UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS: ENDOCRINOLOGIA

AVALIAÇÃO DA FUNÇÃO ENDOTELIAL E ASSOCIAÇÃO COM ANDROGÊNIOS ENDÓGENOS EM PACIENTES PÓS-MENOPÁUSICAS

Maria Augusta Maturana

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Tese apresentada como requisito parcial para a obtenção do título de Doutor em Ciências Médicas: Endocrinologia

Orientadora: Profª. Drª. Poli Mara Spritzer

Co-orientadora: Profª. Drª. Maria Claudia Irigoyen

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À Vitória, minha amada filha.

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Science without religion is lame, religion without science is blind.

Albert Einstein

APRESENTAÇÂO

A disfunção endotelial está implicada na gênese da aterosclerose e provavelmente no aumento da incidência de doença cardiovascular em mulheres na pós- menopausa. Na literatura, ainda há controvérsia a respeito da influência dos androgênios endógenos, como um dos fatores envolvidos num perfil de risco cardiovascular nesta população. Nesta tese, estudamos as associações entre androgênios endógenos, variáveis antropométricas, proteina C-reativa, endotelina-1 e função endotelial numa amostra de pacientes pós-menopáusicas. A apresentação desta tese está organizada em três capítulos, conforme o formato sugerido pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, da Universidade Federal do Rio Grande do Sul.

O primeiro capítulo é constituído por um trabalho de revisão (artigo 1) :*Menopause, Estrogens and Endothelial Dysfunction: Current Concepts*. Este artigo enfoca aspectos pertinentes à menopausa e hipoestrogenismo, endotélio e suas funções na homeostase cardiovascular, e modificações na função endotelial na pós-menopausa. Aborda ainda outros temas, como as ações vasculares e extravasculares do estrogênio endógeno, e implicações do uso da terapia hormonal na pós-menopausa. Este manuscrito, já está aceito para publicação no periódico *Clinics*.

Nos próximos dois capítulos foram inseridos os artigos 2 e 3, referentes a dados originais que constituem a parte experimental e discussão desta tese desenvolvida na Unidade de Endocrinologia Ginecológica do Serviço de Endocrinologia do HCPA.

No artigo 2 "*Relationship between endogenous testosterone, endothelin-1 and C-reactive protein in postmenopausal women*" foram incluídas 53 mulheres na pós-menopausa, com idade superior a 40 anos, excluídas diabéticas, com disfunção tireoidiana ou hepática. Neste trabalho, o objetivo foi testar a influência dos níveis de testosterona endógena em marcadores inflamatórios, de função endotelial, e no perfil metabólico e antropométrico.

O artigo 3 "*Free androgen index and endothelial function in postmenopausal women"*, analisa 26 pacientes pós-menopáusicas, não tabagistas ou hipertensas, com idade inferior a 65 anos, que foram selecionadas entre as pacientes do estudo anterior, com o objetivo de determinar uma associação entre o índice de androgênios livres e marcadores vasomotores endoteliais Os exames de avaliação do endotélio venoso, que fizeram parte deste estudo, foram realizados pelo Dr Marcelo Rubira, no Serviço de Cardiologia do Hospital de Clínicas de Porto Alegre.

Estes dois manuscritos serão enviados para publicação em periódicos indexados internacionais.

SUMÁRIO

ARTIGO 1

Menopause, Estrogens and Endothelial Dysfunction: Current Concepts

Maturana MA, Irigoyen MC, Spritzer PM[1]

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Menopause, Estrogens and Endothelial Dysfunction: Current Concepts

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Running title: Menopause, estrogens and endothelial dysfunction

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Abstract

Menopause is defined as the permanent cessation of menses. Cardiovascular disease is the leading cause of death among postmenopausal women in developed countries. The disparity between the incidence of cardiovascular disease among women in pre- and post-menopause has been ascribed to the actions of endogenous estrogen on the cardiovascular system and, particularly, on the vascular endothelium. The endothelium plays an important role in cardiovascular homeostasis, either in the vascular tonus and regulation or in coagulation and inflammatory response. Endothelial dysfunction is implicated in the genesis of atherosclerosis and other chronic disorders, such as diabetes mellitus and hypertension. The pharmacological use of estrogen exerts influence on the circulating levels of vascular tonus, inflammatory, pro-thrombotic and fibrinolytic markers, but the impact of these changes on the atherosclerotic disease is still uncertain.

Keywords: menopause, endothelial function, cardiovascular disease, estrogen, hormone therapy

Resumo

A menopausa é definida como a cessação permanente das menstruações. A doença cardiovascular é a principal causa de mortalidade em mulheres na pós- menopausa, em países desenvolvidos. A disparidade entre a incidência de doença cardiovascular entre mulheres na pré e pós-menopausa tem sido atribuída a ações do estrogênio endógeno sobre o sistema cardiovascular e, em especial, sobre a função do endotélio vascular. O endotélio tem importante papel na homeostase cardiovascular, seja no controle do tônus e permeabilidade vascular, ou da coagulação e resposta inflamatória. A disfunção endotelial está implicada na gênese da aterosclerose e de outras doenças crônicas, como diabete melito e hipertensão arterial. O uso farmacológico de estrogênio exerce influência sobre concentrações circulantes de marcadores do tônus vascular, inflamatórios, pró-trombóticos e fibrinolíticos, porém o impacto destas alterações sobre a doença aterosclerótica ainda não está determinado.

Palavras chave: menopausa, função endotelial, doença cardiovascular, estrogênio, terapia hormonal

Menopause is defined by the World Health Organization as the permanent cessation of menses as a result of the loss of ovarian follicular function or of surgical removal of ovaries. [1] The mean age for occurrence of natural menopause is around 50 years. [1,2]

The management of the menopausal patient has been a matter of great concern in the last decades, both in terms of epidemiology and public health and in medical scientific research. This is largely due to the improved life expectancy, which allows to predict that the female population can live a third of their lifetime after menopause. Demographic studies indicate that in 1990 about 467 million women were at age 50 or over. For year 2030, the estimate is that this group exceeds 1200 million women. [3]

Hormonal changes accompanying menopause, particularly the decreased levels of estrogen hormones, have a great physiological impact. Estrogen deficiency has been associated with vasomotor symptoms, urogenital atrophy, and cognitive impairment, as well as increased risk of chronic degenerative diseases such as osteoporosis and Alzheimer's disease.

Menopause and Cardiovascular Disease

CVD remains as the leading cause of death in the twenty first century. [4] Despite the advances in this area, CVD is still the main cause of death among women in developed countries. In the United States, over half a million women die of CVD every year, exceeding the number of deaths among men and the sum total of the next leading causes of death among women. Coronary arterial disease (CAD) is responsible for most of the deaths by CVD among women .[5] Moreover, women have a less favorable prognosis than men in the presence of coronary event: 40% of the total coronary events in women are deadly, and 67% of sudden deaths occur in women

without history of coronary disease.[5]

The prevalence of CVD in pre-menopausal women is smaller than in post-menopausal ones, when there is an exponential increase, with the female risk equaling the male one at the age of 70 years. [6] This lag concerning the age period at which the frequency of cardiovascular events increases among women as compared to men has been ascribed to the actions of endogenous estrogen on the cardiovascular system, through mechanisms as yet not completely clarified. On the other hand, the influence of hormone therapy (HT) during the menopause on the cardiovascular comorbidities is not well-established. Observational studies showed a reduction of up to 50% in the risk of CVD in postmenopausal women using HT. [7,8,9]

Nevertheless, the results of two large randomized prospective studies refute the cardioprotective effects of hormone therapy evidenced in observational studies. The Heart and Estrogen/Progestin Replacement Study (HERS) was a randomized clinical trial designed to test the effectiveness of HT in the secondary prevention of coronary heart disease. The results indicate an increase in the coronary events within the first year of follow-up after acute myocardial infarction (AMI) among patients treated with a combination of conjugated estrogens/ medroxy progesterone acetate at fixed doses and mean age of 66.7 years.[10] Recently, the Women's Health Initiative (WHI), a prospective study of primary prevention of CVD, was interrupted early because a higher incidence of cardiovascular events was demonstrated among the women randomized for use of HT, than in the group receiving placebo. In this study, although HT was associated with reduced risk of colorectal cancer and bone fracture, it was associated as well with increased risk of breast cancer.[11] Finally, in 2004 the results of the therapeutic arm of the WHI evaluating the isolated use of conjugated estrogens versus placebo among 10,739 hysterectomized post-menopausal women were published. There was increased risk of CVA (rr=1.39 CI: 110-177) but decreased risk of

hip fracture and null effect on the incidence of CVD, as well as potential reduction in the incidence of breast cancer. Overall, the risk-benefit index was neuter. [12]

Many are the well-known risks for CVD, such as systemic hypertension, smoking, obesity, sedentary life style, dyslipedemia, stress, family history of CVD, diabetes mellitus, and insulin resistance. [6] More recently, endothelial vascular dysfunction has been on the agenda for its association with CVD, as detailed below.

