infarction, coronary heart disease, stroke, and even cardiac mortality, especially in women [6–14].

Exact pathophysiological mechanisms of migraine have 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 the signalling molecule nitric oxide (NO) and might be one of the mediators in migraine initiation and maintenance [16,17]. The second mechanism is thought to originate from brainstem neural activation. It involves the simultaneous trigemino-vascular release of (vasodilatory) neuropeptides, including calcitonin gene-related peptide (CGRP) and pituitary adenylate cyclase-activating peptide (PACAP-38), causing dilation of cranial blood vessels and pain sensation [18–21].

Both mechanisms are also involved in cardiovascular disease. Endothelial dysfunction is an early indicator of cardiovascular risk and a reversible phase of atherosclerosis [22]. Neuropeptides contribute to peripheral vasodilation and are also implicated in cardiovascular health and disease pathogenesis, including vessel remodelling, atherosclerosis and ischemia [23–26].

This is the first study using an innovative method to assess the microvasculature in 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 [27], who may present with ovulatory dysfunction, clinical or biochemical signs of hyperandrogenism and polycystic ovarian morphology [28]. 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 [29,30], including a twofold increase in stroke prevalence, and with cardiovascular risk factors, such as metabolic syndrome and coronary artery disease [31, 32]. While in other PCOS cohorts, no evidence of increased cardiovascular disease (risk), including atherosclerosis, was shown [33,34], both migraine and PCOS could be viewed as two underappreciated and chronic cardiovascular risk factors in which sex steroids play a substantial role and that mainly affect young to middle-aged women. As ageing is one of the determinants of NO bioavailability in the human body, as well as cardiovascular health [35,36], 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 (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. Patients and 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 [37]. 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 [34].

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 [34]. 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 [38]. 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

Table 1
General (including intoxications), cardiometabolic, gynaecological/obstetric and endocrine characteristics of the total study population, that includes women with or without migraine who were diagnosed with PCOS.

	All subjects (N = 49)	Women with migraine (N = 23)	Women without migraine (N = 26)
General parameters and intoxications[a]			
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[b]	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[c]	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[c,d]	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[a,d]	2 (4.2%)	1 (4.5%)	1 (3.8%)
Diabetes Mellitus[a,e]	6 (12.2%)	3 (13.0%)	3 (11.5%)
Framingham Risk Score (10 year Cardiovascular Disease Risk %)[d,e]	4.9% (3.7–8.5)	4.8% (3.4–8.8)	5.6% (3.7–7.8)
Framingham Risk Score categories[d,e]			
Low risk (<10%)	41 (85.4%)	19 (82.6%)	22 (88.0%)
Intermediate riskv(10–19%)	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[a,f]	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[g]	20 (40.8%)	10 (43.5%)	10 (38.5%)
Endocrine parameters[c]			
Estradiol (pmol/L)[h]	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)[i]	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)

Values of all subjects are displayed as means (standard deviation) or medians (interquartile range, $Q_3 - Q_1$) – depending on whether data were normally distributed – or as numbers (percentage).
BMI, Body Mass Index; BP, blood pressure; FAI, Free Androgen Index; SHBG, sex hormone-binding globulin.

[a] Self-reported.
[b] This includes both frequent and (very) infrequent alcohol consumers.
[c] Data are missing in one migraine patient, that has been excluded from the analyses.
[d] Data are missing in one patient without migraine, that has been excluded from the analyses.
[e] Four participants did not know whether they are diabetic. These patients have been considered as non-diabetics, based on their medication use.
[f] This includes OCP use, having a hormone spiral, but also use of hormone therapy during menopause or hormonal treatments for breast cancer.
[g] Unclear, for example due to hormone use, which masks their natural menstrual cycle, or due to a hysterectomy.
[h] 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.
[i] 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.

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 5 min 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 anti-hypertensive 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 [39]. 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) 3 h 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. All measurements were performed at the Erasmus Medical Center in Rotterdam, The Netherlands, during the time period April 2019 and December 2019.

We performed cross-sectional 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 [40]. 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 5-min baseline measurement at 33 °C to standardize the temperature of the skin. Within 10 min 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 [41, 42]. The peak phase is followed by the plateau phase 30–35 min after heating. NO is the key contributor to this plateau phase, responsible for approximately 60% of the response [40,42–44].

