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TITLE:

CLINICAL, BIOCHEMICAL - MOLECULAR AND ETIOLOGICAL ASPECTS OF THE METABOLIC SYNDROME AFTER THE MENOPAUSE

PhD Thesis

Peter Chedraui, MD, Msc

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Tutor: Prof. Tommaso Simoncini, MD, PhD

Università di Pisa, Pisa, Italia

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CHAPTER 1

Introduction

The metabolic syndrome (METS) is a cluster of lipid and non-lipid factors that increase cardiovascular risk [1]. The National Cholesterol Educational Program and its Third Adult Treatment Panel (NCEP ATP-III) have established diagnostic criteria for the METS, which are met when three or more of the following are present: abdominal obesity, decreased high-density lipoprotein cholesterol (HDL-C) levels and increased serum triglycerides (TG), fasting glucose and/or blood pressure levels [2].

A. Prevalence

Prevalence of the METS is higher in women, especially those of Latin America ancestry [3], with an increased frequency observed in relation to age and the menopausal status. Indeed, during the menopausal transition there is an emergence of features related to the METS: obesity, dyslipidemia, diabetes, hyperinsulinism, hypertension and co-morbid conditions [4], possibly but not totally related to increasing estrogenic deficiency [5]. Rates may also vary according to the studied population from 22% [6] to 34% as determined in seven cross-sectional studies of non-diabetic Europeans [7]. This syndrome increases cardiovascular morbidity and mortality and the risk of developing diabetes [8].

B. The effect of the menopause over female weight

The prevalence of abdominal obesity is nearly double that of general obesity, with rates in North American women calculated for 2008 in 65.5% (aged 40-59 years) and 73.8% (60 years or more)[9]. It has been suggested that body mass index (BMI) but not menopausal status determines central adiposity in postmenopausal women. However, there is substantial evidence that the perimenopause is associated with a more rapid increase in fat mass and redistribution of fat to the abdomen, resulting in a transition from a gynoid to an android pattern of fat distribution and an increase in total body fat [10]. Moreover, postmenopausal women have greater amounts of intra-abdominal fat as compared to premenopausal ones, as determined with several radiological modalities [11]. Waist circumference represents both

subcutaneous and visceral adipose tissue depot size and correlates closely with the risk for cardiovascular disease. In women, it is also closely associated with dyslipidemia [12].

Abdominal fat is considered an endocrine organ as it has the capacity to secrete adipokines and other active substances closely related to metabolic diseases: insulin resistance, type 2 diabetes and the METS [13]. Both, the menopausal transition and aging, are associated with changes in adipose tissue metabolism, which contribute to the accumulation of body fat after menopause [14]. Deleterious changes in inflammatory markers and adipokines correlate strongly with increased visceral adiposity after the menopause [15].

Waist circumference significantly changes in relation to the time since final menstrual period. Moreover, significant increases in central abdominal fat have been reported from longitudinal studies in Caucasian and Asian women [16]. It has been observed that when non-obese premenopausal women are followed-up for several years, significant increases in total, percentage and truncal and visceral fat mass occur [16]. Women who later became peri or postmenopausal displayed a significant increase in visceral fat compared with baseline [16].

C. The impact of increased weight over menopausal symptoms

Severity and prevalence of menopausal symptoms relate several factors. These include not only the hormonal changes imposed by the transition, but also psychosocial factors. As weight increased during the menopausal transition, so do menopausal symptoms. Obesity is an independent risk factor for more severe menopausal symptoms [17,18].

Reduction of weight, BMI and abdominal circumference have been associated with a significant reduction in vasomotor symptoms women who are overweight and obese [19]. The combination of dietary modification and exercise also has positive effects on health related quality of life (HRQOL) and psychological health, which may be greater than that from exercise or diet alone [20]. Improvements in weight, aerobic fitness and psychosocial factors may mediate some of the effects of these interventions on HRQOL [20]. Weight loss in

overweight and obese women improves psychological wellbeing, HRQOL, self-esteem and health practices [21]. In addition, dietary weight loss and exercise exert a positive effect over insulin resistance in postmenopausal women, which together with a decrease in menopausal symptoms may potentially decrease cardiovascular risk.

D. The METS a state of pro-inflammation and endothelial dysfunction

The METS is associated with increased inflammation, endothelial dysfunction, oxidative stress and abnormalities in both the macro- and microvasculature [22]. Adipocytes and adipose-tissue macrophages are involved in the production of IL-6, which is one of the main mediators of chronic inflammation [23]. Elevated IL-6 is an established risk factor for cardiovascular events in women after the menopause; thus, it is interesting to find that the presence of METS, rather than the menopause itself, relates to increased IL-6 levels. IL-6 serum levels are associated with visceral adipose tissue and can influence insulin levels [24]. On the other hand, it has been reported that IL-6 polymorphisms may play a role in the pathogenesis of the METS through the modulation of IL-6 levels [25].

F. Endothelial dysfunction during pregnancy as a model for future METS risk

Preeclampsia is a leading cause of maternal mortality and morbidity worldwide [26]. Epidemiological data indicate that women complicated with preeclampsia are more likely to develop future cardiovascular disease (CVD). Population-based studies relate preeclampsia to an increased risk of later chronic hypertension (RR, 2.00 to 8.00) and cardiovascular morbidity/mortality (RR, 1.3 to 3.07), compared with normotensive pregnancy [27]. Women who develop preeclampsia before 36 weeks of gestation or have multiple hypertensive pregnancies are at highest risk (RR, 3.4 to 8.12). The underlying mechanism for the remote effects of preeclampsia is complex and probably multifactorial. Many risk factors are shared by CVD and preeclampsia, including endothelial dysfunction, obesity, hypertension, hyperglycemia, insulin resistance, and dyslipidemia. Therefore, it has been proposed that the METS may be a possible underlying mechanism common to CVD

and preeclampsia. Follow-up and counseling of women with a history of preeclampsia may offer a window of opportunity for prevention of future disease [27].

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CHAPTER 2

Aim and outline of the thesis

The aim of this thesis was to explore several clinical, biochemical/molecular and etiological aspects of the METS occurring after the menopause. In **chapter 3** the prevalence of the METS and factors relating to each of its composing factors are explored in a sample of postmenopausal Ecuadorian women. The results suggest that the prevalence of the METS was high and associated to metabolic and lipid abnormalities. Abdominal obesity was related to lower education; hence, there is a need to educate high risk populations and increase awareness of the problem.

Clinical aspects of postmenopausal METS are analyzed in **chapter 4.** The first study analyzed the hypothesis that the METS or its components can cause a negative impact on menopausal symptoms in postmenopausal women as determined with a validated tool (The MENQOL). Results confirmed this hypothesis, as menopausal symptoms were significantly related to components of the syndrome. Indeed, abdominal obesity related to hot flushes, depression and muscle and joint pain. Given these results, in a second study it was aimed at determining if the METS or its components would significantly affect sleep (insomnia) in postmenopausal women by means of The Athens Insomnia Scale. Study results indicated that this association was not found yet was related to other psycho-somatic female and partner issues.

Biochemical aspects related to inflammation and endothelial dysfunction encountered among postmenopausal women with the METS are explored in **chapter 5.** Nitric oxide (NO) and pro-inflammatory cytokine levels (IL-6 and TNF-α) were measured in postmenopausal women with and without the METS. Results indicated that those with the METS displayed higher IL-6 and NO levels as compared to those without the syndrome, with significant correlations found between studied analytes and some of the METS components. Finding higher instead of lower NO levels in those with the METS is discussed. Several markers related to angiogenesis, inflammation and endothelial function were measured in the second study using multiplex technology. Higher IL-6 (marker of inflammation) and lower uPA

(marker of endothelial dysfunction) levels were found among those with postmenopausal METS. These were mainly related to metabolic and lipid abnormalities.

Several studies have determined that endothelial dysfunction occurring as a consequence of pregnancy related complications (i.e preeclampsia) may indeed increase the risk of presenting the METS and its composing features and further increase cardiovascular risk. Evaluation of endothelial function from a biomolecular perspective has been performed in women with preeclampsia however only in the maternal side. Studies assessing the umbilical fetal circulation are scarce or lacking. Given our prior results regarding NO and the METS, and as a first step, **chapter 6** aimed at analyzing endothelial dysfunction by measuring NO, ADMA and VEGF levels in fetal circulation of pregnant women with severe preeclampsia and discuss the possible role of gestational endothelial dysfunction in the genesis of postmenopausal METS and further cardiovascular risk. In addition, DNA was extracted from umbilical vein to determine the frequency of VEGF gene single nucleotide polymorphisms. Results found that women with severe preeclampsia displayed higher NO and ADMA fetal circulating levels (vein and artery) and lower VEGF umbilical vein levels. There was a significant trend of finding lower VEGF levels in the presence of -2578 CC and -1154 AG genotypes. As a next step, further research is warranted to confirm these findings and to find a biomolecular marker capable of establishing the link between endothelial dysfunction found in preeclampsia and postmenopausal METS.

Finally, the biological mechanisms involved in cardiovascular disease during mid-life are analyzed in **chapter 7**, by means of a critical review. Particular emphasis is given to biochemical markers and indicators of cardiovascular dysfunction and damage.

CHAPTER 3

The metabolic syndrome during the postmenopause

The metabolic syndrome and its components in postmenopausal women.

Chedraui P. *San Miguel G, Vintimilla-Sigüenza I, Villacreses D, Romero-Huete L, Domínguez A, Jaramillo W, Escobar GS, Pérez-López FR, Genazzani AR, Simoncini T.*

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MENOPAUSE

The metabolic syndrome and its components in postmenopausal women

Peter Chedraui[1], Glenda San Miguel[1], Isabel Vintimilla-Sigüenza[1], Diego Villacreses[1], Lucía Romero-Huete[1], Andrea Domínguez[1], Winston Jaramillo[1], Gustavo S. Escobar[1], Faustino R. Pérez-López[2], Andrea R. Genazzani[3], and Tommaso Simoncini[3]; Research Group for the Omega Women's Health Project

[1]*Institute of Biomedicine, Research Area for Women's Health, Facultad de Ciencias Médicas, Universidad Católica de Santiago de Guayaquil, Ecuador,* [2]*Department of Obstetrics and Gynecology, Facultad de Medicina, Lozano Blesa University Hospital, Universidad de Zaragoza, Spain, and* [3]*Molecular and Cellular Gynecological Endocrinology Laboratory, Department of Reproductive Medicine and Child Development, Division of Obstetrics and Gynecology, University of Pisa, Italy*

Abstract

Background: Prevalence of the metabolic syndrome (METS) increases significantly after the menopause.

Objective: To assess the prevalence of the METS and its components in postmenopausal women. Factors relating to each of the composing items of the METS were also analyzed.

Methods: Natural postmenopausal women (40–65 years) were invited to participate in this cross-sectional study in order to assess the presence of the METS using modified Adult Treatment Panel III (ATP-III) criteria. Participants were also requested to fill out a general socio-demographic questionnaire.

Results: A total of 204 women were surveyed with a median age of 56 years. A 52.9% presented the METS according to modified ATP-III criteria, with 37.3% presenting hyperglycemia, 51.5% hypertension, 58.3% abdominal obesity, 45.6% high triglyceride levels and 56.4% low HDL-C levels. Women with the METS presented a higher rate of dyslipidemia (high triglyceride and low HDL-C levels), hyperglycemia, hypertension and abdominal obesity than those without the syndrome. Those with abdominal obesity and hyperglycemia significantly displayed higher rates of low HDL-C levels (bivariate analysis). Multiple linear regression analysis found a positive correlation between glucose and triglyceride levels. Systolic blood pressure significantly and positively correlated to age and abdominal circumference. Abdominal circumference displayed an inverse correlation with educational level.