The Endothelium

The endothelium is a layer of cells lining all the vessels of the organism and placing itself between the blood and the vascular smooth muscle layer. The endothelial cells form the more exposed surface of the inner vascular layer, presenting selective permeability, of non-thrombogenic character, with exuberant metabolic activity, having also the ability to produce several vasoactive substances. [13]

The main actions exerted by the endothelium can be described as:

- Selective permeability: The endothelium forms a highly selective permeability barrier, regulating the flow of nutrient substances, many biologically active molecules, and blood cells. [14]

- Maintenance of a balance between thrombosis and fibrinolysis: The endothelium normally provides a non-thrombogenic surface due to its ability to form prostaglandin derivatives, especially prostacyclin, which is a potent vasodilator and an effective inhibitor of platelet aggregation.[15] The endothelial cells also secrete agents that are effective for the lysis of coagula, including the plasminogen, but also synthesize pro-coagulant agents such as Von Willebrand factor (vWF). Also, they produce thrombogenic substances such as coagulation factors, adhesion molecules, plasminogen activator inhibitor-1 (PAI-1), and tromboxan A2.[16]

- Inhibition of cell proliferation of vascular smooth muscles, preventing the migration of smooth muscle cells through direct mechanisms, such as synthesis of nitric oxide, which inhibits cell proliferation, and/or indirect ones (platelet action). [17,18]

- Active participation in immune response through the release of factors leading to active contraction, control of plasma extravasation, as well as increase in the expression of integrins, molecules of adhesion and secretion of cytokines, allowing the migration of monocytes, lymphocytes and neutrophils and greater local phagocytic activity.

- Modulation of vascular tonus through the production of numerous vasoactive substances (Table 1). The regulation of the vasomotor tonus is determined by a balance of dilation and constriction. Nitric oxide (NO) is the main mediator of vasomotor tonus in physiological situations. Some stimuli such as the dragging force produced by the pulsatile blood flow, the pressure of blood against the vascular wall, and the shear stress contribute to the basal generation of NO.[19,20,21]

Endothelial Dysfunction

The term endothelial dysfunction is more frequently used to mean reduction in endothelium-dependent vasodilatation, associated with diminished bioactivity of local vasodilating factors, especially NO, but it probably includes such other normal functions of the endothelium as interaction with leukocytes, platelets and regulatory substances. Currently, it is a consensus that endothelial dysfunction is the initial event in atherosclerosis development.[22] Several conditions such as aging, menopause, dyslipedemia, high blood pressure and diabetes mellitus are associated with endothelial dysfunction. [23,24,25,26,27]

Techniques for Evaluation of Endothelial Function

There are many techniques to investigate the endothelium, from those that focus on cell and molecular aspects, through methods involving tissue cultures and molecular biology tools, to the clinical trials applied to human beings, using invasive and non-invasive procedures to evaluate endothelium-dependent vasodilatation, or the determination of plasmatic substances that indicate endothelial activation and damage (Tables 2 and 3).

Intracoronary studies evaluating endothelium-dependent vasodilatation after infusion of acetylcholine are considered gold standard techniques in assessing the endothelial function, but their invasive character precludes their use in large scale. [22] Thus, taking into account that endothelial dysfunction is present at early stages of atherosclerosis, and that it involves several arteries, non-invasive tests in peripheral circulation have been increasingly used. [25] Three techniques are particularly useful to estimate the endothelial dysfunction in peripheral circulation: ultrasonography of brachial artery, impedance pletismography, and dorsal hand vein compliance. Each of these techniques evaluates the endothelial function indirectly by measuring changes to the size of brachial artery, forearm veins, and back of the hand, respectively, after physical (active hyperemia) or pharmacological stimulation (bradykinin or acetylcholine).

Ultrasonography of Brachial Artery

Flow mediated dilatation (FMD) can be measured through high resolution ultrasonography. This technique contrasts the changes to the diameter of the brachial artery in response to the increased flow by reactive hyperemia and sublingual nitrate. Vasodilating responses are expressed as a percentage of change to the size of the vessel (basal and following stimulation). Celermajer et al, in 1994, studied more than 500 healthy individuals using this method and demonstrated negative association

between FMD and a variety of cardiovascular risks, such as smoking, hypertension, age and hypercholesterolemia. [28] Other studies have demonstrated a positive correlation between FMD and central hemodynamic parameters, extension and prognosis of coronary disease. [29,30]

This technique has been used in several studies, but there are limitations related to environmental and individual factors, such as prandial state and arterial size. [31] Also, problems related to reproducibility and intraobserver variation are matters of debate in literature. Rossi et al, 2005, demonstrated variation coefficients of 3.3% and 12.4%, respectively, for basal size and after stimulation, of the brachial artery.[32]

There is as yet no consensus in literature about the parameters of normality for FMD. Ryliskyte et al (2004) analyzed 115 individuals with low cardiovascular risk and found that the only independent predictors of FMD were age and vessel size [33]. These authors thus suggest that in analyzing results, one must take into account normality ranges according to age and the caliber of the tested vessel.

Impedance Pletismography and Dorsal Hand Vein Compliance

Compliance and vein occlusion pletismography uses a sensor of stretching to quantify variations in volume in the forearm or lower limb, taking into account the fact that variations in volume in these parts are dependent on the local blood flow variation. Forearm blood flow (FBF) measurements can be done following ischemic (reactive hyperemia) or pharmacological stimulation, the latter requiring catheterization and drug infusion into brachial artery (invasive). Flow values can be calculated by manual or semi-manual analysis using specific software.

This technique has been validated in several studies as a tool to evaluate the endothelial function: diminished FBF is associated with the increased thickness of the tunica media of the artery, presence of DAC, and risk factors associated with it. [16,34,35]

FBF can also be used to monitor the changes to the endothelial function after interventions on cardiovascular risk factors. [36]

Environmental and physiological variables can influence the magnitude of FBF responses, such as room temperature, age, race, hormonal state, phase of menstrual cycle, anxiety, and prandial state. FBF values variation in a single individual can be about 12.9%, reflecting the influence of these variables. [37,38] To minimize variability the tests must be done in a silent room at stable temperature, and the patient must be fasting.

The dorsal hand vein compliance technique uses measurements of venous diameter variations, obtained through a linear transducer. Venodilatation curves are obtained after infusion of acetylcholine or bradykinin (endothelium-dependent vasodilatation) and nitrate (endothelium-independent vasodilatation) as compared to basal curves [39]. As regards safety, risks and accuracy, it is comparable to impedance pletismography, although it has the advantage of using vasoactive substances at lower doses, thus avoiding potential systemic confounders [22]. Again, the results are influenced by environmental and physiological factors. Greater reproducibility and smaller intra-patient variability of the test are related to pre-constriction of the tested vessel, ideally around 80% of vein constriction in basal state (ED_{80}).[39]

Estrogen, menopause and endothelial function

There is evidence of an association between endothelial dysfunction and reduced endogenous production of estrogens after natural or surgical menopause or premature ovarian failure (POF) in women with or without CAD. [27,40,41,42]

The actions of endogenous estrogens on the cardiovascular system can be mediated directly on the vessels or indirectly through the modulation of cardiovascular risk factors, as well as on the lipid profile (reduction of total cholesterol and LDL,

increase in HDL), as already described more than 20 years ago. [7] More recently, studies have demonstrated as well an antioxidant effect by estrogen, reducing LDL oxidation *in vivo* and *in vitro*.[43]

The direct effects of estrogen on the vascular system and which modulate the vascular tonus comprise 1) acute vasodilatation, increasing the synthesis and bioactivity of nitric oxide; [44,45] 2) long term modulation of vascular tonus, regulating the production of prostaglandins and expression of eNOS and the endothelin gene[46]; 3) inhibition of endothelin-induced vasoconstriction[47]; and 4) inhibition of sympathetic activity [27].