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 g lidocaine/prilocaine cream (both 25 mg/g, EMLA®, Aspen Netherlands b.v., Gorinchem, The Netherlands) at least

1 h prior to the experiment to inhibit neuropeptide release, similar to the protocol applied by Minson et al. [42]. Additionally, iontophoresis at 60 μA during 7 min as previously validated in pilot experiments (data not shown, Perilont LI611, Järfälla, Perimed Sweden [45]) 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). Blockade of neuropeptide release and NO was assumed to be robust and effective by applying EMLA cream and L-NMMA, based on results of previous studies using similar application techniques and compounds [42,46].

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 min 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.

Fig. 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).

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 5 min baseline period, as the main outcome measure for all three conditions (control conditions, after application of EMLA cream, and after iontophoresis of L-NMMA) using the trapezoidal rule. DBF responses after EMLA application and iontophoresis of L-NMMA were expressed as 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 LTH peak phase (defined as the 10-min phase after heating) and plateau phase (defined as the 5-min phase starting 30 min 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 to migraine diagnosis to minimize bias. We handled these outliers by imputation of the concerning value, using
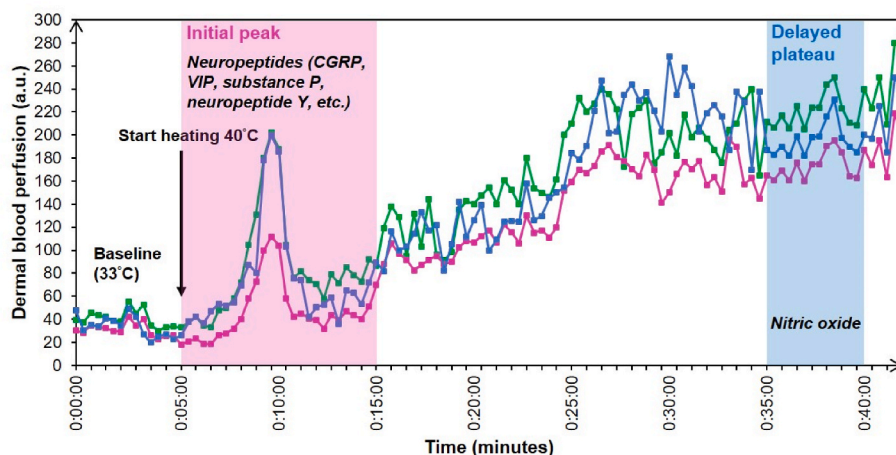

Fig. 1. An example of a response in middle-aged women with PCOS, after induction of LTH on the skin of the forearm under control conditions (green line), application of EMLA (lidocaine/prilocaine) (pink line) and L-NMMA (NO synthase inhibitor) (blue line).

a.u., arbitrary units; CGRP, calcitonin gene-related peptide; *VIP,* vasoactive intestinal polypeptide. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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, Fig. 1) had a 1.5-fold higher baseline of 5 min before heating to 40 °C compared to the LTH response without pretreatment (green line, Fig. 1), we took the average of the DBF measurements of approximately 1.5 min before until 1.5 min after start heating.

2.4.2. EndoPAT measurements

Approximately 5 min after completion of LTH measurements, EndoPAT measurements were performed using the index fingers of both arms. The EndoPAT device measures plethysmographic changes during reperfusion after a 5-min 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 [47,48]. 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 [48]. 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 [49]. In addition, we used the augmentation index (AI), normalized to heart rate of 75 bpm (AI@75), as a measure for arterial stiffness [47].

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) [50]. 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 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. Participants with missing data are excluded from the concerning analysis.

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 <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

In total, we approached 138 of the 200 women with PCOS, who were included previously in the multicentre CREW consortium, as these 138 patients were already registered at the Erasmus Medical Center. Four women did not fulfil our criteria, and 33 women indicated they were unwilling to participate, mainly due to lack of time. Fifty-two women could not be reached (due to missing or changed contact details) or did not respond to our invitation.

Forty-nine women with PCOS patients fulfilled the inclusion criteria and were willing to participate, comprising 23 individuals with migraine and 26 individuals without migraine. The migraine group consisted of 13 individuals with only MA (including 2 individuals suffering from aura without headache), 3 individuals with both MA and MO, and 7 individuals with only MO. 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). Of those who suffered from active migraine, 7 women were in the pre-/perimenopausal stage, 2 women were in the postmenopausal stage, and the menopausal stage was unclear in 8 women. Of those who had a history of migraine, 4 women were in the pre-/perimenopausal stage and the menopausal stage was unclear in 2 women.

