VASCULAR FUNCTION AND CARDIOVASCULAR RISK FACTORS IN WOMEN WITH SEVERE FLUSHING

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Precis: Flushing postmenopausal women appear to have better vascular responses than non-flushing women but paradoxically, such women appear to have worse (not better) CVD risk factors.

ABSTRACT

Background: 70% of postmenopausal women suffer from hot flushes causing significant morbidity in 25%. Oestrogen replacement provides symptom relief, but its use has declined following safety issues and there is, as yet, no good alternative. Pathophysiology is poorly understood, but one proposed mechanism is altered peripheral vascular reactivity. It has recently been suggested that the presence of flushing may be a marker of underlying cardiovascular risk**.**

Aim: To measure i) peripheral vascular reactivity in subcutaneous vessels ii) routine and novel cardiovascular risk factors in postmenopausal women who flush, and compare results to a matched group of women who do not flush.

Methods: 32 postmenopausal women with at least 20 flushes/week and 14 non-flushing postmenopausal women were recruited. Cutaneous microvascular perfusion was measured using laser Doppler imaging and endothelial function was assessed by iontophoresis (administration of vasoactive agents through the skin by an electric current) of acetylcholine [Ach] (endothelial-dependent) and sodium nitroprusside [SNP] (endothelial independent). Blood samples for risk factors were taken following vascular assessment.

Results: Both study groups were well matched demographically. The response of the subcutaneous vessels was greater in women who flushed than those who did not, following administration of both the endothelium-dependent and independent vasodilators, (ACh, p = < 0.001, SNP, p = 0.001, 2-way ANOVA). By contrast, levels of HDL-cholesterol and ApoA1 were significantly lower in the flushing women compared with the control women (P=0.02 and 0.002, respectively), and levels of ***inter-cellular adhesion molecule-1*** (ICAM-1) were higher (P=0.03), findings robust to adjustment for confounders, suggesting an adverse cardiovascular risk profile.

Conclusion: These results confirm a better vascular response in women but paradoxically, such women appear to have worse (not better) CVD risk factors in particular l lower HDL-cholesterol but also higher non-HDL-c to HDL-c ratio and increased ICAM-1. Further studies are needed to assess vascular risk factors in women who flush.

INTRODUCTION

The menopause is defined by the World Health Organisation (WHO) as the permanent cessation of menstrual periods that occurs naturally, or is induced by surgery, chemotherapy, or radiation. The climacteric is the period of transition from regular menstruation to its cessation, and during this time gonadal hormones change substantially.

There are a number of symptoms associated with this period and decreasing oestrogen levels, although some women will experience none of these. They include hot flushes and night sweats (vasomotor symptoms), vaginal symptoms, depression, anxiety, irritability and mood swings (psychological effects), joint pains, migraines or headaches, sleeping problems and urinary incontinence.

Hot flushes are the most commonly reported symptom, occurring in approximately 70% of women (1) causing significant morbidity in 25%, affecting social life and even the ability to work (2). They are periods of intense heat, which are associated with sweating and peripheral vasodilation. Flushing commonly occurs when hypooestrogenism follows a period of oestrogen exposure. If left untreated, hot flushes resolve within one year, or less, in the majority of postmenopausal women. However, a third will report symptoms that last up to 5 years after natural menopause, and in 20% hot flushes persist for up to 15 years (3).

Hot flushes and other related symptoms have been successfully treated with oestrogen for years, effective in over 80% of cases. In 1995, 37% of American women took HRT, principally for this purpose. For this reason few studies were carried out investigating the pathophysiology of flushes. However, following publication of results from studies such as the Women's Health Initiative and Million Women Study there has been renewed interest in this topic since ***hormone replacement therapy*** (HRT) prescription dropped 50%.

The mechanism of flushing is still poorly understood, although hypotheses now exist surrounding both central and peripheral mechanisms.