Conclusion: Prevalence of the METS in this postmenopausal female sample was high and associated to metabolic and lipid derangements. As abdominal obesity was significantly associated to lower education, there is an urgent need of implementing educational programs directed to high-risk populations in order to increase awareness of the problem.

Keywords

Insulin resistance, metabolic syndrome, obesity, postmenopause, risk determinants

History

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Introduction

The metabolic syndrome (METS) is a cluster of lipid and non-lipid factors that increase cardiovascular risk [1,2]. The National Cholesterol Educational Program and its Third Adult Treatment Panel (NCEP ATP-III) have established diagnostic criteria for the METS, which are met when three or more of the following are present: abdominal obesity, decreased high-density lipoprotein cholesterol (HDL-C) levels and increased serum triglycerides (TG), fasting glucose and/or blood pressure levels [3]. Women display higher METS rates, especially those of Latin America ancestry [4], with an increased prevalence seen in relation to age and menopausal status. Indeed, during the menopausal transition there is an emergence of features related to the METS: obesity, atherogenic lipid profiles, diabetes, hyperinsulinism, hypertension and co-morbid conditions [5], possibly but not totally related to increasing estrogenic deficiency [6–8]. Rates may vary according to the studied population from 22% [9] to 34% as determined in seven cross-sectional studies of non-diabetic Europeans [10].

The METS increases cardiovascular morbidity and mortality and the risk of developing diabetes [11]. While some studies report that several behavioral and lifestyle factors (i.e. physical activity, sleep alterations and tobacco and alcohol consumption) relate to the METS, others have found inconsistent results [8,12–18]. The aim of this research was to assess the prevalence of the METS and its components in postmenopausal women. Factors relating to each of the composing items of the METS were also analyzed.

Methods

Study design and participants

This was a cross-sectional study carried out from December 2011 to June 2012 at the *Instituto de Biomedicina, Facultad de*

Address for correspondence: Peter Chedraui MD, MSc. Institute of Biomedicine, Research Area for Women's Health, Facultad de Ciencias Médicas, Universidad Católica de Santiago de Guayaquil, Guayaquil, Ecuador. Tel: +(5934) 220-6958. Fax: +(5934) 220-6958. E-mail: peter.chedraui@cu.ucsg.edu.ec

Ciencias Médicas, Universidad Católica de Santiago de Guayaquil, Guayaquil, Ecuador. Primarily, the initiative aimed at assessing the prevalence of the METS and its components among natural postmenopausal Hispanic women (40 to 65 years), recruited through newspaper advertising, who were not taking hormone therapy (HT). Secondarily, menopausal symptoms, insomnia and mood problems were assessed. Women who were taking phytoestrogens or drugs intended to decrease lipid levels were excluded from the study. Eligible women were requested to attend the Institute to be informed about the study, its purposes and provide written consent of participation. Those consenting and fulfilling the inclusion criteria were asked to return after an 8 hour overnight fast, moment in which socio-demographic data, waist circumference, weight, height and blood pressure measurements were recorded. In addition a 10–15 ml peripheral venous blood sample was obtained to provide plasma, serum and white cells. In a subsequent appointment, woman were counseled and managed according to the results and invited to participate in educational group sessions aimed to discuss topics related to the menopause, the METS, its risk determinants and cardiovascular risk implications. Research protocol of the study was reviewed and approved by the Scientific Research Committee of the Institute of Biomedicine.

General questionnaire

An itemized questionnaire was designed to assess and record all general data. Prior to its implementation, the tool was validated in 50 women and included the following female data: age (years), marital status, educational level (years), parity, time since menopause onset (years), perceived healthiness, active sexual status (yes/no) and current partner status (yes/no). Lifestyle and other personal factors included in this section were smoking habit, alcohol and coffee consumption, psychotropic drug use and sedentarism. Studied women were further categorized as early (1 to 4 years) and late postmenopausal (≥5 years).

Diagnostic criteria for the METS

The METS was defined using NCEP ATP-III diagnostic criteria [19] modified by the American Heart Association and the National Heart, Lung, and Blood Institute [20]. This was the case if three or more of five criteria were encountered: abdominal obesity (waist circumference >88 cm), increased TG (≥150 mg/dL), decreased HDL-C (<50 mg/dL), high fasting glucose levels (≥100 mg/dL) and increased blood pressure (≥130/85 mmHg) [20]. Of importance is highlighting the fact that modified ATP-III criteria only includes the change of cut-off value for hyperglycemia (from 110 to 100 mg/dL) and that the waist circumference cut-off value of 88 cm has recently been reported optimal for defining the METS in postmenopausal Latin American women [21]. Women taking oral hypoglycemic or antihypertensive medication were considered, respectively, as diabetic or hypertense independent of the serum or blood pressure findings. After a 10 min resting period in sitting position, mean blood pressure was determined by performing two separate determinations 10 min apart.

Weight (kg) and height (m) were recorded for each participant. Body mass index (BMI) was calculated as weight (in kg) divided by the square of height (in m). Obesity was defined as a BMI ≥30 kg/m^2 [22]. Waist circumference expressed in centimeters was obtained from women in supine position. Subjects were defined as sedentary if carrying out less than 15 min of physical activity twice per week [23].

Serum assays

Blood samples withdrawn from each participant were centrifuged at 5 °C for 10 min at 3000 rpm. Obtained serum, plasma and white cells were decanted into 0.5 ml aliquots which were then stored at −70 °C until further analysis. TG, HDL-C and glucose levels were assayed using the enzymatic colorimetric method with a Hitachi 717 automatic photometric analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (IBM SPSS, Armonk, NY, USA). Data are presented as medians, frequencies, percentages, beta coefficients and 95% confidence intervals. The Kolmogorov–Smirnov test was used to determine the normality of data distribution. The chi-square test was used to compare percentages.

Multiple linear regression analysis was performed to assess variables relating to each of the composing items of the METS. The dependent variables were each of the METS components. These models were constructed from independent variables achieving $p = 0.20$ during bivariate analysis. Independent variables tested during bivariate analysis included among the most important: age, parity, educational level, marital status, time since the menopause onset and each of the METS diagnostic items. Entry of variables into the model was performed using a forward/backward stepwise procedure. A p value less than 0.05 was considered statistically significant.

Sample size calculation

We have previously reported that 41.5% of postmenopausal women present the METS [24]. Hence, a minimal sample size of 179 participants was calculated assuming that 35% of surveyed women would present the METS with a 7% desired precision and a 95% confidence level.

Results

During the study period, a total of 207 natural postmenopausal women were invited to participate. Three did not meet inclusion criteria; hence, statistical analysis was performed on 204 complete surveys. Median age of surveyed women was 56 years. Prevalence of the METS and its components in relation to general demographic data is presented on Table 1. A 52.9% of studied women presented the METS according to modified ATP III criteria, with 37.3% presenting hyperglycemia, 51.5% hypertension, 58.3% abdominal obesity (38.2 according to BMI values), 45.6% high TG levels and 56.4% low HDL-C levels. During bivariate analysis, it was found that hypertension rate was significantly higher in relation to age and years since the menopause. There was a significant inverse relation between abdominal obesity and years of education. Women who were younger displayed higher rates of obesity defined through BMI values.

Lipid and metabolic profile of women with the METS and three of its main risk determinants is depicted on Table 2. Women with the METS presented a higher rate dyslipidemia (high TG and low HDL-C levels), hyperglycemia, hypertension and obesity (abdominal and BMI assessed) than those without the syndrome. Those with abdominal obesity and hyperglycemia significantly displayed higher rates of low HDL-C levels.

Factors relating to each of the composing items of the METS (Multiple linear regression analysis) are presented on Table 3. A positive significant correlation was found between glucose and TG levels. Systolic blood pressure significantly and positively correlated to age and abdominal circumference. Abdominal circumference displayed an inverse correlation with educational level. There was a non-significant correlation between abdominal circumference and HDL-C levels ($p = 0.096$).

Table 1. Prevalence of the metabolic syndrome and its components in relation to general demographic data ($n = 204$).

Parameter	All $n = 204$	Metabolic syndrome 108 (52.9)	Hyperglycemia[a] 76 (37.3)	Hypertension 105 (51.5)	Abdominal obesity 119 (58.3)	Triglycerides >150 mg/dL 93 (45.6)	HDL-C <50 mg/dL 115 (56.4)	Obesity BMI ≥30 78 (38.2)
Age								
40–49	19 (9.3)*	6 (31.6)	4 (21.1)	6 (31.6)	13 (68.4)	9 (47.4)	8 (42.1)	11 (57.9)
50–59	126 (61.8)	69 (54.8)	46 (36.5)	61 (48.4)	68 (54.0)	62 (49.2)	75 (59.5)	40 (31.7)
60–65	59 (28.9)	33 (55.9)	26 (44.1)	38 (64.4)	38 (64.4)	22 (37.3)	32 (54.2)	27 (45.8)
p Values**		**0.14**	0.18	**0.02**	0.26	0.31	0.33	**0.03**
Married								
Yes	93 (45.6)	50 (53.8)	34 (36.6)	53 (57.0)	52 (55.9)	37 (39.8)	55 (59.1)	38 (40.9)
No	111 (54.4)	58 (52.3)	42 (37.8)	52 (46.9)	67 (60.4)	56 (50.5)	60 (54.0)	40 (36.0)
p Values		0.82	0.85	0.14	0.52	0.12	0.46	0.48
Parity								
0	17 (8.3)	9 (52.9)	6 (35.3)	10 (58.9)	8 (47.0)	8 (47.0)	8 (47.0)	5 (29.4)
1–5	160 (78.4)	83 (51.9)	58 (36.3)	78 (48.8)	93 (58.1)	71 (44.4)	93 (58.1)	61 (38.1)
>5	27 (13.2)	16 (59.3)	12 (44.4)	17 (63.0)	18 (66.7)	14 (51.9)	14 (51.9)	12 (44.4)
p Values		0.77	0.73	0.32	0.43	0.76	0.59	0.60
Menopause ≥5 years								
Yes	118 (57.9)	64 (54.2)	44 (37.3)	68 (57.6)	72 (61.0)	47 (39.8)	68 (57.6)	48 (40.7)
No	86 (42.1)	44 (51.2)	32 (37.2)	37 (43.0)	47 (54.7)	46 (53.5)	47 (54.7)	30 (34.9)
p Values		0.66	0.99	**0.03**	0.36	0.06	0.67	0.40
Education								
0–6	73 (35.8)	41 (56.2)	30 (41.1)	39 (53.4)	47 (64.4)	31 (42.5)	41 (56.2)	29 (39.7)
7–12	68 (33.3)	37 (54.4)	25 (36.8)	41 (60.3)	46 (67.6)	35 (51.5)	39 (57.4)	30 (44.1)
≥13	63 (30.9)	30 (47.6)	21 (33.3)	25 (39.7)	26 (41.3)	27 (42.9)	35 (55.6)	19 (30.1)
p Values		0.58	0.64	0.14	**0.003**	0.49	0.97	0.24
Sedentary								
Yes	69 (33.8)	38 (55.1)	23 (33.3)	39 (56.5)	39 (56.5)	30 (43.5)	42 (60.9)	27 (39.1)
No	135 (66.2)	70 (51.9)	53 (39.3)	66 (48.9)	80 (59.3)	63 (46.7)	73 (54.1)	51 (37.8)
p Values		0.66	0.40	0.30	0.70	0.66	0.35	0.85
Current partner								
Yes	127 (62.3)	64 (50.4)	43 (33.9)	68 (53.5)	72 (56.7)	53 (41.7)	70 (55.1)	51 (40.1)
No	77 (37.7)	44 (57.1)	33 (42.9)	37 (48.0)	47 (61.0)	40 (51.9)	45 (58.4)	27 (35.0)
p Values		0.34	0.19	0.44	0.54	0.15	0.64	0.46
Sexually active								
Yes	100 (49.0)	50 (50.0)	36 (36.0)	51 (51.0)	53 (53.0)	44 (44.0)	57 (57.0)	36 (36.0)
No	104 (51.0)	58 (55.8)	40 (38.5)	54 (51.9)	66 (63.5)	49 (47.1)	58 (55.8)	42 (40.4)
p Values		0.40	0.71	0.89	0.13	0.65	0.85	0.51

*Data are presented as *n* (%); **p values as determined with the chi-square test; [a] women with a fasting glycemia >100 mg/dL.; HDL-C, high density lipoprotein cholesterol; BMI, body mass index.
Bold values indicate significant p values.