In addition to these actions on the vascular tonus, estrogen exerts an antiproliferative action on the vascular smooth layer. [48] Also, it appears to have a major role in vascular remodeling, inhibiting the proliferation of the inner layer after injury [49] and increasing the expression of contractile proteins in the myocardium. [50]

In other tissues, such as the liver, estrogen can mediate both beneficial (expression of genes of apoproteins that improve the lipid profile) and adverse effects (increase in the expression of pro-coagulant factors and decrease of fibrinolytic factors). [51]

From the clinical point of view, our group has recently reported, in post-menopausal women who were not on hormone therapy, a positive association between nitric oxide and free estradiol level, confirming the influence of this steroid on the endothelial function. [52]

On the other hand, androgen and sex hormone binding globulin (SHBG) levels have been associated with risk of CVD in pre- and postmenopausal women. [53,54] The increase in circulating androgens appears to be associated with insulin resistance and a predictor of diabetes mellitus. [55,56,57] In a previous study, we demonstrated a positive association, independent of obesity, between testosterone levels and hyperinsulinemia

in post-menopausal women with no clinical evidence of CVD. [58]

Hormone Therapy and Endothelial Function

Disturbances in the endothelial function have an important role in the physiopathology of atherosclerosis, and several lines of evidence suggest that interventions in the endothelial function could modify the progress rates of atherosclerotic disease and the risk of cardiovascular events. A number of studies in the literature have tested the impact of the use of HT on the endothelial function using inflammatory, fibrinolytic/thrombogenic markers and functional methods. [40,41,59,60,61]

Results from the main randomized studies, HERS 2 and WHI, indicate increased risk of venous thromboembolism with the use of HT. [8,62] It is important to highlight that these studies were criticized in at least two respects: patient selection, which included women in average 10 years older than the age at which HT is usually recommended (WHI), and the use in both studies of a regimen of HT with drugs, administration route and fixed doses.

The administration route of HT appears to affect the fibrinolytic activity and the coagulation markers. The oral route for estrogen therapy is associated with changes in the levels of coagulation and fibrinolysis markers, especially at the early period of use. [63,64,65] Studies using the estrogen therapy by transdermal route, however, did not confirm these findings. [66,67] Moreover, Scarabin et al (2003), in a multicenter case-control study, evaluated 155 post-menopausal women hospitalized because of venous thromboembolism (VTE) and demonstrated increased risk for VTE with oral, but not with transdermal, therapy. [68] Our group showed, in postmenopausal patients, that reduction of antithrombin III, usually seen with oral estrogen therapy, did not occur with the use of estradiol-17β in the form of' percutaneous gel both alone [69] or in association with micronized progesterone [71] (Figure 1). Plasma rennin activity was also similar

before and during this non-oral hormone therapy (Figure 1). These findings have also shown that progesterone does not appreciably attenuate estradiol-induced beneficial effects.

In addition, we have recently shown that, in a sample of hypertensive post-menopausal women, the association of non-oral estradiol-17b and low doses of vaginal micronized progesterone for one year were both effective on climacteric symptoms and safe on maintaining blood pressure control and preventing endometrial hyperplasia .[70,71] Moreover, in those patients, serial echocardiograph scans showed no change in left ventricle mass, but a significant reduction in the thickness of the left ventricle posterior wall was observed [71] (Table 4).

On the other hand, a number of studies have been calling attention to the effect of HT in endothelial inflammatory markers, such as decreased expression of adhesion molecules like ICAM-1, VICAM-1 and E-selectin. [72,73,74]

Studies using the oral route have evidenced an increase in the circulating levels of C-reactive protein (CRP), a well-known inflammatory marker. [73,74,75] However, the clinical significance of these results has not been totally clarified. Other trials with non-oral routes reported, on the contrary, stability in CRP levels during HT. [73,76]

Acute non-oral use of estradiol may increase endothelium-dependent vasodilatation. [77,78] Saitta et al. 2001 compared the effects of using 17-β estradiol+ norethisterone, raloxifen or placebo for 6 months in postmenopausal patients [79] . Treated women, in both groups, showed significant increase in endothelium-dependent vasodilation measured by ultrasonography of brachial artery and in the plasmatic nitrite/nitrate levels, not observed in placebo users. A significant increase in the NO metabolites levels has also been shown by Balci et al (2005) with the use of transdermal 17β estradiol, 100 μg/week for 3 months.[80]

Although there is some evidence towards a favorable action of HT on the vasomotor endothelial function, the long-term benefits on the natural history of atherosclerotic disease are yet unpredictable. Ceballos et al, 2000, showed a significant increase in endothelium-dependent vasodilation in menopausal patients treated with a combination of transdermal 17β estradiol and micronized progesterone, but this benefit was lost after 6 months of drug discontinuation. [81]

Few studies in literature have boarded the effect of progestogens on cardiovascular risk and despite well-recognized benefits of estrogens, controversy surrounds the risks and negative aspects of combined estrogen and progestogen use in HT.

The vascular actions of progestins and progesterone are mediated by progesterone receptors, expressed in endothelial cells and the vascular smooth muscle as well as through down-regulation of the estradiol receptor. [45] Concerning progesterone, evidences suggest that the natural molecule facilitates the inhibitory effects of estrogen on vascular smooth muscle proliferation [82] and may induce endothelium-dependent vascular relaxation. [45,77] In addition, natural progesterone used in HT appears to preserve the beneficial actions of estrogen. [8,70,71]

In turn, progestins present different pharmacological profiles according to their molecular structure, dosage and presence of comorbidities. As with estrogens, the various progestins used in HT may differ significantly as to how closely they mimic their natural counterparts. For instance, progestin molecules with androgenic properties may antagonize estrogen-dependent beneficial effects on lipids [83] and a new molecule with antimineralocorticoid activity may reduce blood pressure in postmenopausal women with hypertension. [84]

Progestins added to estrogen therapy seem to increase inflammatory markers. [72] In addition, medroxyprogesterone acetate associated to conjugated equine

estrogens produce no effects [85] or inhibit endothelium-dependent vasodilatation stimulated by estrogens. [86] These observations have been considered to explain, at least in part, the adverse results observed on the large prospective, randomized, placebo-controlled trials of combined HT: WHI and HERS studies. However, the extent to which findings of these studies of medroxyprogesterone acetate and conjugated equine estrogens apply to other HT formulations is unclear at present.

Endothelial Dysfunction and CVD Predictors in Menopause

Data from prospective trials have been confirming the hypothesis that endothelial dysfunction precedes the emergence of chronic disorders. The MONICA/KORA study (Monitoring of Trends and Determinants in Cardiovascular Disease/Cooperative Research in the Region of Augsburg), which involved more than 2,000 patients, found an association between increased levels of E-selectin and I-CAM and increased risk of Diabetes Mellitus Type 2 (DM-2). [87] Rossi et al (2005), in a follow-up study of 840 postmenopausal women, showed an adjusted relative risk for DM-2 of 5.87 (confidence interval of 95%:4.34-8.10) in patients with the lower tertile of FMD ($\leq$4.3).[32]

In addition to effects on endothelial markers, changes in other factors of cardiovascular risk have been associated with the menopausal transition, such as the lipid profile [88], weight, and body fat distribution. [89,90] The association between prevalence of cardiovascular risk during peri-menopause and post-menopause and intimal-medial thickness (IMT) of the carotid was monitored in 314 women by Matthews et al (2001).[88] In pre-menopausal patients, arterial and pulse pressure, LDL, HDL, triglycerides, and BMI values were predictors of TMT and the presence of atherosclerotic plaque, after 5 years of menses cessation. In post-menopausal patients, only the increase in pulse pressure was a predictor of IMT.[91] These findings

support the notion that women at higher risk of CVD can be identified during pre-menopause, and it is in this period that strategies must be implemented to prevent the development of atherosclerosis in the postmenopausal years.

Endothelium dysfunction can be curtailed by non-pharmacological measures, such as physical activity[92] and weight loss[93], or pharmacological ones, such as statins and angiotensin converting enzyme (ACE). [94.95] The indication of HT use must be individualized, taking into account the presence of climacteric symptoms and their impact on inflammatory and coagulation markers, since long-term benefits on atherosclerotic vascular disease have not yet been determined.

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Legend of Figure 1

Renin activity and antithrombin III in 20 postmenopausal women with mild to moderate hypertension, before and during 1 year of non-oral natural estradiol and low dose of micronized progesterone.

Table 1. Endothelial-derived vasoactive factors [21]

Vasodilator Factors	Vasoconstrictor Factors
Nitric oxide (NO)	Endothelin (ET)
Endothelium-derived hyperpolarizing factor (EDHF)	Prostanoids (PGH_2, TXA_2, O_2)
Prostacyclin (PGI_2)	Angiotensin (AII)
Acetylcholine	
Bradykinin	

Table 2. Markers of Endothelial function [24,91]

Endothelial Function	Marker
Coagulation	Fibrinogen
	vWF
	TXA_2
Fibrinolysis	t-PA
	PAI-1
Inflammation	CRP
	E-selectin
	fibrinogen
	ICAMs and VICAMs
	IL-6
Vascular Tonus	Plasma Markers
	ET-1
	NO

vWF: von Willebrand factor; $TXA_{2;}$ tromboxan; $A_{2;}$ tPA: tissue plasminogen activator; PAI-1: plasminogen activation inhibitor-1; CRP: C-reactive protein; ICAMs: intercellular adhesion molecules; VICAMs: vascular cell adhesion molecules; IL-6: interleukin 6; ET-1: endothelin-1; NO: Nitric Oxide

Table 3. Functional Tests for endothelial-dependent vasodilation evaluation [24, 90]

	Functional Tests
Invasive	Coronary angiography
	Pletismography
Non Invasive	Positron emission tomography
	FMD
	Brachial artery ultra-son
	Pletismography and dorsal hand vein compliance method

FMD: flow-mediated vasodilation

Table 4. Echocardiographic variables in 20 hypertensive post-menopausal women [71]

	Before HT	6m	12m	P
Left ventricle posterior wall (mm)	9.1 ±0.4	8.3 ± 0.3	8.0 ± 0.2	0.042
Ejection fraction (%)	71.3 ± 1.3	67.9 ± 1.2	69.4 ± 1.5	NS
Interventricular septum (mm)	9.2 ± 0.4	8.9 ± 0.4	8.2 ±0.2	NS

Blood pressure control was achieved by administration of amlodipine at individually adjusted doses. Hormone therapy was introduced in a cyclic regimen (21 of 28 days) with percutaneous estradiol (1.5 mg/day) and vaginal micronized progesterone (100 mg/day).