It has been shown that postmenopausal women who flush have a diminished vasoconstrictor response to cold (4) and that they have increased blood flow to the forearm and hand during a flushing episode (5) This altered (potentially heightened) peripheral vascular reactivity is another proposed mechanism responsible for the pathophysiology underlying hot flushes. In this study, we aimed to assess vascular function in postmenopausal women who flush and compare it with postmenopausal women who do not flush.

In addition we aimed to study factors which might influence vascular reactivity, and to examine associations between hot flushes and endothelial function and several circulating cardiovascular disease (CVD) risk factors. These included lipids and apolipoproteins, inflammatory markers and intracellular adhesion molecule-1 (ICAM-1), the latter being linked to vascular dysfunction and higher risk of CVD and especially diabetes in several studies (6).

MATERIALS AND METHODS

Study Participants

A total of 32 postmenopausal women who each experienced at least 20 flushes/day and 14 non-flushing women were recruited to participate in this study. Recruitment of volunteers was facilitated by Scottish media coverage as well as taking place within relevant out-patient clinics. A number of women (cases and controls) were also recruited from the West of Scotland Breast Screening Centre in Glasgow.

Participating women were all aged 50-65 years, non-smokers, not known to be hypertensive, non-diabetic and not taking any drugs which could affect vascular function. These strict recruitment criteria minimised potential for confounding by several common factors. Menopausal status was determined by either an FSH greater than 20 Units/Litre or amenorrhoea for 1 year or longer.

Design and Procedures

All study participants were assessed using Laser Doppler Imaging (LDI) with iontophoresis of vasoactive compounds. Blood was obtained at the time of LDI assessment. Each participant in the flushing group was requested to keep a 'Hot Flush' diary for 4 weeks prior to assessment.

All work was performed according to the Declaration of Helsinki with approval granted by the institutional ethics committee (REC 01/50704/43). All patients gave written informed consent.

LASER DOPPLER IMAGING WITH IONTOPHORESIS

Non-invasive skin perfusion can be measured using Laser Doppler Iontophoresis (LDI) (7).

Iontophoresis is a technique which allows for transdermal delivery of vasodilator agents acetylcholine (ACh) and sodium nitroprusside (SNP) across the skin under the influence of an applied current. In the past iontophoresis has been used in conjunction with laser Doppler flowmetry, a non-invasive method for assessing microvascular perfusion at a single point (8). More-recently, iontophoresis has been combined with ***laser Doppler imaging***, which reduces measurement variability (9-10). This is because unlike laser Doppler flowmetry, laser Doppler imaging measures perfusion across many points (11), and an average measure of perfusion can be computed for any chosen area.

Iontophoresis of acetylcholine (ACh) at the anode tests endothelial function since its vasodilator action involves binding to muscarinic receptors on endothelial cells, with subsequent generation of NO. It is therefore said to be 'endothelium dependent'. Vasodilatation is ultimately mediated by action of NO on vascular smooth muscle (via the cGMP pathway) and so iontophoresis of an NO donor, sodium nitroprusside (SNP), delivered at the cathode, is used as an 'endothelium-independent' control to test the integrity of vascular smooth muscle.

Drug delivery is achieved using a battery-powered constant-current iontophoresis controller (MIC-1e; Moor Instruments Ltd., Axminster, U.K.). The chambers used for iontophoresis (ION 6; Moor Instruments Ltd.) are constructed of Perspex (internal diameter 22mm; area 3.8cm^2) with an internal platinum wire electrode. Two chambers are attached to the skin of the volar aspect of the forearm by means of double-sided adhesive discs, avoiding hair, broken skin, and superficial veins. The chambers are connected to the anode and cathode connections on the iontophoresis controller and the voltage across the chambers is monitored. A thermometer is also attached to the arm in order to measure skin temperature.

2.5ml of 1% ACh (Sigma) is introduced to the anodal chamber and 2.5ml of 1% SNP (Sigma) is introduced to the cathodal chamber. The vehicle for these drugs is 0.5% sodium chloride (NaCl). Both of these agents are delivered simultaneously during each period of current administration. Fluid is prevented from escaping by placing circular 32mm coverslips over the chambers.

The iontophoresis protocol involves incremental current delivery with four scans at 5µA, four at 10µA, four at 15µA and two at 20µA, giving a total charge of 8mC.