Table 2. Lipid and metabolic profile of women with the metabolic syndrome and three of its main risk factors.

Parameter	Triglycerides ≥150 mg/dL, $n = 93$	HDL-C <50 mg/dL $n = 115$	Hyperglycemia $n = 76$	Hypertension $n = 105$	Abdominal Obesity $n = 119$	Obesity BMI ≥30 $n = 78$
Metabolic syndrome						
Yes $n = 108$	77 (71.3)	93 (86.1)	61 (56.5)	73 (67.6)	81 (75.0)	53 (49.1)
No $n = 96$	16 (16.7)	22 (22.9)	15 (15.6)	32 (33.3)	38 (39.6)	25 (26.0)
p Values*	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.001**
Hyperglycemia						
Yes $n = 76$	41 (53.9)	50 (65.8)		43 (56.6)	50 (65.8)	34 (44.7)
No $n = 128$	52 (40.6)	65 (50.8)	–	62 (48.4)	69 (53.9)	44 (34.4)
p Values	0.06	**0.03**	–	0.26	0.09	0.14
Hypertension						
Yes $n = 105$	45 (42.9)	64 (61.0)	43 (41.0)		65 (61.9)	46 (43.8)
No $n = 99$	48 (48.5)	51 (51.5)	33 (33.3)	–	54 (54.5)	32 (32.3)
p Values	0.42	0.17	0.26	–	0.28	0.09
Abdominal obesity					–	–
Yes $n = 119$	56 (47.1)	76 (63.9)	50 (42.0)	65 (54.6)	–	–
No $n = 85$	37 (43.5)	39 (45.9)	26 (30.6)	40 (47.1)		
p Values	0.61	**0.01**	0.09	0.28		
Obesity BMI ≥30						
Yes $n = 78$	33 (42.3)	50 (64.1)	34 (43.6)	46 (59.0)		
No $n = 126$	60 (47.6)	65 (51.6)	42 (33.3)	59 (46.8)	–	–
p Values	0.45	0.08	0.14	0.09	–	–

*p value as determined with the chi-square test; BMI, body mass index.
Bold values indicate significant p values.

Table 3. Factors relating to each of the items composing the METS: multiple linear regression analysis.

Risk factor	Regression coefficients	Standard error	95% CI	p Value
Glucose levels (mg/dL)				
Triglyceride levels (mg/dL)	0.10	0.04	0.01–0.18	0.01
$r = 0.69$, adjusted $r^2 = 0.68$, $p < 0.0001$				
Systolic blood pressure (mm Hg)*				
Age (years)	1.21	0.25	0.72–1.71	0.0001
Abdominal circumference (cm)	0.43	0.10	0.24–0.63	0.0001
$r = 0.78$, adjusted $r^2 = 0.77$, $p < 0.0001$				
Triglyceride levels (mg/dL)				
Glucose levels (mg/dL)	0.27	0.11	0.05–0.50	0.01
$r = 0.68$, adjusted $r^2 = 0.67$, $p < 0.0001$				
Abdominal circumference (cm)				
Educational level (years)	−0.44	0.15	−0.75 to −0.14	0.004
Systolic blood pressure (mm Hg)	0.17	0.04	0.08–0.25	0.0001
$r = 0.65$, adjusted $r^2 = 0.60$, $p < 0.0001$				
HDL-C levels (mg/dL)				
Abdominal circumference (cm)	−0.14	0.08	−0.32–0.02	0.09
$r = 0.39$, adjusted $r^2 = 0.40$, $p = 0.096$				

CI, confidence intervals; *similar model was found using diastolic blood pressure as a dependant variable.

Discussion

Body weight and composition are the consequences of a complex balance between energy intake and expenditure. Weight gain may relate to lifestyle, dietary and behavioral factors as well as hormone-dependant life events such as pregnancy and the menopause. Indeed, estrogens regulate reproduction and body weight through actions exerted on alpha estrogen receptors (ER-α). In female mice, deletion of brain ER-α leads to hyperphagia and abdominal obesity. The simultaneous deletion of hypothalamic pro-opiomelanocortin and ER-α neurons produces hyperphagia without changes in energy expenditure and fat visceral adiposity [25,26]. Although in humans social-economical and lifestyle factors may determine conditions associated to the METS, in general it is well known that estrogens found in the human brain play a key role in body weight gain. Despite all the aforementioned, human body weight gain and obesity is difficult to be explained by a single mechanism, hormonal or not [27]. In this study, although the METS (as a defined category) was not related to any of the studied socio-demographic variables, this was not the case for some of its composing items (Table 1). Indeed, upon bivariate analysis, it was found that hypertension rate was significantly associated to age and years since the menopause. After multiple linear regression analysis, time since the menopause onset was excluded from the final model (confounding factor); age, however, persisted in this model, and abdominal circumference appeared as a factor related to higher systolic blood pressure. There is controversy in the literature on whether age or years since the menopause are in fact causing the metabolic changes observed during the menopausal transition; our study seems to favor age. In Spanish postmenopausal women, BMI increased with age, time since menopause and parity, with significant correlations found with hormonal and metabolic parameters [28].

The prevalence of the METS was found to be high in the present cohort, although in accordance to what has been previously described among postmenopausal women in Ecuador [24] and Latin America [4]. As a region, Latin America is known to be increasingly adopting lifestyle patterns of developed societies, especially those from the United States, including dietary and sedentary habits. Consequently, body weight in individuals of this region has been increasing in recent years to alarming records [21]. Supporting this fact was finding abdominal obesity as the most frequently observed component of the METS. This confirms its importance as a central key factor in the definition of the METS and from which many of the metabolic and lipid derangements can be explained and lead to increased cardiovascular risk [8,20]. Indeed, our multiple linear regression analysis found that abdominal obesity (expressed as higher abdominal circumference) significantly and positively correlated to higher systolic blood pressure readings and lower HDL-C levels. It has been reported that among mid-aged men in the highest visceral fat content tertile, abdominal obesity is associated with higher odds ratio for coronary heart disease, the METS and hypertriacylglycerolemia than those in the lowest tertile. In women, visceral fat content positively correlated with C-reactive protein and triacylglycerol concentrations and abnormal homeostasis model assessment (HOMA) values [29]. In our study, glucose and TG levels displayed a significant positive correlation. In diabetic subjects, such correlation has also been documented [30]. TGs are the major storage form of body fat while blood sugar is the amount of glucose in the bloodstream. There is a correlation between TG levels and insulin resistance, and many individuals with diabetes in fact have high TG levels. In addition, dietary excessive carbohydrate intake may elevate blood TG. Contrary to this, in postmenopausal women restrictive short- and long-term diets change body composition and the metabolic profile, including an improvement of both fasting plasma TG and glucose levels [31].

Although definitions for the METS (and its components) have changed throughout time, currently no single criteria can be considered as definitive. For instance, our research found that 37.3% of women display hyperglycemia according to modified ATP-III criteria (cut-off 100 mg/dL), value which is higher than 25% when the original ATP-III criteria is used (cut-off 110 mg/dL). As a consequence, METS prevalence also increases from 50.5% to 52.9%. Defining the METS as a category imposes limitations when it comes to analyzing related factors or comparing analyte levels. To highlight the latter, we have previously reported no differences in pro-inflammatory cytokine levels when comparing those with and without the METS. Contrary to this, cytokine levels significantly correlated with several of its independent composing items expressed as numeric variables and not categories (i.e. abdominal circumference and blood pressure levels) [32]. For this reason, in our study multiple linear regression analysis was performed for the assessment of factors relating to each of the composing items of the METS expressed as independent continuous variables and not categories.

Finally, our study found an inverse correlation between abdominal circumference values and educational level. This highlights the need for education and awareness of the problem and the encouraging of shifting to healthy lifestyle habits. Regarding education, we have previously reported that postmenopausal women with the METS display lower knowledge of the menopause, the METS and related themes. After participating in educational sessions, their knowledge increased significantly [33]. Moreover, we have envisioned that similar educational programs should be implemented not only in postmenopausal women but also in pre- and perimenopausal ones as a cost-effective measure of decreasing the METS prevalence and associated complications [8]. This must be seen as a pivotal measure beyond therapeutical ones which sometimes are shadowed by poverty conditions such as those seen in Ecuador and other developing countries of Latin America and the world. Regarding healthy lifestyle habits, it has been reported that unbalanced diets and a sedentary life in the postmenopause may increase weight gain and relate to adverse outcomes [34]. Diet components may reduce or increase the METS prevalence and subrogate endpoints [35–37]. In this sense, the advantages of a Mediterranean and prudent diets have previously been reported [34,36,38]. Exercise is also needed to decrease obesity and the inflammatory status associated with the METS and obesity [39].

As for the limitations of our study one can mention its cross-sectional design which does not allow determining causality. As no associations were found between the METS (as a category) and the various socio-demographical studied variables (excluding collinear composing items), no regression model was constructed for the METS, yet only for its composing factors. In addition, only studying postmenopausal women may be seen as limitation. It would have been interesting to have included pre- and perimenopausal ones in order to analyze the individual effect that the different menopausal stages would have had over METS prevalence. Future research designs should take this into account and also explore new variables or more details related to those already included in this study such as dietary components, exercise intensity, other healthy lifestyle factors, just to mention some.

Despite the aforementioned limitations, our study renders prevalence data on the METS and its components in a female Hispanic postmenopausal developing country population, providing a highlight on the importance of educating high-risk populations.

In conclusion, prevalence of the METS in this postmenopausal female sample was high and associated to metabolic and lipid derangements. As abdominal obesity was significantly associated to lower education, there is an urgent need of implementing educational programs directed to high-risk populations in order to increase awareness of the problem.

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Declaration of interest

Authors declare having no declaration of interest.

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CHAPTER 4

Clinical aspects of the metabolic syndrome after the menopause

Menopausal symptoms and associated risk factors among postmenopausal women screened for the metabolic syndrome.

Chedraui P*, Hidalgo L, Chavez D, Morocho N, Alvarado M, Huc A.*

Arch Gynecol Obstet 2007;275:161-8.

Assessment of insomnia and related risk factors in postmenopausal women screened for the metabolic syndrome.

Chedraui P, *San Miguel G, Villacreses D, Dominguez A, Jaramillo W, Escobar GS, Pérez-López FR, Genazzani AR, Simoncini T.*

Maturitas 2013;74:154-9.