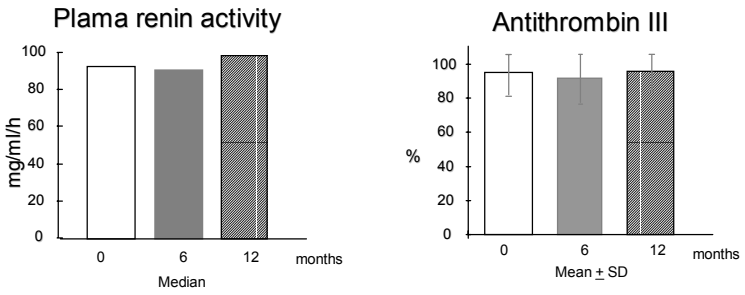
Plama renin activity
100
80
60
40
20
0
mg/ml/h
0
6
12
months
Median
Antithrombin III
100
80
60
40
20
0
%
0
6
12
months
Mean ± SD

Figure 1

ARTIGO 2

Relationship between endogenous testosterone, endothelin-1 and C-reactive protein in postmenopausal women

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Short title: ET-1, CRP and testosterone

Key words: menopause, androgens, endothelin-1, C - reactive protein, cardiovascular risk factors, endothelial dysfunction

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ABSTRACT

Objective: To verify whether endogenous testosterone levels are correlated with endothelial dysfunction markers and metabolic profile in 53 postmenopausal women.

Methods: Total testosterone (TT), endothelin-1 (ET-1), C-reactive protein (CRP) and metabolic and haemostatic variables were determined.

Results: Mean age was 55 (±5) years. Median time since menopause was 5.5 (3-8) years. Body mass index and waist circumference were significantly higher in women with TT ≥ 0.49 ng/mL (group mean) than in women with TT ≤ 0.49 ng/mL ($p < 0.005$). Median CRP levels were greater in women with higher TT [1.170 (0.175-2.360) *versus* 0.175 (0.175-0.610 mg/L, p= 0.039]. Median ET-1 levels were also higher in women with TT ≥ 0.49 ng/mL [0.84 (0.81-0.97) *versus* 0.81 (0.74-0.84) pg/mL, p=0.012]. TT was associated with CRP (r=0.466, p=0.008) and ET-1 (r=0.365, p=0.024). These correlations were independent of time since menopause and central obesity.

Conclusions: A positive association was observed between testosterone and endothelial dysfunction markers. Endogenous testosterone in recently postmenopausal women, even within normal limits, may be part of a proatherogenic profile. Longitudinal studies are needed to determine if androgenicity represents a risk factor for cardiovascular disease and to establish the clinical relevance of its association with ET-1 and CRP in this population.

INTRODUCTION

Although much progress has been made in the prevention and treatment of cardiovascular disease (CVD), it is still the leading cause of death among postmenopausal women in developed countries.[1] Changes in endothelial function play an important role in the pathophysiology of atherosclerosis,[2] and there is evidence suggesting that interventions to improve endothelial function may impact the progression and the risk of cardiovascular events.[2-5]

Circulating inflammatory markers are regarded as manifestations of endothelial dysfunction and have also been linked to CVD. C-reactive protein (CRP), a reliable and easily measured marker of inflammation, has been described as a predictor of cardiovascular events in postmenopausal women.[6-8] Another inflammation marker, endothelin 1 (ET-1), a peptide isolated from endothelial cells, presents a powerful vasoconstrictor action. Increased levels of ET-1 have been observed in states of insulin resistance and in early endothelial dysfunction.[9] Also, a positive association between ET-1 levels and androgenicity has been described in women with polycystic ovary syndrome (PCOS), suggesting a correlation with the early-onset endothelial dysfunction found in these patients.[10]

In turn, endogenous androgens are thought to be potential mediators of CV risk in women at midlife, in addition to having been associated with CRP [15-16]. Therefore, the aim of the present study was to investigate the relationship between testosterone, CRP and ET-1 levels and the metabolic profile in a group of postmenopausal women.

SUBJECTS AND METHODS

Subjects

The study was carried out with women consulting for climacteric symptoms at the Gynecological Endocrinology Unit at Hospital de Clínicas de Porto Alegre, Brazil. Fifty-three postmenopausal women fulfilling all the inclusion criteria were consecutively enrolled in the study. Inclusion criteria were as follows: 1) menopause, defined as last menstrual period at least 1 year before the beginning of the study plus follicle stimulating hormone (FSH) levels higher than 35 IU/L; 2) more than 40 years of age; 3) no use of any medication known to interfere with hormonal, glucose or lipoprotein levels in the past 3 months and 4) no use of steroidal or nonsteroidal anti-inflammatory drugs in the last 15 days. Diabetic patients or patients with thyroid, hepatic or renal dysfunction were excluded. Five patients were smokers. The study protocol was approved by the local Ethics Committee, and written, informed consent was obtained from every subject.

Study protocol

Anthropometric measurements included body weight, height, waist circumference (waist measured at the midpoint between the lower rib margin and the iliac crest), hip circumference (recorded at the level of the greater trochanter), waist-to-hip ratio (WHR), and body mass index (BMI, current measured weight in kg divided by height in m^2). Blood pressure was measured in the supine position after a 10-minute rest. The same calibrated mercury manometer attached to a 12.5 x 23 cm inflatable cuff was used in all patients by the same operator, who adopted the 5th Korotkoff sound to determine diastolic pressure. Hypertension was defined as systolic blood pressure $\geq$ 140 mmHg, diastolic blood pressure $\geq$ 90 mmHg, or current use of antihypertensive drugs.[11]

After the patients were submitted to a 3-day 300 g carbohydrate diet, two blood samples were drawn from an antecubital vein for determination of plasma glucose and insulin: one after overnight fasting, and another 2 hours after the ingestion of 75 g of glucose. FSH, luteinizing hormone (LH), estradiol, total testosterone (TT), sex hormone binding globulin (SHBG), dehydroepiandrosterone sulphate (DHEAS), fibrinogen, ET-1, CRP, total and HDL-cholesterol and triglycerides were also determined using the fasting blood sample. All samples were obtained between 8 and 10 a.m. Free androgen index (FAI) and homeostasis model assessment (HOMA) index were calculated as previously reported.[12]

Assays

Total cholesterol, HDL-cholesterol, triglycerides, and glucose were determined by colorimetric-enzymatic methods using the Bayer 1650 Advia System. LDL cholesterol was determined indirectly using the formula LDL = total cholesterol - HDL - triglycerides / 5. Serum LH and FSH were measured by electrochemiluminescence immunoassay (ECLIA), with intra and interassay coefficients of variation (CV) of 1.8% and 4.8%, respectively, for LH, and 1.8% and 3.3% for FSH. The sensitivity of the assays was 0.12 IU/L for LH and 0.05 IU/L for FSH. TT levels were measured with the RIA method (ICM, Costa Mesa, CA) with an assay sensitivity < 0.2 ng/mL and intra and interassay CV of 10% and 11.3%, respectively. Free testosterone index was estimated by dividing TT (nmol/L) by SHBG (nmol/L) x 100. Estradiol was measured by ECLIA (Roche Diagnostics, Mannheim, Germany), with an assay sensitivity of 5.0 pg/mL and intra and interassay CV of 5.7% and 6.4%. SHBG was measured by chemoluminescence enzyme immunoassay (DPC, Los Angeles, CA), with an assay sensitivity of 0.2 nmol/L and intra and interassay CV of 6.1% and 8.0%, respectively. Serum insulin levels were measured using ECLIA (Roche Diagnostics, Mannheim, Germany), with sensitivity of 0.200 μIU/ml and intra and interassay CV of 2.0% and

4.3%, respectively. SDHEA was measured by ECLIA (Roche Diagnostics, Mannheim, Germany), with sensitivity of 0.10 μg/dL and intra and interassay CV of 2.8% and 6.5%, respectively. Fibrinogen was measured by the coagulometric method (Diagnostica Stago, Asnières, France), with sensitivity of 4 s and intra and interassay CV of 3.3% and 10.0%, respectively. CRP was assayed using stored specimens, with a validated high-sensitivity nephelometric method (Dade Behring Marburg, Marburg, Germany). Sensitivity was 0.175 mg/L and intra and interassay CV were 4.4% and 5.7%, respectively. For data analysis, individual results below the limit of sensitivity were considered as equal to 0.175 mg/L. ET-1 was assayed using a luminoimmunoassay (R&D Systems) in stored EDTA plasma samples, with sensitivity of 0.5 pg/mL in our laboratory, and intra and interassay CV of 4.6% and 6.5%, respectively.