The laser doppler imager (Moor Instruments, UK) is equipped with a red laser (wavelength 633nm, power 1mW, beam diameter 1mm). The laser is scanned in a raster fashion over both chambers and through the coverslips. The backscattered light is collected by photodetectors and converted into a signal proportional to perfusion in arbitrary perfusion (flux) units (PU) that is displayed as a colour-coded image on a monitor. Perfusion measurements are obtained using the imager manufacturer's image analysis software by outlining a region of interest (ROI) around the internal circumference of the chamber. Statistical analysis of the ROI is subsequently performed to yield the median flux value across approximately 700 measurement points. Twenty repetitive scans are taken during each LDI assessment, the first being a control (before current administration), followed by the incremental current protocol as described above (fourteen scans), and followed by a further five scans with no

current administration. An assessment of the overall response to the drugs is obtained by calculating the area under the curve (AUC).

This technique is reproducible with between-day and within-day coefficients of variation of 6.4 ±33% and 8.9 ±5.3% respectively. Variability being reduced by averaging perfusion over a large skin area (12).

All participants fasted for at least 5 hours prior to assessment (water only permitted). Prior to the procedure, patients were allowed to acclimatize for 15 minutes in a temperature-controlled room. Participants all lie in a semi-recumbent position with the flexor aspect of the forearm exposed on an arm rest.

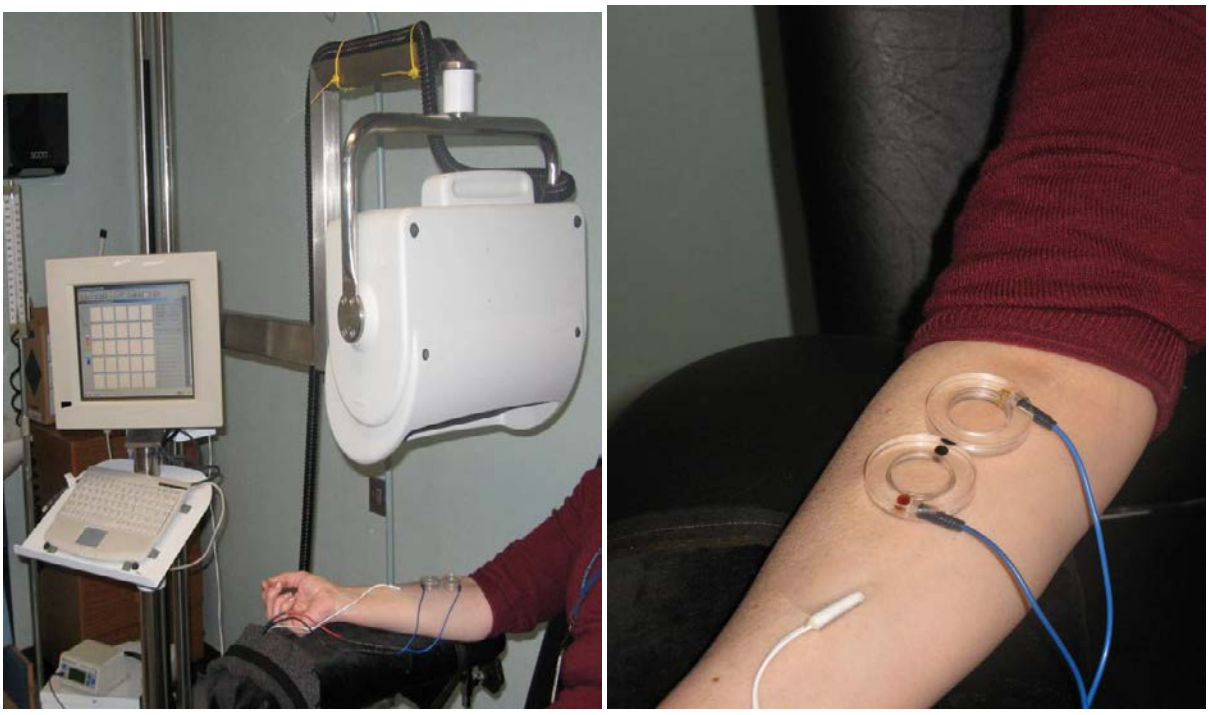

Figure 1: Image of laser Doppler iontophoresis equipment.