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ORIGINAL ARTICLE

Menopausal symptoms and associated risk factors among postmenopausal women screened for the metabolic syndrome

P. Chedraui · L. Hidalgo · D. Chavez · N. Morocho · M. Alvarado · A. Huc

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Abstract
Background Although the frequency of menopausal symptoms may vary according to the studied population, in general severe intensity has been related to lower quality of life.
Objective To assess the frequency of menopausal symptoms and involved risk factors in an Ecuadorian postmenopausal population.
Methods Postmenopausal women that participated in a metabolic syndrome screening program were interviewed with the Menopause-specific quality of life questionnaire (MENQOL) in order to determine the most frequently presenting menopausal symptoms and correlate these symptoms with socio-demographic data and the main results of the screening program.
Results Three hundred and twenty-five postmenopausal women ($n = 325$) were surveyed with the MENQOL. Mean age of participants was 55.9 ± 8.1 years (median: 54 years). The most frequently presenting symptoms were: hot flushes (53.3%), sweating (49.2%), poor memory (80.6%), feeling depressed (67.4%), aching in muscles and joints (84%), drying of their skin (85.5%), avoiding intimacy (76.2%) and change in their sexual desire (76.5%). Multivariate analysis determined that abdominal obesity was a significant risk factor for presenting hot flushes, depression and muscle and joint pain. High triglyceride levels were associated to higher rates of sweating and depression. While women with basal hyperglycemia were associated to dry skin and changes in sexual desire in a higher proportion, those who were older and with more years of menopause onset were related less frequently to vasomotor symptoms. Older age was also significantly associated in a higher rate to dry skin.
Conclusion In this postmenopausal Ecuadorian population, the frequency of menopausal symptoms, as assessed with the MENQOL, was found to be relatively similar to other Latin and non-Latin American populations and associated to age, hormonal status and related metabolic conditions.

Keywords Menopausal symptoms · Quality of life · Health assessment · Latin America · Metabolic syndrome

P. Chedraui (✉) · L. Hidalgo · D. Chavez · N. Morocho · M. Alvarado · A. Huc
Facultad de Ciencias Médicas,
Universidad Católica de Santiago de Guayaquil,
Institute of Biomedicine, P.O. Box 09-01-4671,
Guayaquil, Ecuador
e-mail: peterchedraui@yahoo.com

P. Chedraui · L. Hidalgo · N. Morocho
Foundation for Health and Well Being in the Climacteric
"FUCLIM", Guayaquil, Ecuador

Introduction

The menopausal transition is characterized by a progressive decrease of estrogenic secretion which in turn increases the frequency of related signs and symptoms [5, 6]. After the menopause onset, complete cease of estrogen secretion increases severity of these symptoms, thus impairing female quality of life (QoL) [2, 6]. Age at menopause presentation in Ecuador, as in other Latin American countries, has been recently determined to occur earlier than in women from Europe and USA, fact that has been related to, among other factors, higher altitude residency and lower educational and economical income

[4, 30]. Therefore, the earlier the onset of the menopause the longer the exposition to the negative effects of hypoestrogenism. It is a general consensus that the transition through the climacteric phase causes physiological and psycho-social changes which in turn affect female QoL. Particularly in Ecuador, these changes have been described among *climacteric* (vasomotor symptoms, headaches, decreased libido and loss of bone mass) and *postmenopausal women* (increased prevalence of hyperglycemia, hypertension and dyslipidemias) [10, 14, 15, 30].

Up-to-date several menopause specific health related quality of life tools have been proposed [35]. In a recent study carried out in Ecuador, using one of these tools, the Greene Climacteric Scale, among women of low socio-economic income aged 40–65 years, it was found that the most frequently and intensive presenting symptoms of the 21 composing the scale were: difficulty in concentrating (87%), feeling unhappy or distressed (82%), headaches (83.9%), and hot flushes (82%). Older age, higher parity and lower schooling were associated to higher symptom intensity [30]. Frequency of hot flashes in this low income series was found to be higher than percentages found among other Latin American populations, i.e.: Chilean (77%), Mexican (73%) and Brazilian (70%) [1, 22, 27]. Additionally the rate of women presenting loss of interest in sex was found to be high (75.8%), more prevalent among premenopausal women and related to higher parity. This rate was also found to be higher than that found among healthy middle aged Chilean women (51%) [8].

The menopause-specific quality of life questionnaire (MENQOL) is another specific tool, based upon women's own perspective, designed to measure the influence of age/menopause related issues over female QoL. [17]. This tool has been validated among a Hispanic Chilean population [2] and not only allows the measuring of QoL in terms of domain and total scores (means and medians) yet one may access the frequency of each of the menopausal symptoms composing the questionnaire (percentages).

The objective of the present research was to assess the frequency of menopausal symptoms and related risk factors in an Ecuadorian postmenopausal population that participated in a metabolic screening program with the use of the MENQOL instrument.

Methods

Subjects

This cross-sectional study was approved by the Institutional Review Board of the Medical Faculty of the Universidad Católica de Santiago de Guayaquil, Ecuador and carried out at its Institute of Biomedicine from February 1st to March 31st 2005 primarily as a metabolic syndrome screening program aimed to determine the prevalence of this entity and related risk factors among postmenopausal women. Before the initiation of the study, the screening program was advertised through a newspaper add in which postmenopausal women (>1 year of amenorrhea), ≥ 40 years, non hormone therapy (HT) users and with intact uterus were recruited and asked to participate in the program [10]. Women who were eligible attended the Institute to be informed about the study, its purposes and sign consent for participation. Those consenting and fulfilling inclusion criteria were asked to return with an 8 h overnight fasting period after which socio-demographic data, as well as waist circumference and blood pressure measurements were recorded. Additionally a blood sample was also obtained at this point of the study for serum glucose and lipid profile analysis, data that is presented in detail elsewhere [10]. As a secondary objective QoL was measured among participants with the menopause-specific quality of life questionnaire (MENQOL).

The metabolic syndrome (MS) was diagnosed, according the 3rd Adult Treatment Panel (ATPIII) criteria, as women presenting three or more of five risk determinants: abdominal obesity (waist circumference > 88 cm), increased serum triglycerides (≥150 mg/dL), decreased high density lipoprotein cholesterol (<50 mg/dL), high fasting glucose (≥110 mg/dL) and increased blood pressure (≥130/85 mmHg) [13]. Body mass index (BMI) was calculated as weight in kilos divided by squared height in meters. Women with a BMI ≥30 were considered as obese. Definitions regarding race, rural residency, economic income, sedentary habit and other basal socio-demographic features of our population are described elsewhere [9, 16, 30].

Quality of life assessing tool

For this study, QoL was assessed using the MENQOL, proposed by Hilditch et al. [17] at the University of Toronto, in its Spanish version which has been validated among a Hispanic Chilean population [2]. The questionnaire is composed of 29 items grouped in four domains: vasomotor, psycho-social, physical and sexual. Each item can be checked as non-present or present. In the latter case, the item is graded according to its severity from 0 to 6. No total score is available, rather a mean score within each domain is

generated according to each subject's response. This manuscript presents data assessed with the MENQOL in terms of frequencies of all the items contained in each of the domains of the questionnaire as well as the relation between the most prevalent symptoms and socio-demographic data and the main findings of the MS screening program. Data presented as total scores, medians as well as the factors related to higher scores are presented in detail elsewhere [11].

Statistical analysis

Analysis of data was performed with the use of EPI-INFO 2000 statistical software (Centers for Disease Control and Prevention, Atlanta, GA, USA/World Health Organization, Geneva, Switzerland). Data is presented as means, medians, standard deviations, percentages, odds ratios and confidence intervals. Comparison of categorical data was performed with χ^2-test (univariate analysis). Multivariate analysis was performed to determine risk factors for presenting each of the most frequently presenting items per domain of the MENQOL. A $p < 0.05$ was considered as statistically significant.

Results

A total of 325 ($n = 325$) postmenopausal women fulfilling inclusion criteria participated in the MS screening program during the study period. Participant's age ranged from 40 to 70 years (mean: 55.9 ± 8.1; median: 54 years) of which 21.2% were aged 40–49 years; 51.4% (50–59 years) and 27.4% (60–70 years). Women in 56% of cases had a parity of 4 or more (median).

Frequency of the 29 items (menopausal symptoms) contained in each of the domains of the MENQOL questionnaire among all subject ($n = 325$) is depicted on Table 1. The two most frequent symptoms found within each of the domains of the MENQOL were: hot flushes (53.3%) and sweating (49.2%) (vasomotor); poor memory (80.6%) and feeling depressed (67.4%) (psycho-social); aching in muscles and joints (84%) and dry skin (85.5%) (physical). Only 206 women were sexually active at the moment of the survey. Of the three items contained in the sexual domain, the two most relevant ones found within these sexually active women were: avoiding intimacy (155/206: 76.2%) and change in their sexual desire (160/209: 76.5%). In regard to the latter, three women not having sexual activity responded to this item of the MENQOL.

Table 1 Menopausal symptoms among postmenopausal women ($n = 325$) within each domain of the MENQOL questionnaire

Domain	No. of cases (%)
Vasomotor	
1. Hot flushes or flashes	170 (53.3)
2. Night sweats	159 (48.9)
3. Sweating	160 (49.2)
Psycho-social	
4. Being dissatisfied with my personal life	147 (45.2)
5. Feeling anxious or nervous	213 (65.5)
6. Experiencing poor memory	262 (80.6)
7. Accomplishing less than I used to	218 (67)
8. Feeling depressed, down or blue	219 (67.4)
9. Being impatient with other people	182 (56)
10. Feelings of wanting to be alone	164 (50.5)
Physical	
11. Flatulence (wind) or gas pains	230 (70.8)
12. Aching in muscles and joints	274 (84.3)
13. Feeling tired or worn out	258 (79.4)
14. Difficulty sleeping	141 (43.4)
15. Aches in back of neck or head	206 (63.4)
16. Decrease in physical strength	262 (80.6)
17. Decrease in stamina	254 (78.1)
18. Feeling a lack of energy	255 (78.5)
19. Drying skin	278 (85.5)
20. Weight gain	192 (59.1)
21. Increased facial hair	63 (19.4)
22. Changes in appearance, texture or tone of your skin	232 (71.4)
23. Feeling bloated	107 (53.3)
24. Low backache	203 (62.5)
25. Frequent urination	172 (52.9)
26. Involuntary urination when laughing or coughing	167 (51.4)
Sexual	$n = 206$[a]
27. Change in your sexual desire	160/209 (76.5)[b]
28. Vaginal dryness during intercourse	153 (74.3)
29. Avoiding intimacy	155 (75.2)

[a] Only those sexually active

[b] Three non-sexually active subjects also responded

Socio-demographic data as well as the findings of the metabolic screening program in relation to the two most frequently found symptoms per domain (univariate analysis) is depicted in Table 2. During univariate analysis, hot flushes were less frequently encountered among older women (41.9% vs. 64.2%, $p < 0.05$). Women with five or more years of menopause onset were found to have lower rates of hot flushes and sweating (42% vs. 65.3% and 44.2% vs. 55.5%, $p < 0.05$, respectively). Women found to have high basal triglyceride levels had higher rates of sweating and depression (54% vs. 42.9% and 73.5% vs. 59.3%, $p < 0.05$, respectively). Women with abdominal obesity (waist circumference > 88 cm) were found to have

Table 2 Socio-demographic data and findings of the metabolic screening program in relation to the most frequent symptoms per domain: univariate analysis