Statistical analysis

Results are expressed as means ± SD or median and interquartile range. Comparisons between the two group means were analyzed by Student's t-test; comparisons between median values were analyzed with Mann-Whitney's U test. Spearman's rank or Pearson's correlation coefficient were calculated between variables using a two-tailed significance test for variables with a Gaussian or non-Gaussian distribution, respectively. Partial correlations of TT with ET-1 and CRP were calculated (adjusted for waist circumference and time since menopause).

Comparisons between ratios were carried out using the chi-square test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA). Data were considered to be significant at $p<0.05$. Calculation of the sample size was based on the higher ET-1 levels observed in the pilot study in women with circulating TT ≥ 0.49 pg/mL. The sample had a power of 90%, with a significance level of 0.05.

RESULTS

The mean age of participants was of 55 (± 5) years, the age at menopause was 48 (± 3) years, and the median time since menopause was 5.5 (3-8) years. Table 1 presents the anthropometric, metabolic and hormonal profile of participants, showing that total and LDL cholesterol levels were slightly higher than reference levels. Metabolic syndrome as defined by NCEP-ATP III criteria[13] was diagnosed in eight patients (14.3%). Twelve (21%) had hypertension and 17 (34%) presented impaired glucose tolerance.

Table 2 presents metabolic and haemostatic variables and markers of inflammation and endothelial function stratified by testosterone levels in the two groups. The distribution of metabolic and haemostatic variables and markers of inflammation and endothelial function was analyzed considering TT levels in relation to 0.49 ng/mL, which was the mean value for this group (TT < 0.49 ng/mL or ≥ 0.49 ng/mL) (Table 2). No differences were found in terms of age and time since menopause between the groups. Lipid levels were also similar in both groups. The group with TT ≥0.49 ng/mL had greater BMI and waist circumference than the group with TT <0.49 ng/mL. While fibrinogen was similar, CRP and ET-1 were significantly higher in the group with TT ≥ 0.49 ng/mL.

Positive correlations were observed between TT levels and waist circumference ($r=0.516$, $p<0.001$), systolic ($r=0.475$, $p=0.001$) and diastolic pressure ($r=0.334$, $p=0.019$). TT was associated with CRP and ET-1. These correlations were independent of time since menopause and central obesity as adjusted by waist circumference by waist circumference (Table 3).

DISCUSSION

In the present study, a significant association between endogenous testosterone and ET-1 was found in postmenopausal women. To our knowledge, this is the first time this association is described in recently postmenopausal women presenting hypertension without clinical evidence of cardiovascular disease. The pro-inflammatory marker CRP was also found to be associated with testosterone levels in this population.

Evidence suggests that sex hormones may modulate plasma ET-1 levels. Webb et al.[14] found that 17β- estradiol decreased ET-1 levels in the coronary circulation of postmenopausal women. More recently, Silvestri et al.[15] also showed a reduction of ET-1 levels in postmenopausal women under oral hormone therapy. In turn, high levels of ET-1 were described by Polderman et al.[16] in a study with female-to-male transsexuals treated with testosterone.

Orio et al.[17] have recently described early impairment of endothelial function in young normal-weight PCOS patients without metabolic or cardiovascular disease. The patients presented a significant increase in carotid intima-media wall thickness, a decrease in flow-mediated dilation and increased ET-1 levels.[17] Moreover, the use of the insulin sensitizer metformin in patients with PCOS has been shown to promote overall improvement in androgenic profile, insulin resistance and ET-1 levels, without requiring concomitant changes in body weight.[18] The present study shows that the positive association between endogenous levels of testosterone and ET-1 is independent of central adiposity. Together with the studies by Orio et al.[17,18] this suggests that the association between androgens and ET-1 levels is explained, at least in part, by an obesity-independent mechanism. Further studies are needed to specifically determine the role of metabolic variables in this association.

Fibrinogen has been suggested as an independent risk factor for cardiovascular disease. Framingham Study data indicate that increases in fibrinogen impose an independent increment on cardiovascular risk in both sexes.[19] Additionally, androgens have been associated with some haemostatic factors,[20] but there is controversy concerning the relationship between androgens and fibrinogen.[21,22] In our study, fibrinogen levels were similar in the two groups stratified by testosterone levels. Taken together, these data support the notion that fibrinogen is more related to BMI[23] and lipoprotein levels than to menopausal status,[24] at least when postmenopausal women without clinical cardiovascular disease are considered.

In the present study, TT was independently associated with CRP. CRP is considered an independent predictor of CVD in both males and postmenopausal women.[8,25] Our data confirm the results of a recently published cross-sectional study in which CRP was negatively associated with SHBG and positively associated with bioavailable testosterone after adjustment for age, BMI, physical activity, alcohol consumption and tobacco use.[26] Folsom et al.,[22] analyzing a sub-sample of the ARIC study (n=57), showed that, after adjustment for age, race, and case-control status, mean CRP was 2-fold greater in the highest vs. lowest quartiles of estrone and androstenedione, and CRP was 2-fold lower across quartiles of SHBG. However, because of the sample size, not all these associations reached statistical significance.[22]

Conversely, a few studies disconfirm the association between androgenicity and CRP in specific postmenopausal sub-populations such as older patients referred to coronary angiography, stratified by the presence or absence of coronary artery disease.[27] Joffe et al.[28] found CRP to be negatively and independently correlated with SHBG and testosterone in menopausal women who subsequently developed clinical CVD, but the negative correlation between CRP and testosterone was not present in those who remained CVD-free. However, the patients in that study were older than our patients and around 20% were smokers. In addition, a positive association between

CRP and free androgen index was found when the entire group of women not using hormonal therapy was analyzed. Therefore, it is possible that the association between testosterone and CRP is not linear across the range of CRP values, appearing only with lower CRP levels, as suggested by Crandall et al.[26]

While time since menopause was not controlled in the present study, the patients included were in the early years of menopause, with no clinical evidence of disease but light hypertension in 22% of the sample. Along with the low levels of CRP, this profile may also partially explain the low prevalence of metabolic disturbances such as insulin resistance and dyslipidemia. In a previous study we found an association between insulin resistance and androgenicity.[29] However, in that study patients were older and more obese than those in the present study.

In conclusion, the present results suggest that testosterone levels in recently postmenopausal women, even within normal limits, may indicate a proatherogenic profile. Longitudinal studies are needed to determine if androgenicity represents a risk factor for cardiovascular disease and to establish the clinical relevance of the association between testosterone, ET-1 and CRP in postmenopausal women.

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Table 1. Baseline characteristics of postmenopausal study participants (n=53)

	Mean	**SD**
Age (yr)	55	5
Age at menopause (yr)	48	3
Time since menopause (yr)	5.5	(3-8)*
BMI (kg/m^2)	27	4
Waist circumference (cm)	88	10
Estradiol (pg/mL)	13	7
Testosterone (pg/mL)	0.49	0.23
FAI *	2.9	(2.2-4.6)
Fasting glucose (mg/dL)	93	10
Total cholesterol (mg/dL)	220	37
HDL-C (mg/dL)	57	10
LDL-C (mg/dL)	141	31
Triglycerides (mg/dL)	113	47
Fasting insulin (μIU/mL)	7.6	3
FSH (mIU/mL)	85	33

*Median (and interquartile range); BMI: body mass index.