PLASMA ANALYSIS

At LDI assessment, blood was collected and processed to allow measurements of insulin, glucose, lipids, ***apolipoproteins***, CRP, and ICAM-1.

Samples were spun in the centrifuge at 3000rpm, at 4^{o}C for 10 minutes. The plasma was stored at -80^{o}C in 1ml aliquots, within 1 hour of being taken from the patient.

STATISTICAL ANALYSIS

Measurement of vascular responses was performed using raw values. Comparisons were by General Linear Model. Because of the marked differences in variances between basal perfusion values and the maximal responses to the drugs, but the similar coefficients of variation, $\log_{10}$ transformation of the data was performed to equalise the variances and thereby permit parametric data analysis.

Log_{10} transformation of the plasma data was also performed to allow for parametric data analysis with comparison by students t-test. In addition, plasma data was adjusted for BMI, Age, Years since last menstrual period (LMP) and Parity using general linear model.

Comparison of demographic data was by t-test as data were of a Gaussian distribution.

RESULTS

32 women with severe hot flushing and 14 women with no hot flushing, aged between 50 and 65, who were medically fit and were not taking drugs that might impact on vascular reactivity, were recruited and had LDI assessment of vascular reactivity of the subcutaneous vessels.

A full set of demographic characteristics were available for 29 flushing women and 13 control women, and they were broadly matched, but even so we were careful to adjust CVD risk factor comparisons for age, BMI, parity and time since LMP. (Table 1)

Responses to acetylcholine (ACh) and sodium nitroprusside (SNP)

Vascular reactivity for 32 flushers and 14 controls (non-flushers) was measured using LDI. Data was analysed as described above.

The response of the subcutaneous vessels was greater in women who flushed than in those who did not (Figure 2). The enhanced vascular response occurred following administration of both the endothelium-dependent (ACh) and independent vasodilators (SNP), (ACh, $p = < 0.001$, SNP, $p = 0.001$, 2-way ANOVA).

As both groups appeared to show a consistent difference between ACh and SNP responses, this difference was calculated and the analysis between groups repeated (using the general linear model). However, this calculated difference between the endothelium dependent and independent responses was not found to differ between flushers and controls (P=0.17; Figure 3)

CVD risk factors (Table 2)

Women with flushing had significantly lower HDL-cholesterol levels (P=0.02), lower apolipoprotein A1 (P=0.002), and higher I-CAM levels (P=0.03).

These results were ***not affected by*** adjustment for body mass index (BMI), age, years since last menstrual period (YSLMP) and parity: HDL-cholesterol (P=0.007), Apolipoprotein A1 (P<0.001), and I-CAM (P=0.05).

In the control group HDL-cholesterol also correlated with vascular reactivity as measured by AUC for the ACh response (P=0.01, R-Sq 48.3%), but not in the flush group (data available on request). Again,

this relationship remained significant when adjusted for ***body mass index*** (BMI), age, and ***waist hip ratio*** (WHR), P=0.035, R-Sq 60.7%. Also, Apo B was inversely related to Ach mediated vascular reactivity in the control group (P=0.02, R-Sq 46%, and adjusted P=0.004, R-Sq 80.8%).

DISCUSSION

In this group of postmenopausal women, those with severe flushing have a greater vasodilator response than those women with no flushing. This is in keeping with previous studies showing a diminished vasoconstrictor response, and increased blood flow during a hot flush (4-5). The alteration to their vascular function seems to be an increased vasodilatory response in comparison with asymptomatic women

This increase has been demonstrated to be present in both the endothelial dependent (ACh) and independent (SNP) responses. We can therefore say that there is an increased response of the vascular smooth muscle to the NO donated by SNP compared to asymptomatic postmenopausal women. Sodium nitroprusside dissociates in the circulation to release NO, which activates guanylate cyclase in vascular smooth muscle and increases intracellular production of cGMP. cGMP stimulates calcium movement from the cytoplasm to the endoplasmic reticulum and reduces calcium available to bind with calmodulin. Vascular smooth muscle relaxes and vessels dilate.