	Parameter							
	Hot flushes, 170 (53.3)[a]	Sweating, 160 (49.2)	Poor memory, 262 (80.6)	Depression, 219 (67.4)	Muscle joint ache, 274 (84.3)	Dry skin, 278 (85.5)	Change in sexual desire, 160/209 (76.5)	Avoid intimacy, 155/206 (75.2)
Age ≥ 54 years (median)								
Yes: 174 (53.5)	73 (41.9)**	78 (44.8)	144 (82.8)	113 (64.9)	144 (82.5)	161 (92.5)*	70/91 (76.9)	66/88 (75)
No: 151 (46.5)	97 (64.2)	82 (54.3)	118 (78.1)	106 (70.2)	130 (86)	117 (77.5)	90/118 (76.3)	89/118 (75.4)
Parity > 4 (median)								
Yes: 182 (56)	95 (52.2)	90 (49.4)	152 (83.5)	124 (68.1)	155 (85.1)	152 (83.5)	72/91 (79.1)	72/96 (75)
No: 143 (44)	75 (52.4)	70 (49)	110 (76.9)	95 (66.4)	119 (83.2)	126 (88.1)	88/118 (74.6)	83/110 (75.4)
Mestizo								
Yes: 288 (88.6)	155 (53.8)	144 (50)	234 (81.3)	204 (70.8)*	243 (84.4)	248 (86.1)	144/187 (77)	140/184 (76)
No: 37 (11.4)	15 (40.5)	16 (43.2)	28 (75.7)	15 (40.5)	31 (83.8)	30 (81)	16/22 (72.8)	15/22 (68.2)
Urban residency								
Yes: 299 (92)	158 (52.8)	149 (49.8)	245 (81.9)*	202 (67.5)	252 (84.3)	255 (85.3)	145/191 (75.9)	141/189 (74.5)
No: 26 (8)	12 (46.1)	11 (42.3)	17 (65.4)	17 (65.4)	22 (84.6)	23 (88.5)	15/18 (83.3)	14/17 (82.3)
Menopause ≥ 5 years								
Yes: 181 (55.7)	76 (42)*	80 (44.2)*	145 (80.1)	122 (67.4)	151 (83.4)	162 (89.5)*	78/102 (76.5)	75/100 (75)
No: 144 (44.3)	94 (65.3)	80 (55.5)	117 (81.3)	97 (67.4)	123 (85.4)	116 (80.5)	82/107 (76.6)	80/106 (75.5)
Low income								
Yes: 164 (50.5)	86 (52.4)	82 (50)	133 (81)	114 (69.5)	131 (79.9)*	140 (85.4)	77/101 (76.2)	75/100 (75)
No: 161 (49.5)	84 (52.2)	78 (48.4)	129 (80.1)	105 (65.2)	143 (88.8)	138 (85.7)	83/108 (76.9)	80/106 (75.5)
Sedentary								
Yes: 167 (51.4)	87 (52)	83 (49.7)	136 (81.4)	118 (70.6)	144 (86.2)	77 (46.1)*	78/98 (79.6)	71/96 (76.5)
No: 158 (48.6)	83 (52.5)	77 (48.7)	126 (79.7)	101 (63.9)	130 (82.3)	134 (84.8)	82/111 (73.9)	84/110 (76.4)
Metabolic syndrome								
Yes: 135 (41.5)	70 (51.9)	71 (52.6)	109 (80.7)	97 (71.9)	115 (85.2)	117 (86.6)	66/83 (79.5)	66/82 (80.5)
No: 190 (58.5)	100 (52.6)	89 (46.8)	153 (80.5)	122 (64.2)	159 (83.7)	161 (84.7)	94/126 (74.6)	89/124 (71.8)
Hyperglycemia								
Yes: 54 (16.6)	31 (57.4)	32 (59.2)	44 (81.5)	39 (72.2)	46 (85.2)	52 (96.3)*	33/37 (89.2)*	31/36 (86.1)
No: 271 (83.4)	139 (51.3)	28 (47.2)	218 (80.4)	180 (66.4)	228 (84.1)	226 (83.4)	127/172 (73.8)	124/170 (72.9)
Hypertension								
Yes: 126 (38.8)	63 (50)	64 (50.8)	106 (84.1)	90 (71.4)	107 (84.9)	113 (89.7)	54/73 (74)	54/73 (74)
No: 199 (61.2)	107 (53.8)	96 (48.2)	156 (78.4)	129 (64.8)	167 (83.9)	165 (82.9)	106/136 (77.9)	101/133 (75.9)
Abdominal obesity								
Yes: 176 (54.2)	98 (55.7)	94 (53.4)	142 (80.7)	126 (71.6)	155 (88.1)**	149 (84.6)	84/110 (76.4)	84/109 (77)
No: 149 (45.8)	72 (48.3)	66 (44.3)	120 (80.5)	93 (62.4)	119 (79.9)	129 (86.6)	76/99 (76.8)	71/97 (74)
Obesity BMI ≥ 30								
Yes: 129 (39.7)	66 (51.2)	67 (52)	105 (81.4)	90 (69.8)	114 (88.4)	110 (85.3)	60/78 (76.9)	59/77 (76.6)
No: 196 (60.3)	104 (53)	93 (47.4)	157 (80.1)	129 (65.8)	160 (81.6)	168 (85.7)	100/131 (76.3)	96/129 (74.4)

Table 2 continued

	Parameter							
	Hot flushes, 170 (53.3)[a]	Sweating, 160 (49.2)	Poor memory, 262 (80.6)	Depression, 219 (67.4)	Muscle joint ache, 274 (84.3)	Dry skin, 278 (85.5)	Change in sexual desire, 160/209 (76.5)	Avoid intimacy, 155/206 (75.2)
Triglycerides ≥ 150 mg/dL								
Yes: 185 (56.9)	100 (54)	100 (54)*	151 (81.6)	136 (73.5)*	157 (84.9)	155 (83.8)	89/116 (76.7)	90/115 (78.3)
No: 140 (43.1)	70 (50)	60 (42.9)	111 (79.3)	83 (59.3)	117 (83.6)	123 (87.9)	71/93 (76.3)	65/91 (71.4)

* Significant difference $p < 0.05$ (χ^2 calculation)

[a] Numbers in parenthesis are percentages

higher rates of muscle and joint aches. Older women and those presenting abnormal fasting glucose levels were found to have higher rates of dry skin. Also women with basal hyperglycemia had higher rates of changes in sexual desire when compared to women with normal fasting glucose levels (89.2% vs. 73.8%, $p < 0.05$).

Multivariate analysis was also performed in order to determine risk factors involved for presenting each of the menopausal symptoms analyzed during univariate analysis (Table 3). As one can observe some risk factors found to be significant during univariate analysis were excluded from the regression model such as urban residency, mestizo race and low income whereas abdominal obesity was found as a significant risk factor for presenting hot flushes and depression, situation that was not found during univariate analysis.

Discussion

Although the climacteric phenomena has not been fully studied and explored in Latin America, research, particularly in Ecuador, is currently growing in this field. The menopause as well as other reproductive health issues such as abortions, adolescent pregnancies, sexually transmitted diseases can also be influenced by the marked socio-economical and demographical differences found across Latin American populations. For instance, we have recently determined that the age

Table 3 Risk factors for the most frequently presenting menopausal symptoms: multivariate analysis

Risk factor	
Hot flushes	
Age ≥ 54 years	(0.5: 0.3–0.9)[a,*]
Menopause ≥ 5 years	(0.5: 0.3–0.9)[a,*]
Abdominal obesity[b]	(2.1: 1.1–4.1)[a,*]
Sweating	
Menopause > 5 years	(0.6: 0.4–1)[a,*]
Triglyceride ≥ 150 mg/dL	(1.5: 1–2.4)[a]
Depression	
Abdominal obesity	(1.6: 1–2.6)[a,*]
Triglyceride > 150 mg/dL	(1.8: 1.1–3)[a,*]
Muscle and joint ache	
Abdominal obesity	(1.8: 1–3.4)[a,*]
Dry skin	
Age ≥ 54 years	(3.4: 1.7–6.8)[a,*]
Hyperglycemia[c]	(4.7: 1–20.3)[a,*]
Change in sexual desire	
Hyperglycemia	(2.9: 0.9–10.3)[a,*]

* $p < 0.05$

[a] In parenthesis: odds ratio and confidence interval

[b] Waist circumference > 88 cm

[c] Fasting glucose level ≥ 110 mg/dL

at menopause presentation in Latin America, Ecuador included, occurs earlier than in women from USA and Europe, where living in cities of high altitude and lower economical and educational level were found to be significant associated factors [4].

Physiological and psycho-social changes among climacteric women in Ecuador have recently been described: vasomotor, psychological and somatic symptoms and the loss of bone mass [14, 15, 30]. In a previous study, drawn upon low income climacteric women (pre–peri and postmenopausal) with the use of the Greene Climacteric Scale, we reported that the most frequently and intensive presenting symptoms were: difficulty in concentrating, feeling unhappy or distressed, headaches and hot flushes. In this series, severity of vasomotor symptoms, headaches and loss of sexual interest was found to be related to parity, and educational and economical level [30]. Rates of vasomotor symptoms were found to increase from one menopausal stage to the other and be higher than percentages found among other Latin American populations [1, 22, 27].

The present study focused specifically upon postmenopausal women who had participated in a MS screening program and aimed to primarily determine the prevalence of this entity and to secondarily assess QoL and the most frequently presenting menopausal symptoms contained in the MENQOL, another menopausal specific health related QoL tool recently validated in a Latin American population [2]. According to this tool, the two most frequently found symptoms within the vasomotor domain were hot flushes (53.3%) and sweating (49.2%). These rates were lower than those previously reported: (86.3%) and (69.6%), respectively [30]. Despite this, in general, the frequency of the remaining menopausal symptoms assessed through the MENQOL in the present research displayed similarity to our previous study using the Greene Climacteric Scale. In comparison to menopausal women of other ethnical background, our population presents some similarity as well as differences in relation to the frequency of menopausal symptoms [19, 25, 32].

Pedro et al. [27] reported higher rates of hot flushes and sweating among postmenopausal Brazilian women when compared to our postmenopausal women. These differences as well as those found within our Ecuadorian population regarding vasomotor symptoms warrants further research. However, as described by others, certainly socio-economic as well as demographic differences may explain these differences [29]. For instance, parity in the present study was not found to be related to higher rates of menopausal symptoms, especially vasomotor symptoms and sexual disturbances, as we have previously reported within a specific low income climacteric series [30].

The MS has been defined as a constellation of lipid and non-lipid risk factors that predispose subjects to develop cardiovascular disease [13]. During the menopausal transition there is an emergence of features related to this syndrome such as hypertension, hyperinsulinism, atherogenic lipid profiles and diabetes, which in turn increase cardiovascular risk [7, 31]. Using ATP III criteria, our screening program found that 41.5% had MS, hypertension (38.8%), hyperglycemia (16.6%), hypertriglyceridemia (56.9%) and abdominal obesity (54.2%), high prevalences when compared to other reports [3, 23, 24] and to best of our knowledge the only study addressing these issues among a postmenopausal Latin American population. The most important ATP III diagnostic feature presenting in women with MS was abdominal obesity as it presented in 83.7% of cases of MS. Obesity has demonstrated an increasing trend in Latin America and has been related to poverty and to the increase in the prevalence of diabetes and hypertension.

The present research found that abdominal obesity increased the risk for hot flushes twofold. This is consistent with the findings of others [12, 21, 33, 34]. While high triglyceride levels were found to increase the risk for hot flushes and sweating, older age and those with ≥5 years menopause decreased this risk. Data relating abnormal lipid levels and hot flushes is scarce or non-existent and warrants further research. Using another QoL instrument, the questionnaire on life satisfaction (QLS), obesity was found to impair QoL of healthy postmenopausal women, affecting their physical and psychological well being [20].

The effect of obesity over depressive symptoms in postmenopausal women varies. While one study has found no effect at all [18] another reported different grades of depression, anxiety, anger, distress, low levels of social support, impairing QOL severely [28]. Our data supports the findings of the latter as the rate of depression among our postmenopausal women with abdominal obesity was higher when compared to those non-obese. Additionally they were found to have muscle and joint aches in a higher degree.