Table 2. Distribution of metabolic, haemostatic, inflammatory and vasomotor markers by total testosterone levels

TT levels (n)	**< 0.49 (n=32)**	**≥ 0.49 (n=21)**	**P**
Age (yr)	55 ± 5	54 ± 5	0.846
Time since menopause*	6 (3-8)	5 (3-9)	0.908
Testosterone (pg/mL)	0.34 ± 0.08	0.69 ± 0.18	<0.001
FAI*	2.5 (1,5-3.2)	5.3 (3.39-6.8)	<0.001
HOMA	1.7 ± 0.8	2.7 ± 1.0	0.499
SHBG (nmol/L)*	53 (38-76)	44 (31-73)	0.273
BMI (kg/m^2)	25 ±3	27 ± 2	0.005
Waist circumference (cm)	84 ± 7.9	92 ± 7	0.002
Hypertension (%)†	5 (15)	7 (34)	0.136
Fibrinogen (mg/dl)	323± 122	350 ± 134	0.469
CRP (mg/L)*	0.175 (0.175-0.610)	1.170 (0.175-2.360)	0.039
Endothelin-1 (pg/mL)*	0.81 (0.74-0.84)	0.84 (0.81-0.97)	0.023

TT: total testosterone; BMI: body mass index; CRP: C-reactive protein
Student T test or [a]Mann-Whitney U test (median IQR:25-75%); [b]Chi-Square test

Table 3. Cross-sectional association of total testosterone with ET-1 and CRP levels, adjusted by waist circumference and time since menopause

	Testosterone (pg/mL)	P
ET-1(pg/mL)	0.365	0.024
CRP (us)(mg/L)	0.466	0.008

ARTIGO 3

Free androgen index and endothelial function in postmenopausal women

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Free androgen index and endothelial function in postmenopausal women

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Running title: FAI and endothelial function in menopause

ABSTRACT

Objective: To assess the influence of free androgen index on endothelial function in postmenopausal women.

Design: Cross-sectional study. 26 postmenopausal women were submitted to dorsal hand vein compliance technique. Acetylcholine (Ach) and sodium nitroprusside (NPS) dose-response curves were constructed to test the endothelium-dependent and –independent relaxation, respectively. Patients were stratified in 2 groups according to free androgen index (FAI).

Results: Mean age was 54 years (±4) and median time of menopause was 6 years (interquartile range: 3-9). Waist-to-hip ratio was significantly higher in the group with FAI > 2.5. Maximum vasodilatation (VD) with Ach and with NPS was similar across the groups stratified by FAI. The median dose of Ach for maximum vasodilatation was higher in the FAI group > 2.5 (36 (0.36-360) ng/mim) than in the group of FAI ≤ 2.5 (720 (360- 3600) ng/mim) p=0.005. Positive correlations were observed between Ach doses for maximum VD and FAI (r=0.473, p=0.015), waist (r=0.510, p= 0,011), and waist-to-hip ratio (r=0.479, p= 0.021). Sex hormone binding globulin (SHBG) was negatively correlated with Ach doses (rs= - 0.400 p=0.043).

Conclusion: The results from this study suggest that FAI, even if still in the normal limits, is related to early changes on endothelial function in recently postmenopausal, healthy women. Longitudinal studies are needed to evaluate the clinical relevance of these findings.

Key words: menopause, androgens, vasodilation, vascular endothelium, dorsal hand vein

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death among men and postmenopausal women in underdeveloped (Mansur et al., 2006) and developed countries (Stramba-Badiale et al., 2006; AHA, 2004). Women develop heart disease later in life than men and this gender disparity in cardiovascular disease has been interpreted primarily as reflecting estrogen-mediated protection against atherogenesis (Mendelsohn, 2005).

Recent studies show that androgens can influence cardiovascular risk and vascular function differently according to the gender disparity. In men, testosterone is inversely correlated with cardiovascular risk factors, such as arterial wall thickness (Muller et al., 2004), blood pressure (Svartberg et al., 2004), obesity and metabolic syndrome (Chen et al., 2006), and presence and extension of coronary disease (Dobrzycki et al., 2003). In women, androgen levels are positively correlated with risk factors of CVD in menacme (Coviello et al., 2006; Diamanti-Kandarakis et al., 2006; Sowers et al., 2005), and in postmenopausal women (Golden et al., 2004; Maturana & Spritzer, 2002; Phillips et al., 1997)

Endothelial dysfunction is one of the early signs of cardiovascular damage (Mombouli et al., 1999). In women with hiperandrogenic syndromes, markers of endothelial dysfunction are present, even in the absence of other cardiovascular risk factors (Orio et al, 2004). In postmenopausal women, androgens and SHBG are related to inflammatory endothelial markers (Sutton-Tyrrel et al., 2005; Crandall et al., 2006). Therefore, the aim of the present study was to assess the influence of free androgen index on endothelial function in apparently healthy postmenopausal women.

SUBJECTS and METHODS

Subjects

The study was carried out whith women consulting for climateric symptoms at Gynecological Endocrinology Unit at the Hospital de Clínicas de Porto Alegre, Brazil. Twenty-six postmenopausal women were enrolled in the study. Inclusion criteria were as follows: 1) menopause, defined as last menstrual period at least 1 year before the beginning of the study plus FSH levels higher than 35 IU/L; 2) Age between 40 and 65 years; 3) no use of any medication known to interfere with hormonal, glucose or lipoprotein levels in the past 3 months and 4) no use of steroidal or nonsteroidal anti-inflammatory drugs in the last 15 days. Smokers or patients with high blood pressure, diabetes or thyroid, hepatic or renal dysfunction were excluded. The study protocol was approved by the local Ethical Committee, and written, informed consent was obtained from every subject.

Study protocol

Anthropometric measurements included body weight, height, waist circumference, hip circumference (recorded at the level of the greater trochanter), waist-to-hip ratio (WHR), and BMI (current measured weight in kg divided by height in m^2). Blood pressure was measured after a 10-minute rest, with the woman in the sit position. The same calibrated mercury manometer attached to a 12.5 x 23 cm inflatable cuff was used in all patients by the same operator, who adopted the 5th Korotkoff sound to determine diastolic pressure. Hypertension was defined as systolic blood pressure $\geq$ 140 mmHg, diastolic blood pressure $\geq$ 90 mmHg, or current use of antihypertensive drugs (The JNC 7 Report, 2003).

After the patients were submitted to a 3-day 300 g carbohydrate diet, two blood samples were drawn from an antecubital vein for determination of serum glucose and insulin: one after overnight fasting, and then again 2 hours after the ingestion of 75 g of glucose. All samples were obtained between 8 and 10 a.m. The free androgen index (FAI) and the HOMA index were calculated as described elsewhere (Frajndlich & Spritzer, 2005). Patients were stratified by free androgen index (FAI) using as the cutoff the median FAI obtained in the whole sample of participants.

Assays

Total cholesterol, HDL-cholesterol, triglycerides, and glucose were determined by colorimetric-enzymatic methods using the Bayer 1650 Advia System. LDL cholesterol was determined indirectly using the formula LDL = total cholesterol - HDL - triglycerides / 5. Serum LH and FSH were measured by Electrochemoluminescence Immunoassay (ECLIA), with intra and inter-assay coefficients of variation (CV) of 1.8% and 4.8%, respectively for LH, and 1.8% and 3.3% for FSH. The sensitivity of the assays was 0.12 IU/L for LH and 0.05 IU/L for FSH. TT levels were measured with the RIA method (ICM, Costa Mesa, CA) with an assay sensitivity < 0.2 ng/mL and intra and inter-assay CV of 10% and 11.3%, respectively. Free testosterone index was estimated by dividing TT (nmol/L) by SHBG (nmol/L) x 100. SHBG was measured by chemoluminescence enzyme immunoassay (DPC, Los Angeles, CA), with an assay sensitivity of 0.2 nmol/L and intra and inter-assay CV of 6.1% and 8.0%, respectively. Serum insulin levels were measured using ECLIA (Roche Diagnostics, Mannheim, Germany), with sensitivity of 0.200 μIU/ml and intra and inter-assay CV of 2.0% and 4.3%, respectively.

Dorsal hand vein technique

The dorsal hand vein technique, as used in our laboratory, has been described elsewhere (Sabha et al., 1990). Briefly, a 23 G butterfly needle was inserted into a suitable vein on the back of the hand, with the arm positioned at an upward angle of 30° to allow the complete emptying of the veins. A tripod, holding a linear variable differential transformer (LVDT, Schaevitz Engineering Pennsauken, New Jersey, USA) was mounted on the back of the hand with its central aperture, containing a movable metal core at a distance of 10 mm downstream from the tip of the needle. The signal output of the LVDT, which is linearly proportional to the vertical movement of the core, gave a measurement of the diameter of vein. Readings were taken under a congestive pressure of 40 mm Hg by inflating a blood pressure cuff placed on the upper portion of the arm under study. Results were presented as normalized dose-response curves in which the diameter of the vein during saline infusion is defined as 100% dilation. The vein was pre-constricted to 70% of baseline size by infusing increasing doses of phenylephrine (Winthrop Lab, New York,USA;doses: 12-3166 ng/min). This dose rate of phenylephrine was defined as the ED 70 dose and this degree of constriction was defined as 0% dilation for the purposes of subsequent calculations. The vasodilation effects expressed in this study were calculated as a percentage in the range between 0% and 100% dilation. Drugs were infused using a Harvard infusion pump (Harvard Apparatus, South Natick, MA, USA) at a flow rate of 0.3 mL/min. Blood pressure and heart rate were monitored in the opposite arm with a Dynamap Bllod Pressure Monitor (Critikon, Tampa, FL, USA). After pre-constriction of the vein by phenylephrine, acetylcoline (Ach: 0.36- 3.600 ng/min) (Divisão de Farmácia da Universidade de São Paulo, Brazil) and/or sodium nitroprusside (SNP) (Biolab Sanus Ltda, Brazil) were administered with 5 ng/mim infusion rates, to obtain endothelium dependent or

independent mediated responses, respectively. Infusions at each rate lasted for 5 min with the sphygnomanometer cuff inflated to 45 mmHg for the last 2 min of the infusion.