Endothelium dependent responses are also increased this group of flushing women compared with non-flushing women as a result of administration of acetylcholine which acts to stimulate NO production by endothelial cells, by binding to muscarinic receptors. However, the endothelial produced NO must still act upon vascular smooth muscle to have a response. Therefore, in order to examine the endothelial response in isolation, we must remove the vascular smooth muscle response from the equation. In order to do this, we have analysed the difference in the ACh and SNP response and found no variation in the groups. It is possible, then, that the enhanced response in the flushing group, is due purely to an increased vasomotor smooth muscle response.

It is also possible that there are other endothelial derived factors activated that may cause vasodilation. These include prostacyclins, since administration of aspirin prior to LDI, leads to reduced endothelial dependent responses following both oral (13) and intravenous (14) aspirin, although this has not been demonstrated by all studies (7, 10), and electrically induced vasodilation. However, the vehicle 0.5%NaCl and the chamber size used have been shown to minimise an electrically induced hyperaemic response. Furthermore, application of a local anaesthetic agent in an attempt to eliminate the 'axon reflex' thought to be a possible cause of electrically induced hyperaemia did diminish this response, but also caused vasoconstriction (12), therefore any vasodilatory effects would be superimposed upon already vasoconstricted vessels.

This apparently 'better' vascular response in women who flushed excessively may be anticipated to suggest a protective phenotype against vascular risk since impaired skin microvascular function has been linked to several conditions associated with greater CVD risk to include diabetes (15), maternal obesity (16), and hypercholesterolaemia (17) as well CAD itself (18). However, by contrast, results of recent studies suggest the presence of menopausal flushing may be a marker of cardiovascular risk, as marked by reduced flow-mediated dilation (brachial artery) and greater aortic calcification in flushers in the Women's Health Across the Nation Heart Study (19). To examine these issues in more details, we therefore examined CVD risk factors in these women.

Women who flushed in the present study had lower levels of HDL-cholesterol and ApoA1, and higher levels of ICAM-1 than asymptomatic women. Recent work from the Emerging Risk Factor Collaboration, which represents the more complete lipid-CVD risk analyses performed anywhere, has shown that greater non-HDL-cholesterol (or total cholesterol) but lower HDL-cholesterol are strong and independent risk factors for vascular disease in both men and women (20) thereby in keeping with greater vascular risk over the long term.

The blood and vascular data therefore appear contradictory, but can they be explained?

It could be said that these women have an imbalance between endothelium-derived vasodilators that have anti-thrombotic and antimitogenic properties and vasoconstrictors with proatherogenic activity (21). However, greater vasodilatory response is usually considered a sign of vascular health.

Endothelium-derived NO is now recognised to be an anti-inflammatory and anti-arteriosclerotic molecule; mice lacking the endothelial-type NO synthase gene exhibit hypertension and enhanced vascular remodelling in response to injury (22). One possibility is that these postmenopausal flushing women are at increased risk of cardiovascular disease compared with their non-flushing counterparts and have reduced synthesis of NO as a result of endothelial dysfunction, and as a result of this have increased sensitivity to NO and therefore when exposed to NO, have a greater vasodilatory response.

The study by Bechlioulis and colleagues (23) found that women in the early stages of menopause had endothelial dysfunction, but that this was not associated with a change in plaque size, as the carotid intima-media thickness was comparable to that in the premenopausal controls. Perhaps what we are seeing with our results is an increased response to NO due to increased sensitivity as a result of longer term underlying endothelial dysfunction leading to decreased synthesis.

And although there appeared to be no endothelial dependent variation between groups when we examined the difference in independent and dependent responses, it is possible that the increased response that was smooth muscle driven, is superimposed on a background of early endothelial dysfunction that will become more apparent with time.