Table 1 and the Supplementary Tables 1 and 2 present the baseline characteristics, various cardiometabolic, endocrine and gynaecological/obstetric parameters for all participants and for both women with or without migraine separately. No profound 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.

3.1. LTH measurements

LTH measurements were performed in all 49 participants. The measurement of one participant was shorter than 40 min. 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, has 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 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 10-min peak phase or in the mean AUC of the plateau phase (Table 2).

Table 2
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 analysed, as displayed by the *p*-values.

	All subjects (n = 49)	Women with migraine (n = 23)	Women without migraine (n = 26)	*p*-value
CONTROL CONDITIONS				
Total LTH response	$2.00*10^8$	$1.80*10^8$	$2.10*10^8$	**0.280**
in ms*a.u. [IQR]	$[1.47*10^8–2.48*10^8]$	$[1.37*10^8– 2.47*10^8]$	$[1.59*10^8– 2.55*10^8]$	
Peak phase	$3.78*10^7$	$3.64*10^7$	$4.08*10^7$	0.421
in ms*a.u. [IQR]	$[3.01*10^7–4.89*10^7]$	$[2.78*10^7–4.46*10^7]$	$[3.15*10^7–5.06*10^7]$	
Plateau phase	$3.71*10^7$	$3.41*10^7$	$3.97*10^7$	0.141
in ms*a.u. (SD)	$(1.29*10^7)$	$(1.09*10^7)$	$(1.42*10^7)$	
EMLA CREAM CONDITIONS				
Total LTH response	76.53	86.69	67.94	**0.014***
in % (SD)	(26.70)	(26.48)	(24.15)	
Peak phase	55.35	61.16	54.07	0.655
in % [IQR]	[41.45–77.31]	[40.48–79.44]	[44.13–72.24]	
Plateau phase	78.07	83.16	73.17	0.039*
in % [IQR]	[62.25–97.77]	[73.17–109.51]	[54.31–92.01]	
L-NMMA CONDITIONS				
Total LTH response in % [IQR]	98.31	**91.50**	**98.31**	**0.720**
	[72.97–117.20]	**[66.39–128.30]**	**[78.54–110.48]**	
Peak phase	107.66 [81.02–128.68]	107.66	105.70	0.613
in % [IQR]		[74.42–131.48])	[81.18–116.53]	
Plateau phase	88.46	87.63	88.46	0.927
in % [IQR]	[76.47–109.75]	[63.04–125.33]	[79.48–101.90]	

Values of all subjects are displayed as means (standard deviation) or medians (interquartile range, $Q_3 – Q_1$) – depending on whether data were normally distributed. Differences between participants with and 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.
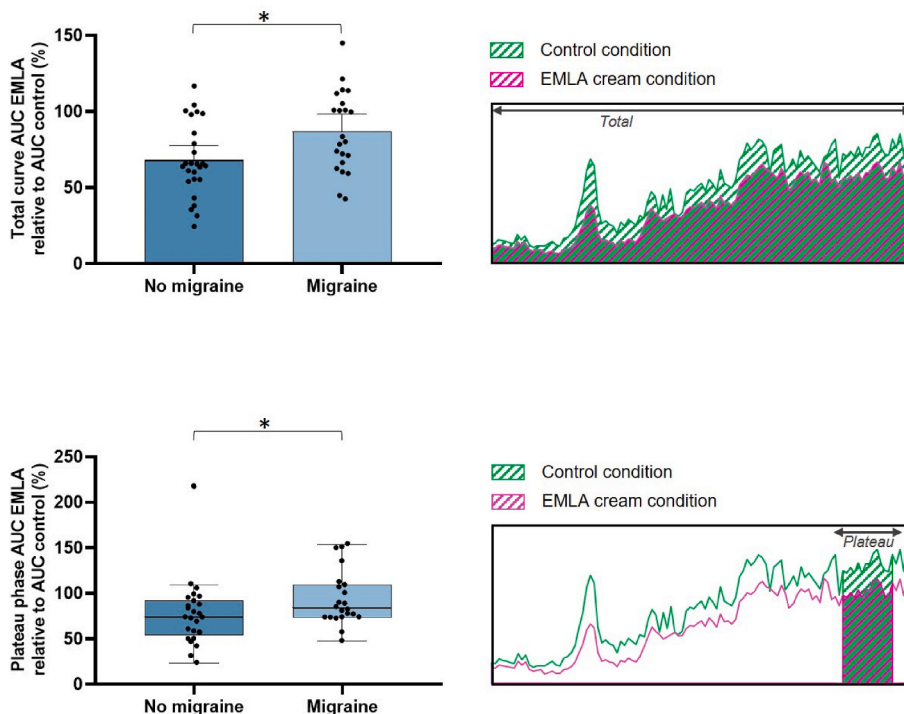