Statistical analysis

Results are expressed as means ± SD or median and interquartile range. Comparisons between the two group means were analyzed by Student's t-test; comparisons between median values were analyzed with Mann-Whitney's U test. Spearman's rank or Pearson's correlation coefficients were calculated between variables using a two-tailed significance test for variables with a Gaussian or non-Gaussian distribution, respectively. Analyses were performed using the Statistical Package for Social Sciences (SPSS, Chicago, IL, USA). Data were considered to be significant at $p<0.05$.

RESULTS

The mean age of participants was 54 ± 4 years, the age of menopause was 48 ± 3 years, and median time after menopause was 6 (3-9) years. Mean systolic pressure was 123 ±8 mmHg and mean diastolic pressure was 80 ± 6 mmHg. The mean fasting glucose was 94± 10 mg/dl and HDL-C was 57 ± 10 mg/dl. Mean testosterone levels were 0.41 ±0.21 ng/mL and median SHBG levels were 54 (34-78) nmol/L.

Table 1 presents antrophometric, metabolic and vasodilatation variables related to endothelial function in the two groups stratified by FAI median. While waist and metabolic variables were similar in the two groups, WHR was higher in the group with FAI > 2.5. Ach induced maximum flow-dilatation did not differ between the groups, but women with FAI > 2.5 needed median doses of acetylcholine that were 20 times greater than those used by the group with FAI < 2.5 to obtain a similar venodilation in response to Ach infusion.

A positive correlation was found between Ach doses for maximum VD and FAI (rs=0.473 p=0.015) (Figure 1).

DISCUSSION

The results of the present study show an association between androgenicity and a state of decreased endothelial sensitivity to acetylcholine in post-menopausal women without clinical evidence of associated diseases and low cardiovascular risk. Women with FAI > 2.5 needed median doses of acetylcholine that were 20 times greater than those used by the group with FAI < 2.5 to obtain a similar venodilation in response to Ach infusion. To our knowledge, this is the first time this association is reported in postmenopausal women.

Literature data have shown that natural menopause is positively associated with changes on endothelial function (Taddei et al., 1996; Sanada et al., 2003; Lima et al., 2005), although the presence and number of risk factors seem to determine the degree of endothelial dysfunction (Ishibashi et al., 2006).

Although the present study was performed in a carefully selected healthy postmenopausal women group, the analysis of the impact of the free androgen index on venodilation by the dorsal hand vein technique does not exclude the presence of other factors that potentially alter vascular reactivity (Grundy et al., 1998; Zhang et al., 2006;Creatsas et al, 2005; Knopp, 2002).

Evidences for a role of testosterone in the development of cardiovascular disease have increased in the last decades (Liu et al., 2003; Thompson & Khalil, 2003). Recent studies show that testosterone levels in men are inversely related to cardiovascular risk factors such as arterial wall thickness (Muller et al., 2004), blood pressure (Svartberg et al., 2004), obesity and metabolic syndrome (Chen et al., 2006) and presence and extension of coronary disease (Dobrzycki et al., 2003). In postmenopausal women, endogenous androgen levels have been associated with insulin resistance, diabetes, central obesity and hypertension (Maturana & Spritzer, 2002; Ding et al. 2006; Phillips et al.1997), as well as with cardiovascular outcomes (Rexrode et al., 2003).

In men it is proposed that the positive influence of testosterone upon vascular reactivity may be observed only when vascular reactivity is sufficiently impaired, as in the case in atherosclerotic vessels (Webb et al., 1999). In turn, there are few studies evaluating testosterone levels and vascular reactivity in women. Subcutaneous physiological testosterone therapy has been shown to increase brachial artery vasodilation in women already receiving long-term oestrogen replacement therapy (Worboys et al, 2001). Similar responses have been described in experimental studies (Honda et al., 1999; Jones et al., 2004). In our population, although women were separated in two groups with high and low FAI levels, both values were still in normal limits. It is possible that if the spectrum of FAI levels included subjects with higher levels of FAI we could detect some differences in maximum venodilation responses.

Endothelial cells release substances acting directly on vascular smooth muscle (VSM) cells, causing either relaxation or contraction. Several endothelium-derived substances causing smooth muscle relaxation have been isolated: nitric oxide (NO), prostacyclin (PGI_2), epoxyeicosatrienoic acids and endothelium-derived hyperpolarizing factor (EDHF). Endothelium-derived vasoconstricting factors are also secreted, such as prostaglandins, reactive oxygen radicals and endothelin-1 (Luz et al., 2003; Maturana et al., 2006 submitted).

In the present study, the response to Ach was used to estimate endothelial functionality, whereas the response to SNP tests the integrity of smooth muscle function. Ach interacts with M_3 muscarinic receptor on the endothelial surface, which initiates a sequence of intracellular events leading to NO synthesis, although prostacyclin and hiperpolarizing factor release may also be induced. NO diffuses across the endothelial cell and basement membrane, binds to guanylate cyclase, leading to an increase in intracellular cyclic guanosine monophosphate and, ultimately, smoth muscle relaxation and vasodilation. By contrast, SNP decomposes to release

NO, which interacts with the vascular smooth muscle guanyl cyclase to produce vasodilation in an endothelium-independent way.

Since testosterone modulates the expression of muscarinic acetylcholine receptor (mAChR) subtypes in different tissues, including smooth muscle cells (Maróstica et al., 2005; Bush & Borda, 2003), we can speculate that such mechanism could be involved in the present observation that women with FAI > 2.5 needed median doses of acetylcholine significantly greater than those used by the group with FAI < 2.5 to obtain a similar venodilation in response to Ach infusion: women with higher bioactive androgen concentrations could have different expression of vascular muscarinic receptor which differently modulates venodilation response.

Vascular tone is defined as the degree of constriction of a blood vessel relative to its maximal diameter in the dilated state. Under basal conditions, most resistance and capacitance vessels exhibit some degree of smooth muscle contraction that determines the diameter or tone of the vessel. Vascular tone is influenced by both the endothelium and VSM. Gender differences in vascular tone have been described in a multitude of vascular beds in both human and experimental animals, suggesting that sex hormones can be involved (Orshall et al., 2004)

Effects of testosterone are found to be both endothelium dependent and independent (Khalil, 2005; Jones et al., 2004; Honda et al., 1999; Webb 1999). In addition, androgen receptors are expressed in endothelium and VSM of vascular tissue (Khalil, 2005)

Androgens have been related to cardiovascular risk factors and endothelial dysfunction in women in reproductive years (Meyer et al, 2005; Coviello et al, 2006; Orio et al, 2004, Nácul et al, 2006) and in post-menopause (Liu et al., 2003; Reckelhoff & Forteppiani, 2004). In women with polycystic ovary syndrome (PCOS) impaired endothelial function and decreased endothelium-dependent vasodilation have been shown (Tarkun et al., 2004). Recent studies have also shown that the endothelial

dysfunction coexist and is influenced by low-grade chronic inflammation in PCOS (Diamanti-Kandarakis et al., 2006; Brinkwhorth et al., 2006; Carmina et al, 2006; Blake & Ridker, 2002) as well as in post-menopausal women (Joffe et al., 2006; Crandall et al, 2006). We have recently shown that androgenicity is related to higher reactive C protein and endothelin-1 in postmenopausal women (Maturana et al, submitted 2006)

In conclusion, data of the present study indicate that endogenous androgens may be associated with a reduced vasodilation response to acetylcholine, an early marker of endothelial dysfunction in apparently healthy postmenopausal women. This suggests that androgenicity may be part of a risk profile related to endothelial dysfunction and preclinical cardiovascular disease, observed in the post-menopause.