As with Findings from the Study of Women's Health Across the Nation Heart Study (19), Gambacciani (24) found arterial blood flow was altered only in women with hot flushes. This too, is in support of Bechlioulis (23) as they also demonstrated that severity of flushes was the most important independent predictor of endothelial dysfunction.

However, ***flow mediated dilation*** (FMD) is expressed as percentage change meaning that a larger percentage change will occur with a small diameter increase in a narrow vessel, than the same increase

in a vessel with a higher baseline lumen diameter. It is possible then that flushers overall had more dilated brachial arteries to begin with, which would be consistent with our data.

Also, it is possible that smaller diameter cutaneous vessels and the vasodilation that has been found to be associated with hypooestrogenism (25), cannot be extrapolated to larger vessels like those studied in flow mediated dilation.

And while measures of peripheral vascular function are used as surrogate markers for coronary vascular function, there is a paucity of data showing any clear correlation. This may be as a result of the difference in size of vessels studied. However there is evidence demonstrating that peripheral vascular function acts a prognostic tool. Impaired brachial artery flow mediated dilation predicts cardiac events in hypertensive postmenopausal women(26).

Women with climacteric symptoms have a lower level of plasma antioxidant activity (27), and oxidative stress is associated with cardiovascular risk. Leal also demonstrated that HRT decreases oxidative stress in addition to decreasing number of flushes. A recent review (28) suggested that a possible reason for the opposite effects of the beneficial effect of HRT in younger women within the first year of use (29) and detrimental effects in women distant to the menopause may be as a result of genuinely altered vascular reactivity making them vulnerable to the effects of HRT.

Although total cholesterol, elevated LDL-C and low HDL-C are well established risk factors for cardiovascular disease (CVD), an association with endothelial dysfunction has not been consistently demonstrated. It is also possible that there is an uncoupling of central and peripheral control, with impulses from the hypothalamus overshadowing peripheral responses. Whatever, the mechanisms for this discrepancy between microvascular function and CVD risk factors, in particular lipids, the link between ***CVD risk factors*** and outcomes is of course far better established and thus the results more informative about future risk.

CONCLUSIONS

The present study demonstrated a difference in the peripheral vascular reactivity of postmenopausal women who flush when compared with postmenopausal women who do not flush, and an adverse cardiovascular risk profile in this group of flushing women. Our results suggest a need for larger studies to examine the CVD risk profile of women with history of severe flushing.

Limitations

These were all lean, Caucasian women; therefore care must be taken when extrapolating these results to the general population.

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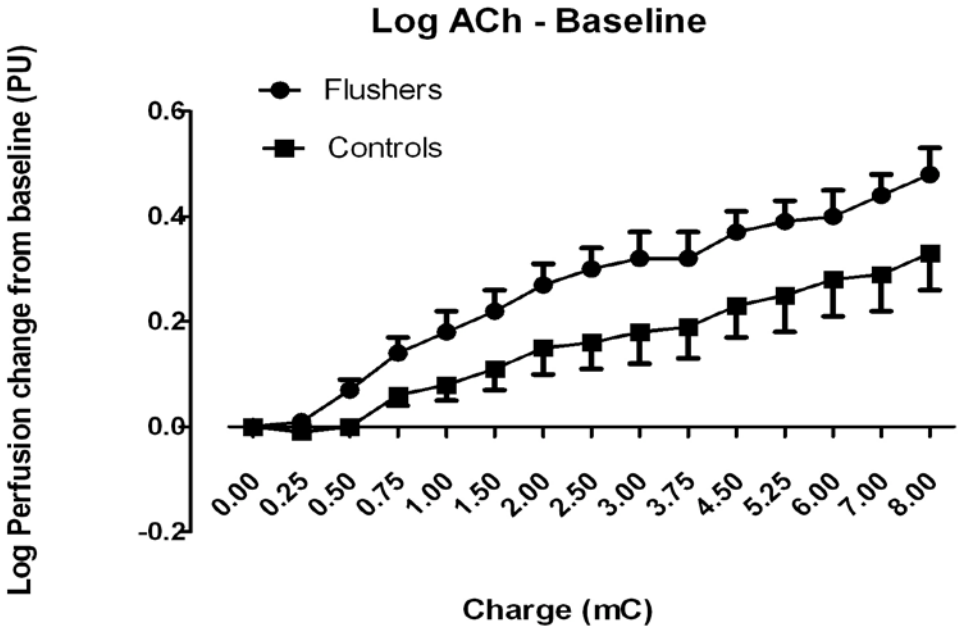
FIGURES