Finally an increased risk for dry skin and changes in sexual desire were found among women exhibiting high fasting glucose levels (≥110 mg/dL). Older age was also related to a higher frequency of dry skin. Important to mention is that in a recent study it was reported that women who are Hispanic, obese, and/or diabetic were associated to an increased risk for vaginal dryness, irritation or itching, discharge and dysuria factors that increase sexual impairment [26].

In conclusion, in this postmenopausal Ecuadorian population, the frequency of menopausal symptoms, as assessed with the MENQOL, was found to be relatively similar to those from other Latin and non-Latin American populations and associated to age, hormonal status and related metabolic conditions. As abdominal obesity in our population was found to be an associated risk factor for the presentation of several menopausal symptoms, changes in lifestyle and dietary habits should be encouraged as an important and cost-effective measure, which in turn will improve QoL and decrease further cardiovascular risk.

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Assessment of insomnia and related risk factors in postmenopausal women screened for the metabolic syndrome

Peter Chedraui [a,*], Glenda San Miguel [a], Diego Villacreses [a], Andrea Dominguez [a], Winston Jaramillo [a], Gustavo S. Escobar [a], Faustino R. Pérez-López [b], Andrea R. Genazzani [c], Tommaso Simoncini [c], Research Group for the Omega Women's Health Project

[a] Institute of Biomedicine, Facultad de Ciencias Médicas, Universidad Católica de Santiago de Guayaquil, Ecuador
[b] Department of Obstetrics and Gynecology, Facultad de Medicina, Lozano Blesa University Hospital, Universidad de Zaragoza, Spain
[c] Molecular and Cellular Gynecological Endocrinology Laboratory, Department of Reproductive Medicine and Child Development, Division of Obstetrics and Gynecology, University of Pisa, Italy

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ABSTRACT

Background: Sleep disturbances are common during female mid life. Nevertheless, there is limited avail able information linking sleep characteristics to the menopause and the metabolic syndrome (METS).
Objective: To assess insomnia prevalence and related risk factors in postmenopausal women screened for the METS.
Methods: In this cross sectional study 204 natural postmenopausal women participating in a METS screening program filled out the Athens insomnia scale (AIS), the hospital anxiety and depression scale (HADS) and a general socio-demographic questionnaire. Criteria of the Adult Treatment Panel III (ATP-III) were used to define the METS.
Results: Median age of the whole sample was 56 years. A 50.5% of women had the METS, 57.4% hot flushes, 58.3% were abdominally obese, 51.5% hypertension, 25.0% hyperglycemia, 15.7% depressed mood and 29.9% anxiety. A 33.8% presented insomnia according to the AIS (scores 6 or more). The AIS displayed a high internal consistency as computed Cronbach's alpha was determined to be 0.86. Multiple linear regression analysis determined that male premature ejaculation, female psychotropic drug use, hot flush intensity, mood morbidity (higher total HADS scores) and higher parity positively and significantly correlated to higher AIS scores (more insomnia).
Conclusion: In this postmenopausal sample insomnia was not related to the METS or its components yet to other psycho somatic female and partner issues.

1. Introduction

Mid-life is a time related to several bio-psycho and social changes. Progressive decline in estrogen levels is frequently related to hot flushes, urogenital discomfort, muscle-skeletal limitations, depression and sleep disorders [1–4]. Regarding the latter, postmenopausal women have more sleep disturbances than premenopausal ones [5,6]. Indeed they report few hours of sleep and more fatigue or difficulty in initiating and maintaining sleep [7]. Kravitz et al. [8] have reported that 38% of women aged 40–55 present sleep difficulties significantly related to the menopause. Using the insomnia severity index (ISI) we have previously reported that 46.7% of postmenopausal Ecuadorian women report insomnia [9]. Using a different cut-off value for the same ISI, 36.6% of mid-aged Spanish women present insomnia [10]. Given the heterogeneity of sleep disturbances, various instruments have been designed to quantitatively and qualitatively assess sleep quality and its impact on every-day life [11]. One such test is the Athens insomnia scale (AIS) which has been developed to quantify sleep difficulty based on the international classification of diseases (ICD) in reference to mental and behavioral disorders [12]. Using the AIS, a recent large multinational Latin American study reported a 57.7% prevalence of insomnia among mid-aged women [13].

Sleep disorders have not only been associated to age and the menopause yet also to a number of chronic entities including cardiovascular disease, diabetes, obesity, the metabolic syndrome (METS) and mood disorders [14–18]. In this sense important to bear in mind is that the prevalence of the METS increases after the menopause [19] and that individually each of its components have also been related to sleep problems [20–22]. Despite the

* Corresponding author at: Instituto de Biomedicina, Facultad de Ciencias Médicas, Universidad Católica de Santiago de Guayaquil, PO Box 09-01-4671, Guayaquil, Ecuador. Tel.: +593 4 220 6958; fax: +593 4 220 6958.
E-mail address: peter.chedraui@cu.ucsg.edu.ec (P. Chedraui).

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aforementioned, there is limited available information linking sleep characteristics to the menopause and the METS. Thus, the aim of the following research was to assess insomnia prevalence and related risk factors using the AIS in postmenopausal women screened for the METS.

2. Methods

2.1. Participants and study design

This cross-sectional study was carried out from December 2011 to June 2012 at the Institute of Biomedicine of the Medical Faculty of the *Universidad Católica de Santiago de Guayaquil*, Guayaquil, Ecuador. The initiative aimed at assessing menopause related quality of life (QoL) and the prevalence of the metabolic syndrome (METS), insomnia and mood problems among non hormone therapy (HT) using natural postmenopausal women (40–65 years), recruited through newspaper advertising. Women taking phytoestrogens or drugs intended to decrease lipid levels were excluded from the study. The study protocol was approved by the Medical Faculty's Bioethics Committee. Eligible women were asked to attend the Institute to be informed about the study, its purposes and provide written consent of participation. Those consenting and fulfilling the inclusion criteria were asked to return after an 8 h overnight fast, moment in which socio-demographic data, waist circumference, weight, height and blood pressure measurements were recorded. Also a 10–15 mL peripheral venous blood sample was obtained to provide plasma, serum and white cells. Women were counseled and managed according to the results and participated in educational group sessions aimed to discuss topics related to the menopause, the METS, its risk determinants and cardiovascular risk implications.

2.2. General questionnaire

An itemized questionnaire was constructed to assess and record all general data. This tool was validated in 50 women before being implemented and included the following female data: age, marital status, educational level, parity, years since menopause onset, perceived healthiness and current partner status (yes/no). Lifestyle and other personal factors included in this section were smoking habit, alcohol and coffee consumption, psychotropic drug use, and sedentarism. Postmenopausal women were further categorized as early postmenopausal (1–4 years) and late postmenopausal (≥5 years). Women provided the information related to their partner including: age, educational level, unfaithfulness (yes/no), alcoholism (yes/no), and the presence of sexual dysfunction (erectile dysfunction and or premature ejaculation). Definitions for alcoholism, erectile dysfunction, and premature ejaculation have previously been described [4]. The presence and severity of hot flushes were assessed with item 1 of the menopause rating scale (MRS) as described elsewhere [23].

2.3. Diagnostic criteria for the metabolic syndrome

NCEP-ATP-III diagnostic criteria were used to define the METS [24]. This was the case if three or more of five criteria were encountered: abdominal obesity (waist circumference > 88 cm), increased serum triglycerides (TG) (≥150 mg/dL), decreased high density lipoprotein cholesterol (HDL-C) (<50 mg/dL), high fasting glucose (≥110 mg/dL) and increased blood pressure (≥130/85 mmHg) [24]. A waist circumference cut off value of 88 cm has recently been reported optimal for defining the METS in postmenopausal Latin American women [25]. Women taking oral hypoglycemic or antihypertensive medication prescribed by a physician were considered, respectively, as diabetic or hypertense independent of the serum or blood pressure findings. After a 10 min resting period in sitting position, mean blood pressure was determined by performing two separate determinations 10 min apart.

Weight (kg) and height (m) were recorded and body mass index (BMI) calculated for each participant as weight (in kg) divided by the square of height (in m). Obesity was defined as a BMI ≥30 kg/m^2 [26]. Waist circumference expressed in centimeters was obtained from women in supine position. Subjects were defined as sedentary if carrying out less than 15 min of physical activity twice per week [27].

2.4. Validated tools

2.4.1. The Athens insomnia scale (AIS)

The AIS [28] is a self-administered psychometric instrument designed to quantify sleep difficulty based on the ICD [12]. It consists of eight items: the *first four* assess sleep induction, night awakenings, early awakenings and sufficiency of total sleep duration. The *fifth item* assesses sleep quality, and the *last three* the impact of insomnia over day time performance. Items can be rated from 0 to 3, higher scores denoting more impaired sleep. The total score (sum of all rated items) may range from 0 to 24, with scores of 6 or more used to define insomnia. This study used the Spanish language validated AIS [29].

2.4.2. The hospital anxiety and depression scale (HADS)

This tool was developed to identify cases of anxiety and depression in non-psychiatric settings [30]. It includes 14 items, seven for anxiety (odd numbered items scored 3–0) and seven for depression (even numbered items scored from 0 to 3). Items of each sub-scale are summed up to provide a total anxiety and depression score. A total cut off value of 8 or more on each sub-scale was used to identify cases of anxiety and depressed mood. The total score for the HADS was computed by summing scores obtained on each sub-scale. These values, representing global mood morbidity, were used as an independent variable for the multiple linear regression analysis.

2.5. Serum assays

Blood samples withdrawn from each participant were centrifuged at 5 °C for 10 min at 3000 rpm. Obtained serum, plasma and white cells were decanted into 0.5 mL aliquots and then stored at −70 °C. TG, HDL-C and glucose levels were assayed using the enzymatic colorimetric method with a Hitachi 717 automatic photometric analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

2.6. Statistical analysis

Statistical analysis was performed using the SPSS version 19.0 (IBM, Armonk, NY, USA). Data are presented as medians (interquartile ranges [IQR]), percentiles (p25–p75), percentages, beta coefficients and 95% confidence intervals. The Kolmogorov–Smirnov test was used to determine the normality of data distribution and the Bartlett test to evaluate the homogeneity of the measured variance. According to this, non-parametric continuous data were compared with the Mann–Whitney *U* test (two independent samples) or the Kruskal–Wallis test (various independent samples). Spearman Rho coefficients were calculated to determine correlations between total AIS scores and various numeric variables (bivariate analysis). Cronbach's alphas were computed for the AIS and HADS to determine their internal consistency.

Multiple linear regression analysis was performed to assess variables related to higher AIS scores and, hence, worse

Table 1
Baseline characteristics of studied women and their partners and AIS total scores according to these features.