Fig. 2A. Bar plot showing the significant difference of the AUC of the total LTH response between subjects without migraine (n = 26) and with migraine (n = 23) after inhibition by EMLA cream (pink area). This is expressed as a percentage relative to the responses under control conditions (green area) (error bars: 95% CI). The right-sided graph represents an example of a single LTH response under EMLA conditions. Differences between participants with and without migraine were tested with the Student's T-test, considering the normal distribution of the data in both groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2B. Box plot that depicts the significant difference of the AUC in the plateau phase of the LTH response between subjects without migraine (n = 26) and with migraine (n = 23) 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. Differences between participants with and without migraine were tested with the Mann-Whitney U test, considering the skewed distribution of the data in both groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.1.1. 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 (Fig. 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 (Fig. 2B).

3.1.2. 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 min 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. These measurements were not performed in the remaining 6 women, as they did not give consent for this part of the study and due to technical issues. The occlusion period was shorter than 5 min in one participant (approximately 2:15 min), 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 Materials).

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. We found no differences in outcomes of EndoPAT measurements and LTH responses under control or L-NMMA conditions between women with and without migraine and comorbid PCOS. However, the EMLA condition revealed a significantly lower neuropeptide action in women with migraine compared to women without migraine.

A variety of studies have reported measurements of the endothelial function as a measure of cardiovascular risk using and validating the EndoPAT method in different populations [51,52]. 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 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 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 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 [53]. 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 [54]. 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. [55].

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 [44,56]. EDHFs have been described to compensate the loss of NO synthesis in humans [57].

It has been generally accepted that NO accounts for approximately

60% of the plateau phase of LTH [42–44], 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 is 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 modified iontophoresis protocol that was applied to this specific study population for the first time that further hampered proper analyses [45].

We also did not observe any 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 [58]. 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 [44]. Another explanation for the low NO bioavailability in these women with PCOS might be related to their low estrogen levels [59], masking potential differences between women with or without migraine that might be present in women with a normal hormone profile. Indeed, several studies describe increased endothelial dysfunction in women with PCOS; however, contrasting views exist on whether this association is dependent on general risk factors, like obesity [28,60,61].

The decreased action of neuropeptides observed in the current study may partly explain the increased cardiovascular risk in female migraine patients [10,62], as certain neuropeptides, for example CGRP, are proposed to exhibit cardioprotective effects in cardiovascular disease [63].

It is noteworthy that most studies measure and compare neuropeptide or biomarker levels (often in peripheral blood or urine) between patients with and without migraine – often without other comorbidities (e.g. Refs. [54,64]). The fact that we rather compared the activity of neuropeptide and NO while accounting for a comorbidity (PCOS) hampers a proper comparison with previous studies and highlights the importance of further studies.

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 [47,49]. 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 [34]. 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 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. The fact that the vast majority (87.8%) had been pregnant at least once during lifetime might also support the hypothesis that our population consisted of mainly (health-conscious) women who were under (long-term) medical check or follow-up. 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 [65,66]. 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 (Fig. 3). 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 [67]. In addition, our findings could be relevant in view of the cardiovascular safety of novel anti-migraine medications that may interfere with neuropeptide function [24].