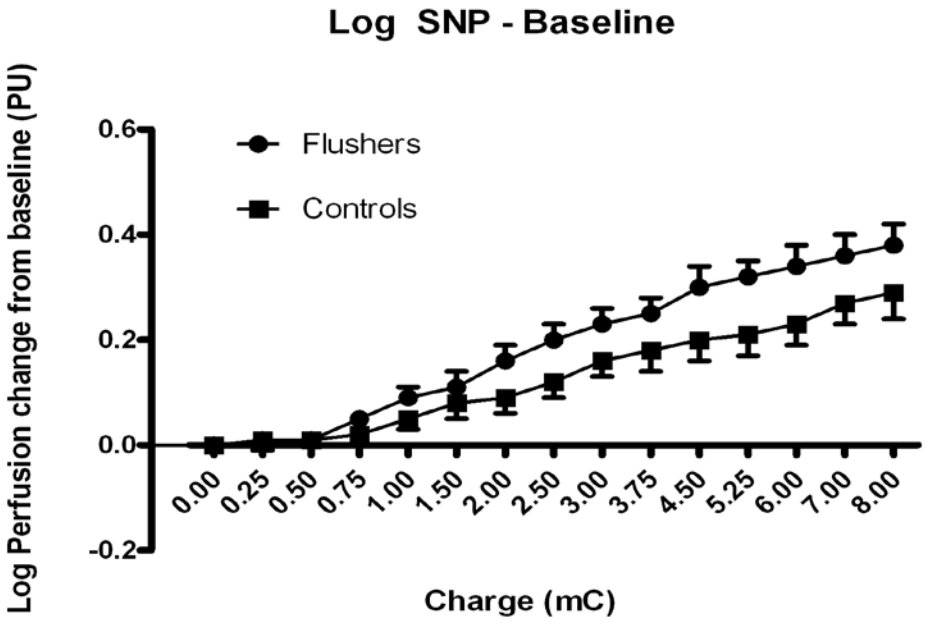


Figure 2: Dose Response Curves
Log perfusion change from baseline in perfusion (flux) units with increasing charge for acetylcholine and sodium nitroprusside in control women compared with flushing women. P<0.001 and P=0.001 respectively. Data are mean ± SEM.