Female data	*n* = 204	Total AIS scores
Age (years)	56 [7; 53–60]	
40–49	19 (9.3)	3 [5; 1–6]
50–59	126 (61.8)	3 [5; 2–7]
60–65	59 (28.9)	4 [5; 2–7]
		$p = 0.54^*$
Educational level (years)	10 [9; 6–15]	
0–6	73 (35.8)	4 [6; 2–8]
7–12	68 (33.3)	4 [5; 2–7]
≥13	63 (30.9)	2 [5; 1–6]
		$p = 0.10$
Marital status		
Married	93 (45.6)	3 [5; 2–7]
Single	37 (18.1)	3 [4; 1–5]
Widowed	18 (8.8)	6.5 [9; 2–11]
Divorced	31 (15.2)	3 [6; 1–7]
Cohabiting	25 (12.3)	4 [6; 2–8]
		$p = 0.09$
Parity	3 [2; 2–4]	
0	17 (8.3)	3 [3; 2–5]
1–5	160 (78.4)	3 [6; 1–7]
>5	27 (13.2)	7 [10; 2 12]
		$p = 0.006$
Time since menopause onset (years)	7.5 [10; 3–13]	
1–4	86 (42.1)	4 [6; 1–7]
≥5	118 (57.9)	3 [5; 2–7]
		$p = 0.75$
Tobacco consumption		
Yes	4 (2.0)	84.5 [21; 70 91]
No	200 (98.0)	88 [16; 81–97]
		$p = 0.23$
Hot flushes present		
Yes	117 (57.4)	4 [6; 2–8]
No	87 (42.6)	3 [5; 1–6]
		$p = 0.04$
Depression (HADS ≥ 8; depression subscale)		
Yes	32 (15.7)	5 [6; 3–9]
No	172 (84.3)	3 [6; 1 7]
		$p = 0.01$
Anxiety (HADS ≥ 8; anxiety subscale)		
Yes	61 (29.9)	6 [6; 3–9]
No	143 (70.1)	3 [5; 1–6]
		$p = 0.0001$
Psychotropic use		
Yes	17 (8.3)	5 [7; 3–10]
No	187 (91.7)	3 [6; 1–7]
		$p = 0.02$
Healthiness (perception)		
Yes	160 (78.4)	3 [5; 1–6]
No	44 (21.6)	5 [6; 2–8]
		$p = 0.07$
Sedentarism		
Yes	69 (33.8)	3 [5; 1–6]
No	135 (66.2)	4 [5; 2 7]
		$p = 0.04$
Currently has partner		
Yes	127 (62.3)	3 [6; 1–7]
No	77 (37.7)	4 [5; 2–7]
		$p = 0.42$
Sexually active		
Yes	100 (49.0)	3 [6; 1–7]
No	104 (51.0)	4 [5; 2–7]
		$p = 0.57$
Diagnostic features of the METS		
Abdominal circumference (cm)	91.0 [15; 84 99]	
Abdominal obesity (waist > 88 cm)		
Yes	119 (58.3)	4 [5; 2–7]
No	85 (41.7)	3 [6; 1–7]
		$p = 0.91$
Glycemia (mg/dL)	94.0 [22; 88–110]	
Hyperglicemia (≥110 mg/dL)		
Yes	51 (25.0)	4 [5; 2–7]
No	153 (75.0)	3 [6; 1–7]
		$p = 0.38$
Systolic blood pressure (mmHg)	123.5 [26; 114–140]	
Diastolic blood pressure (mmHg)	80.0 [12; 70–82]	
Hypertension (>130/85 mmHg)		
Yes	105 (51.5)	4 [5; 2–7]
No	99 (48.5)	3 [5; 2–7]
		$p = 0.55$
Triglycerides (mg/dL)	143.0 [96; 104–200]	
Hypertriglyceridemia (≥150 mg/dL)		
Yes	93 (45.6)	4 [6; 1–7]
No	111 (54.5)	3 [5; 2–7]
		$p = 0.54$
HDL-C (mg/dL)	46.9 [21; 40–61]	
Low HDL-C (<50 mg/dL)		
Yes	115 (56.4)	3 [6; 1–7]
No	89 (43.6)	4 [5; 2–7]
		$p = 0.32$
Body mass index (BMI, kg/m²)	28.2 [6.5; 25–31.5]	
(BMI ≥ 30.0)		
	78 (38.2)	3.5 [4; 2–6]
	126 (61.8)	3.5 [6; 1–7]
		$p = 0.70$
METS		
3 or more positive items		
	103 (50.5)	4 [6; 1–7]
	101 (49.5)	3 [5; 2 7]
		$p = 0.55$
Partner	*n* = 127	
Alcohol abuse		
Yes	40 (31.5)	3 [5; 2–7]
No	87 (68.5)	3 [6; 1–7]
		$p = 0.77$
Erectile dysfunction		
Yes	44 (34.6)	5 [7; 2–9]
No	83 (65.4)	3 [5; 1 6]
		$p = 0.02$
Premature ejaculation		
Yes	32 (25.2)	4.5 [8; 2–10]
No	95 (74.8)	3 [5; 1–6]
		$p = 0.07$

Data are presented as medians [inter quartile ranges; p25–p75] or percentages *n* (%); variables not displayed were not significant; AIS, Athens insomnia scale; HADS, hospital anxiety and depression scale; METS, metabolic syndrome; HDL-C, high density lipoprotein cholesterol.

* *p* values determined with the Mann–Whitney or the Kruskal–Wallis test according to case.

insomnia. Two regression models were generated, *the first* including all surveyed women and *the second* only those with a partner. For each model a primary regression model was generated using a forward/backward stepwise procedure and included all potential interaction variables. This model was constructed from independent variables achieving $p = 0.10$ during bivariate analysis. Subsequently a final reduced best fit model was generated without interaction variables. The dependent variable was the total AIS score. Independent variables tested during bivariate analysis included: age, parity, educational level, marital status, time since the menopause, presence of the METS and its diagnostic features, healthiness status, sedentarism, smoking habit, hot flush presence, global mood morbidity, coffee and psychotropic drug consumption, partner variables, among the most important. A *p* value less than 0.05 was considered statistically significant.

Table 2
Sleep problems identified with each of the items of the Athens insomnia scale and in accordance to the presence of mood morbidity ($n = 204$).

AIS items	n (%)[a]	Mood morbidity[b] $n = 80$	No mood morbidity $n = 124$
Difficulty with sleep induction (item 1)	90 (44.1)	44 (55)	46 (37.0)[†]
Awakening during the night (item 2)	101 (49.5)	52 (65)	49 (39.5)[†]
Early morning awakening (item 3)	81 (39.7)	39 (48.8)	42 (33.9)[†]
Insufficient total sleep time (item 4)	96 (47.0)	48 (60)	48 (38.7)[†]
Insufficient overall quality of sleep (item 5)	70 (34.3)	41 (51.3)	29 (23.4)[†]
Decreased well being during the day (item 6)	78 (38.2)	44 (55)	34 (27.4)[†]
Decreased functioning during the day (item 7)	73 (35.8)	40 (50)	33 (26.6)[†]
Sleepiness during the day (item 8)	133 (65.2)	65 (81.3)	68 (54.9)[†]
Insomnia (total score ≥ 6)	69 (33.8)	42 (52.5)	27 (21.8)[†]

[a] Percentage of women obtaining scores from 1 to 3 inclusive.
[b] As defined by a total HADS score > 8 (median).
[†] $p < 0.05$ as determined with the chi square test when comparing to the mood morbidity group.

2.6.1. Sample size calculation

A minimal sample size of 179 participants was calculated assuming that 35% of surveyed women would present insomnia with a 7% desired precision and a 95% confidence level.

3. Results

During the study period a total of 207 natural postmenopausal women were invited to participate. Three did not meet inclusion criteria; hence statistical analysis was performed on 204 complete surveys. Baseline characteristics of studied women and their partners and AIS total scores according to these features are depicted in Table 1. Median age for the whole sample was 56 years. A 57.4% of surveyed women displayed hot flushes, 15.7% depressed mood, 29.9% anxiety, 8.3% used psychotropic drugs, 62.3% currently had a partner, 58.3% were abdominally obese (38.2% according to BMI value) and 50.5% displayed METS (ATP III criteria). More than 40% of women had hypertension or dyslipidemia (low HDL-C or high TG levels). Regarding the partner, 31.5% abused alcohol, 34.6% displayed erectile dysfunction and 25.2% premature ejaculation. AIS total scores were found to be significantly higher in relation to parity, sedentarism, psychotropic drug use, male erectile dysfunction and the presence of hot flushes or mood morbidity (bivariate analysis).

Sleep problems identified with each of the items of the AIS and in accordance to the presence of mood morbidity are presented in Table 2. A 33.8% of all surveyed women presented insomnia defined as a total AIS score of 6 or more. More than 30% of women presented some degree of sleep problems identified with one of the eight items composing the AIS, diurnal sleepiness (65.2%) and night awakenings (49.5%) being the most prevalent ones. Insomnia and sleep problems (individually identified with each items of the AIS) were significantly more prevalent in women with mood morbidity. Spearman correlations are presented in Table 3. Mild significant correlations were found between total AIS scores and female educational level (inverse) and parity and hot flush severity (positive). A moderate positive correlation was found with total HADS scores. The AIS and HADS displayed a high internal consistency as computed Cronbach's alphas were determined to be high (0.86 and 0.80, respectively).

The final multivariate regression models for factors related to higher total AIS scores (more insomnia) are presented in Table 4. Psychotropic drug use, hot flush intensity, mood morbidity (higher total HADS scores) and higher parity were positively and significantly correlated to higher AIS scores (more insomnia). A similar model was determined when only women with a partner were analyzed which included male premature ejaculation, mood morbidity and parity.

4. Discussion

The menopausal transition is frequently associated to hot flushes, night sweats, painful joints, poor self-rated health and sleeping problems [31]. Sleep disorders are highly prevalent among mid-aged subjects, with a higher rate seen in women [32,33]. Some studies have evidenced a significant relationship between sleep problems and the menopause [3,9,13] whereas others not [34]. Although various questionnaires have been designed to allow subjective sleep assessment (most convenient for epidemiological studies), objective sleep evaluation requires the use of polysomnography [35].

The present study aimed at assessing insomnia and related risk factors in a sample of postmenopausal women invited to participate in a METS screening program. Studied women displayed features such as low education, high parity and a high prevalence of the METS and its components, which is consistent with a previous Ecuadorian [19] and Latin American report [36]. We have previously reported that mid-aged women present insomnia in 41.5% and sleepiness in 33.6% as measured with the ISI [9] and the Epworth sleepiness scale [3] respectively. In these studies, rates increased in relation to the menopausal status. Despite this, each tool explores a specific component of sleep. Contrary to this, the AIS has the property of assessing different aspects of sleep and also sleepiness or daytime functioning [28]. In the present research the AIS tool displayed a good reliability (Cronbach's alpha was high: 0.86) and determined that one third to a half of women displayed some type of sleep disturbance: insomnia (33.8%), decreased

Table 3
Correlation coefficients between total AIS scores and various numeric variables (bivariate analysis).

Parameters	Coefficient	p value
Female age (years)	0.07	0.28
Educational level (years)	−0.16	0.01
Time since menopause onset (years)	0.05	0.43
Parity	0.18	0.01
Hot flush severity	0.19	0.006
HADS total score	0.37	0.0001
Monthly coital frequency	−0.05	0.46
Abdominal circumference (cm)	0.06	0.37
Glycemia (mg/dL)	0.08	0.25
Systolic blood pressure (mmHg)	0.11	0.10
Diastolic blood pressure (mmHg)	0.03	0.62
Triglycerides (mg/dL)	0.02	0.68
HDL-C (mg/dL)	0.08	0.20
BMI	−0.01	0.90
Number of METS items	0.02	0.76
Partner age (years)	0.02	0.78
Partner educational level (years)	−0.16	0.07

AIS, Athens insomnia scale; BMI, body mass index; HDL-C, high density lipoprotein cholesterol.

Table 4
Factors related to higher total AIS scores among women (all and with a partner): final multivariate regression models.