Acknowledgements

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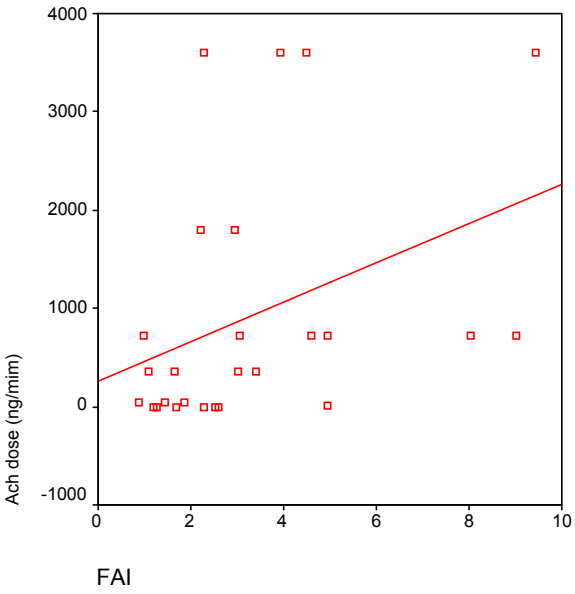
Table 1. Distribution of anthropometric, metabolic and vasomotion markers in full sample and by FAI median

FAI Median	**Full sample**	**1** ≤ **2.5 (n=12)**	**2** **> 2.5 (n=14)**	P
Waist (cm)	86 (8)	82 (8)	89 (8)	0.073
RCQ	0.84 (0.07)	0.81 (0.06)	0.86 (0.07)	0.049
Testosterone	0.41 (±0.21)	0.29 (±0.06)	0.51 (±0.26)	0.009
FAI	2.5 (1.1-2.1)	1.5 (1.1-2.1)	4.7 (2.9-5.7)	< 0.001
Total Cholesterol (mg/dL)	221 (42)	217 (40)	224 (44)	0.647
Triglycerides (mg/dL	107 (50)	101 (48)	113 (53)	0.537
HOMA [a]	1.6 (1-2.5)	1.4(1-2.2)	2.2 (1-3.4)	0.301
MaxVD (%)	80 (49)	73 (58)	89 (41)	0.217
Ach Max VD (ng/min) [a]	360 (2.8-990)	36 (0.36-360)	720 (360-3600)	**0.005**
Max. VD with SNP (%)	146 (55)	144 (58)	145 (57)	0.938

Values are expressed as mean and SD (Student T-test) or median and interquartile range (Mann-Whitney U test) [a] MaxVD: maximum venodilation with acetylcholine; Ach Max VD: acetylcholine doses for maximum VD; VC: venoconstriction after phenylephrine; Max VD with SNP: maximum venodilation with sodium nitroprusside

Figure 1. Correlation between Ach dose for maximum VD and FAI



rs=0.473 p=0.015

ANEXOS

HOSPITAL DE CLÍNICAS DE PORTO ALEGRE

UNIDADE DE ENDOCRINOLOGIA GINECOLÓGICA

DATA: _____

I. IDENTIFICAÇÃO:

Registro :_____

Nome:_____ _____

DN: ___ /___/____ Idade: _____

Cor: B( ) P( ) PD( )

Endereço: rua_____ Nº _____ apto:_____

Bairro:_____ _____CEP_____Cidade:_____

Telefone:_____--

2 ANTECEDENTES GINECO - OBSTÉTRICOS:

Menarca: _____

DUM:____/____/____ Idade de menopausa _____ Tempo de amenorréia:_____

Tipo menstrual no último ano antes de cessar menstruação:

( ) regular: ____I____ dias ( ) irregular ( ) não lembra

Paridade:G___P___A___ Infertilidade: ( ) sim ( ) não

Método (s) anticoncepcional(is): ( ) ACO Tempo de uso:_____

( ) DIU ( ) Outro ou nenhum

Terapia hormonal prévia: ( ) NÃO

( ) SIM Tipo: _____ Tempo de uso:_____

Tipo: _____ Tempo de uso: _____

Ano que usou:_____

Tempo decorrido desde o último tratamento: _____

Sintomas: ( ) Fogachos ( ) Insônia ( ) Dispareunia ( ) IU repetição ( )

Outro_____

3. HISTÓRIA PREGRESSA:

1. DCV: ( ) Sim ( ) Não

( ) HAS ( ) Cerebrovascular ( ) outra Qual: _____

2. Outra:

( ) Diabete melito ( ) tireoideopatia

( ) câncer ( ) Outro

Especificar:_____

4. MEDICAÇÕES EM USO:

_____

5. Tabagismo: ( ) Não

( ) Sim cig/dia:_____ tempo de uso: _____

( ) História de uso tempo de uso: _____

tempo desde que cessou uso: _____

6. Álcoolismo: ( ) sim ( )não

7. História familiar (doença cardiovascular, DM, tireoideopatia, CA ginecológico - mama, útero, colo, etc):

:_____

B. EXAME FÍSICO:

1. CV:_____ FC: _____

Circunferência do braço :_____

PA (braço direito, sentada, após 5 min de repouso): 1ª:: _____

2ª (corrigida): _____

Tireóide:_____

Mamas: Palpação:_____

Galactorréia: ( ) sim ( ) não

2. MEDIDAS ANTROPOMÉTRICAS:

peso:_____alt:_____ IMC:_____

cint:_____quadril:_____ RCQ:_____

C. AVALIAÇÃO LABORATORIAL:

glicemia jejum_____glicemia após 75 g de glicose: _____

Insulina em jejum:_____Insulina após 75 g de glicose: _____

colesterol total:_____HDL:_____ LDL:_____

triglicerídeos:_____

T4: _____ TSH: _____ SDHEA: _____

LH:_____ FSH:_____ E2:_____

TT: _____ IAL:_____ SHBG: _____

Óxido Nítrico_____

Fibrinogênio: _____ Fator VII:_____ von Willebrand:_____

FV _____ PAI-1:_____

AntitrombinaIII:_____Proteína C:_____ Proteína S:_____

FUNÇÃO ENDOTELIAL, PARÂMETROS ANTROPOMÉTRICOS, METABÓLICOS E HORMONAIS EM PACIENTES PÓS-MENOPAUSICAS

Unidade de Endocrinologia Ginecológica/ Serviço de Endocrinologia do HCPA

TERMO DE CONSENTIMENTO

Este estudo tem como objetivo estudar a associação entre alterações no endotélio (camada interna dos vasos sangüíneos) e idade, tempo decorrido desde a menopausa, níveis de glicose, lipídeos, hormônios e substâncias que participam na coagulação sangüinea, fatores de grande importância no aparecimento da doença cardiovascular.

Durante a consulta, será obtido seu histórico médico e verificados sua pressão arterial, peso, altura e as medidas da cintura e quadril, incluídos no exame físico e serão solicitados exames. Serão coletadas amostras de sangue que fazem parte da rotina de avaliação para a mulher na pós-menopausa. Uma fração da amostra de sangue será congelada e utilizada posteriormente para dosagens que não são realizadas rotineiramente no laboratório do Hospital de Clínicas.

Faremos também ecografia transvaginal e mamografia bilateral, que fazem parte da rotina de avaliação em pacientes na pós-menopausa.

Para avaliação da função do endotélio, será realizado através de um exame chamado pletismografia. Para isto, será puncionada uma veia em cima da sua mão direita, onde será colocado um soro e depois injetadas algumas medicações. Estas medicações serão muito diluídas. Assim, a dose que será feita é muito pequena e o efeito causado por elas será única e exclusivamente no local da punção, não tendo nenhum efeito no seu organismo, ou seja, você não corre nenhum risco ao recebê-las nestas doses. As medicações irão fazer com que a sua veia fique mais dilatada (aberta, com uso de acetilcolina ou bradicinina) ou mais estreita (fechada, com o uso de fenilefrina). Em cima da sua mão será colocado um aparelho que vai registrar o movimento desta veia quando injetadas estas medicações. Este exame irá durar em média 3 horas, e a senhora ficará confortavelmente deitado durante todo o procedimento. Os riscos a que a senhora ficará exposta serão mínimos, incluindo dor tipo “picada de mosquito” na hora da punção e algum hematoma (mancha roxa) que poderá surgir após a retirada da agulha da sua veia.

Os resultados dos exames ficarão a sua disposição. Caso queira retirar-se do estudo, estará livre para fazê-lo em qualquer momento que desejar, sem que isso venha a implicar na interrupção de seu tratamento. Os dados coletados, além de serem utilizados para indicação de um melhor tratamento para cada paciente, serão usados para fins de pesquisa e futuras publicações, reservando-se os preceitos da ética. Se necessário, a senhora poderá entrar em contato com a Drª Maria Augusta Maturana ou com a Drª Poli Mara Spritzer, coordenadora desta pesquisa, pelo telefone (051) 33168245.

Eu, ...concordo voluntariamente em participar desta pesquisa e autorizo a utilização dos dados coletados durante a investigação, estando ciente que serão utilizados com finalidade de pesquisa científica e respeitando os preceitos da ética.

_____

Paciente

Porto Alegre,_____ de _____de _____.