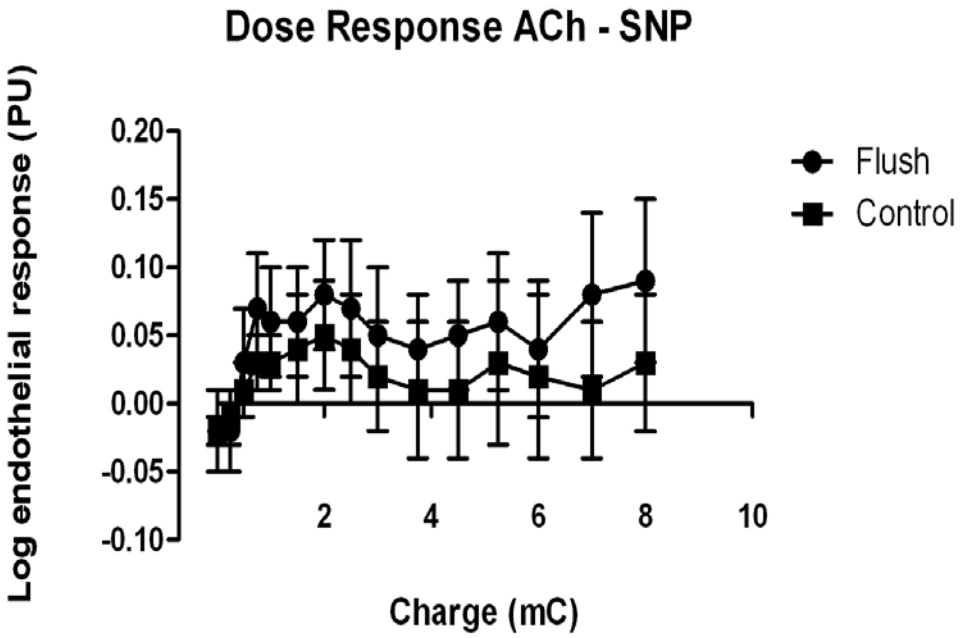


Figure 3: Dose Response Curve
Log endothelial response (difference between endothelium dependent and independent responses). P=0.17. Data are mean ± SEM.

TABLES

	Flush	Control	P
n	**29**	**13**	
Age	**56 (53-61)**	**54 (52-57)**	**0.25**
Years since ***last menstrual period*** (LMP)	**8 (1-12)**	**5 (1-6)**	**0.15**
Smoking	**0**	**0**	**-**
Body Mass Index (BMI)	**25.1 (24.3-28.1)**	**23.0 (20.9-31.9)**	**0.28**
Parity	**2 (0-3)**	**2 (2-3)**	**0.74**

Table 1 Demographic characteristics of study subjects
Data are median (interquartile range). Statistical analysis was performed using the Mann-Whitney U test. All women are non-smokers and not known to be hypertensive (see Figure 2.)

	FLUSHING	CONTROL	P	Adjusted P	R-Sq (%)
Cholesterol (mmol/l)	5.77 (5.01; 6.35)	5.93 (5.05; 6.57)	0.91	0.97	6.86
Triglyceride (mmol/l)	1.17 (0.82; 1.73)	1.09 (0.82; 1.53)	0.83	0.97	11.33
Low Density Lipoprotein (LDL)-cholesterol (mmol/l)	3.51 (2.92; 4.42)	3.17 (2.63; 4.30)	0.29	0.29	14.14
High Density Lipoprotein (HDL)-cholesterol (mmol/l)	1.46 (1.21; 1.76)	1.84 (1.51; 2.10)	**0.01**	**0.01**	40.17
Total Cholesterol to HDL-***Cholesterol*** ratio	3.77 (3.08; 4.74)	2.98 (2.59; 3.73)	**0.02**	**0.04**	26.33
Apolipoprotein A1 (mg/dl)	155.30 (146.15; 170.65)	194.80 (172.40; 200.30)	**<0.001**	**<0.001**	94.81
Apolipoprotein B (mg/dl)	78.30 (71.00; 104.65)	80.40 (62.95; 100.50)	0.23	0.29	11.01
*__Systolic Blood Pressure__	126.__07__ ± 2.67	120.__25__ ± 4.29	0.25		
*__Diastolic Blood Pressure__	70.48 ± 2.11	72.66 ± 2.34	0.61		
Insulin (mu/l)	5.69 (3.48; 9.16)	6.17 (5.27; 8.68)	0.59	0.67	22.99
Glucose (mmol/l)	5.30 (5.10; 5.60)	5.60 (5.25; 5.85)	0.68	0.69	29.14
Adiponectin (mg/ml)	11.6 (8.6; 15.1)	11.7 (9.7; 14.7)	0.38	0.48	11.51
C-Reactive Protein (CRP) (mg/l)	1.70 (0.64; 5.84)	0.90 (0.35; 1.42)	0.07	0.18	19.7
Inter-Cellular Adhesion Molecule 1(ICAM-1) (ng/ml)	243 (202; 263)	187 (164; 228)	**0.02**	**0.05**	18.87

Table 2 CVD risk factors.
Data are median (interquartile range). Statistical analysis was performed using students t-test on log transformed data. *Data are median ± SD

Adjusted P calculated using general linear model to adjust for BMI, Age, Years since last menstrual period (LMP) and Parity.

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