Fig. 3. Graphical abstract.
In the current cross-sectional study, we compared the microvascular status between middle-aged women with and without migraine and comorbid Polycystic Ovary Syndrome (PCOS). By using a sophisticated Local Thermal Hyperemia (LTH) method, we compared the contribution of neuropeptides and nitric oxide in the peripheral vascular system. We identified a putative mechanism underlying the increased cardiovascular risk of migraine patients with comorbid PCOS – i.e. a lower neuropeptide action in migraine patients compared with those without migraine. This association of migraine with an affected peripheral neuropeptide activity is not reflected by traditional measures of cardiovascular risk (e.g. scores that include smoking or an unfavorable cholesterol profile) or atherosclerosis. While larger studies are warranted, our observations provide a potential mechanism supporting previous findings that migraine may be independent from these traditional risk factors and, therefore, challenge our current ideas on how to assess and mitigate the cardiovascular risk in migraine patients.
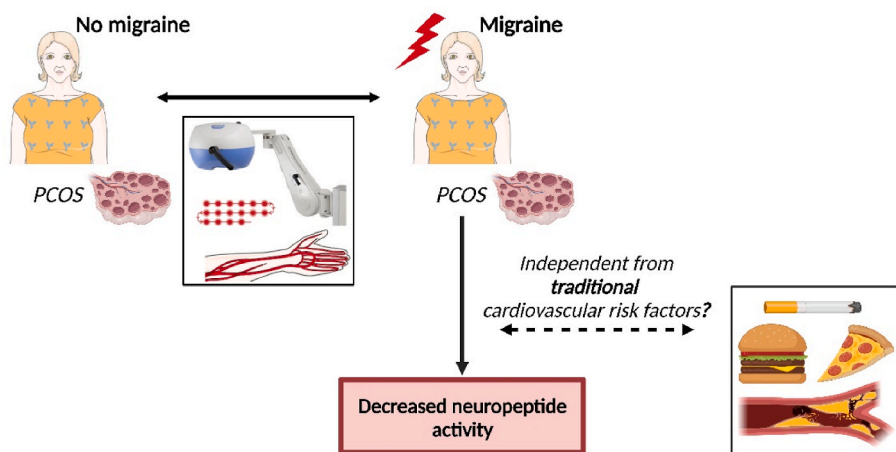
As may be expected with our relatively small and homogenous study population, we did not find a correlation between FRS and outcome measures of microvascular function as assessed with the different techniques. The absence of a correlation between FRS and EndoPAT outcomes is in accordance with a previous study which found that the predictive value of the reactive hyperemia signal on late cardiovascular adverse events went beyond traditional FRS in a similar age group [68]. 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 strenghts

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 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 [69]. 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 [70]. 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 [70,71].

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 [72,73]. This high prevalence is a surprising finding, as a previous case-control study by Pourabolghasem et al. [74] 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 [75]. 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 [10,76]. Interestingly, the prevalence of MA in our population of women with PCOS far exceeded that of the general migraine population (70% *versus* 33%). This small sample size may also not support precise conclusions.

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. Indeed, as described previously, a certain form of selection bias might have taken place – considering the only moderately unfavourable cardiometabolic profile of these women with PCOS, as described elsewhere [34]. Our research population might, therefore, not necessarily serve as a representative reflection of the "average" woman with PCOS. However, this further underscores the importance of our findings of a decreased neuropeptide activity in migraine patients, which we even found in a population without an increased prevalence of cardiovascular risk factors. It is also yet unknown to what extent these results are generalizable to other (middle-aged) women with migraine, and replication of our findings in larger settings is warranted.

Further investigation of the prevalence of migraine (and also migraine 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 while considering the effect of the menstrual cycle.

As the endothelium and peripheral neuropeptides play a pivotal role in the development of an increased risk for 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.

4.5. Conclusions

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. 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 (Fig. 3). Lastly, it indicates that yet underexposed peripheral systems could serve as compensatory mechanisms for the decreased peripheral neuropeptide activity in patients with migraine. Further research is warranted to elucidate the role of neuropeptides (in relation to) the decreased prevalence of atherosclerosis in in younger as well as endocrinologically healthy female and male individuals with migraine.

CRediT authorship contribution statement

Linda Al-Hassany: Investigation, Formal analysis, Visualization, Data curation, Project administration, Writing - original draft, Writing - review & editing. **Katie M. Linstra:** Project administration, Writing - review & editing. **Cindy Meun:** Project administration, Data curation, Writing - review & editing. **Jeffrey van den Berg:** Investigation, Writing - review & editing. **Eric Boersma:** Conceptualization, Formal analysis, Funding acquisition, Writing - review & editing. **A.H. Jan Danser:** Resources, Writing - review & editing. **Bart C.J.M. Fauser:** Conceptualization, Funding acquisition, Writing - review & editing. **Joop S.E. Laven:** Conceptualization, Funding acquisition, Writing - review & editing. **Marieke J.H. Wermer:** Conceptualization, Funding acquisition, Writing - review & editing. **Gisela M. Terwindt:** Conceptualization, Funding acquisition, Investigation, Writing - review & editing. **Antoinette MaassenVanDenBrink:** Conceptualization, Methodology, Resources, Visualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Linda Al-Hassany, Katie M. Linstra, Cindy Meun, Jeffrey van den Berg, Eric H. Boersma, A.H. Jan Danser, and Joop S. Laven declares no conflict of interest with respect to the research, authorship, and/or publication of this article.

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Appendix A. Supplementary data

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