Model considering all studied women					
Factors	Beta coefficient	Standard error	95% CI	t	p value
Psychotropic use	2.78	0.97	0.85–4.71	2.85	0.005
Hot flush intensity	0.67	0.28	0.16–1.23	2.47	0.01
HADS total score (mood morbidity)	0.18	0.04	0.10–0.26	4.43	0.0001
Parity	0.41	0.11	0.19–0.63	3.71	0.0001
$r^2 = 0.56$; adjusted $r^2 = 0.54$, $p < 0.0001$					
Model considering only women with a partner					
Factors	Beta coefficient	Standard error	95% CI	t	p value
Male premature ejaculation	1.95	0.80	0.35–3.55	2.41	0.01
HADS total score (mood morbidity)	0.24	0.05	0.13–0.36	4.32	0.001
Parity	0.64	0.19	0.25–1.02	3.27	0.001
$r^2 = 0.51$; adjusted $r^2 = 0.50$, $p < 0.0001$					

AIS, Athens insomnia scale; CI, confidence intervals.

daytime functioning (35.8%) and sleepiness (65.2%). These figures are consistent with those recently reported among Latin American mid-aged women [13]. However, although many mid-aged women report vasomotor symptoms together with sleep problems, when covariates are assessed this association seems to depend more on the awareness and the recall of vasomotor symptoms than on their physiologic occurrence [37].

Despite finding a high prevalence for the METS and its components these were not found to be related to insomnia. This is contrary to what can be found in the literature [22,38]. Our data supports the theory that psycho-somatic and social problems as opposed to the metabolic profile, seems to be more relevant in our population in favoring sleep disturbances. Indeed, our regression model found that non metabolic aspects related to higher AIS scores and hence more insomnia.

Mood morbidity is highly frequent during the menopausal transition [39,40]. As reported with the HADS, 15.7% and 29.9% of our postmenopausal women displayed depressed mood and anxiety respectively and 8.3% were on psychotropic drug treatment. These were found to be independent risk factors related to higher total AIS scores and hence more insomnia. Insomnia and sleep problems (individually identified with each item of the AIS) were significantly more prevalent in women with mood morbidity. This is in agreement with other reports [39,41]. Indeed, in the general population, anxiety has been associated to a 4 fold increase in the risk for insomnia [42]. In correlation with the present study we have previously reported that psychotropic drug use related to insomnia as assessed with the ISI [9]. Interestingly, depression occurs more commonly during the menopausal transition in women with vasomotor symptoms than in those without [43]; however most women with these symptoms do not develop depression. Mood morbidity and hot flush intensity were both significant and independent risk factors for insomnia in our regression model. It seems, though, that the changes observed during the menopause may represent a window of vulnerability to mood disorders or aggravation in those with minor depressive disorders [39,44]. In addition, as objectively measured, sleep quality is worse in depressed women as compared to non-depressed ones [45].

The relationship between hot flushes and sleep disturbances has been reported by other investigators [8,9,46], as well as the direct association between hot flush severity and insomnia prevalence [5]. This is in agreement with the findings of the present study in which hot flush intensity positively correlated with higher AIS total scores (more insomnia).

Finally the present study also found that insomnia (higher AIS scores) was significantly related to higher parity and male premature ejaculation. This is not the first Hispanic study providing evidence that male issues are key aspects related to female midlife health. For instance, while Arakane et al. [9], reported insomnia related to male erectile dysfunction and unfaithfulness, Cuadros et al. [10] found this correlation only with male unfaithfulness. Sexual dysfunction in men has been associated to male physical and psychological health issues such as diabetes, cardiovascular disease, metabolic syndrome, impaired quality of life and depression [47]. Its relationship to female sleep problems remains to be ascertained. In our study, women with higher parity displayed increased sleep problems; nevertheless the exact involved mechanism is difficult to ascertain but may rely on stress perhaps related to still having to take care of young children.

As for the limitations of the present study one can mention *first* its cross-sectional design and *second*, as this was a local sample, data cannot therefore be extrapolated to the rest of the Ecuadorian or Latin American population. Although women provided partner data this was recall and did not include all socio-demographical partner features. This may also be seen as a limitation. Despite these potential limitations, it is important to mention that sleep data coming from Hispanic populations is scarce and our study may indeed be the first to provide this data from Latin American postmenopausal women, specifically exploring the relationship between the menopause and the METS, which may be considered as a strength. Although the METS was excluded as a possible risk factor for insomnia more research is warranted in this regard.

In conclusion, in this postmenopausal sample insomnia was not related to the METS or its components yet to other psycho-somatic female and partner issues.

Contributors

Peter Chedraui, Faustino R. Pérez-López, Tommaso Simoncini and Andrea Genazzani were involved in the conception and design of the study. Glenda San Miguel, Winston Jaramillo, Andrea Dominguez and Diego Villacreses conducted the clinical surveys. Gustavo Escobar performed biochemical assays. Peter Chedraui performed the statistical analysis. Faustino R. Pérez-López, Peter Chedraui and Tommaso Simoncini performed drafting of the manuscript. All authors were involved in critically revising the manuscript for its intellectual content and approving the final version.

Competing interest

The authors declare no conflict of interests.

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Provenance and peer review

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Chapter 5

Inflammation and endothelial dysfunction in postmenopausal metabolic syndrome

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Nitric oxide and pro-inflammatory cytokine serum levels in postmenopausal women with the metabolic syndrome

Peter Chedraui[1], Gustavo S. Escobar[1], Cecibel Ramírez[1], Faustino R. Pérez-López[2], Luis Hidalgo[1], Paolo Mannella[3], Andrea Genazzani[3] & Tommaso Simoncini[3]

[1]Institute of Biomedicine, Medical Faculty, Catholic University of Santiago of Guayaquil, Guayaquil, Ecuador, [2]Department of Obstetrics and Gynecology, Medical Faculty, University of Zaragoza, Clinical Hospital of Zaragoza, Zaragoza, Spain, and [3]Molecular and Cellular Gynecological Endocrinology Laboratory, Department of Reproductive Medicine and Child Development, Division of Obstetrics and Gynecology, University of Pisa, Pisa, Italy

***Background*: The metabolic syndrome (METS) increases after the menopause which may enhance cardiovascular risk in part explained by a pro-inflammatory state. *Objective*: Measure nitric oxide (NO), tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) serum levels in postmenopausal women with and without the METS (Adult Treatment Panel III criteria). *Methods*: Analyte levels were compared among those with and without the syndrome and each of its diagnostic components. Rho Spearman coefficients were also calculated to determine correlations between analyte levels and various numeric variables. *Results*: Median age of all studied women ($n = 88$) was 54.4 years, 62.5% had abdominal obesity, 14.8% hyperglycemia, 59.1% high triglycerides (TG) and 44.3% hypertension. Women with the METS ($n = 44$) displayed higher body mass index values and higher rates of abdominal obesity, hyperglycemia, hypertriglyceridemia, hypertension and low HDL-C levels. Median NO and IL-6 levels were significantly higher in women with the METS as compared to controls ($p < 0.05$). Independent of presenting the METS, analytes were higher in those displaying abdominal obesity (IL-6), hypertension (IL-6 and TNF-α) and more METS diagnostic criteria and abnormal HDL-C, TG and glucose levels (NO). Both cytokines positively correlated with the number of METS criteria, age and time since menopause, IL-6 positively with waist circumference and TNF-α positively with blood pressure levels. NO levels inversely correlated with HDL-C values and positively with the number of METS criteria, glucose, and TG levels; correlation with the latter being the highest ($r^2 = 0.65$, $p = 0.0001$). *Conclusion*: Postmenopausal women with the METS displayed higher IL-6 and NO levels, with significant correlations found between studied analytes and some of the components of the syndrome.**

Keywords: Cytokines, inflammation, interleukin 6, metabolic syndrome, nitric oxide, obesity, postmenopause, tumor necrosis factor-α

Introduction

Cardiovascular risk increases with age and in relation to obesity, hypertension, insulin resistance and dyslipidemia. These entities have commonly been grouped under the definition of the metabolic syndrome (METS) [1–3]. Diagnostic criteria for this syndrome have been proposed by The Third Report of the US National Cholesterol Program (NCEP) Adult Treatment Panel (ATP-III) [1]. Overall prevalence of the METS is about 20–30% (men and women), with an increasing trend worldwide [4,5], especially in women after the menopause [6,7]. It has been previously reported in Ecuador that 41.6% of postmenopausal women present this syndrome [8].

The METS could be related to endothelial dysfunction and hence to abnormal NO and cytokine production [9,10]. NO effects include: vasodilatation, anti-platelet aggregation, and antioxidant, anti-adhesive and anti-proliferative actions [11]. Overproduction of pro-inflammatory cytokines occurs in the adipose tissue of patients with the METS and is associated to insulin resistance [10]. Insulin sensitivity and NO production suggest a link between endothelial function and insulin secretion [12]. In patients with the METS reduced NO release may contribute to atherogenesis and increased thrombus production [13]. Hence it is likely that adequate NO production may exert beneficial effects in individuals with this syndrome [14].

Several studies seem to point out to the fact that NO and cytokine secretion among postmenopausal women with the METS may be altered [15–18]. The aim of the present research was to measure nitric oxide (NO), tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) serum levels in postmenopausal women with and without the METS (ATP-III criteria).

Methods

Study design and participants

NO, IL-6 and TNF-α were measured in the serum of a total of 88 natural postmenopausal women (amenorrhea >1 year), aged 40 or more, non hormone therapy (HT) users. Women had previously participated in a METS screening program at the Institute of Biomedicine of the Universidad Católica of Guayaquil Ecuador [8]. A control was selected for each METS case matched for age and time since the menopause. Comparisons were performed according to the presence or not of the METS and of each of its diagnostic components. All participants were informed about the research, its purposes and provided written consent. Those taking drugs intended to

Correspondence: Peter Chedraui MD, MSc, Instituto de Biomedicina, Facultad de Ciencias Médicas, Universidad Católica de Santiago de Guayaquil, PO Box: 09-01-4671, Guayaquil, Ecuador. Tel/Fax: +(5934) 220–6958. E-mail: peter.chedraui@cu.ucsg.edu.ec

decrease lipid levels were excluded. Subjects were defined as sedentary if they performed less than 15 min of physical activity twice per week [19]. Research protocol was approved by the Bioethics Committee of the Medical Faculty of the Universidad Católica de Santiago de Guayaquil, Ecuador.

Diagnostic criteria for the metabolic syndrome

NCEP-ATP-III diagnostic criteria were used to define the METS [1]. This was the case if three or more of five criteria were found: abdominal obesity (waist circumference >88 cm), increased serum triglycerides (TG) (≥150 mg/dL), decreased high density lipoprotein cholesterol (HDL-C) (<50 mg/dL), high fasting glucose (>110 mg/dL) and increased blood pressure (>130/85 mmHg) [1]. Additionally, ATP III cut-off values set at 240 and 160 mg/dL were used to categorize women with high total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels, respectively [1]. Women taking oral hypoglycaemic or antihypertensive medication prescribed by a physician were considered as diabetic or hypertense independent of the serum or blood pressure findings. After a 10 min resting period in sitting position, mean blood pressure was determined by performing two separate determinations 10 min apart.

Weight (kg) and height (m) were recorded and body mass index (BMI) calculated for each participant as weight (in kg) divided by the square of height (in meter). Obesity was defined as a BMI ≥30 kg/m^2 [20]. Waist circumference expressed in centimeters was obtained from women in supine position.

Serum assays

A peripheral blood sample of 10–15 mL was withdrawn from each participant after an 8 h overnight fast and centrifuged at 5°C for 10 min at 3000 rpm. Obtained serum was decanted into 1.5 and 2.0 mL aliquots and stored at −70°C until analysis.

Biochemical and cytokine assays

TC, TG, LDL-C, HDL-C and glucose levels were assayed with a Hitachi 717 automatic photometric analyzer (Roche Diagnostics GmbH, Mannheim, Germany). IL-6 and TNF-α were assayed with an Immulite 1000 (Siemens Medical Solutions Diagnostics, Los Angeles, USA) using enzyme amplified chemiluminescence method. Analytic sensitivity was 1.6 and 2 pg/mL for IL-6 and TNF-α, respectively.

Nitrite assay

Nitric oxide production was determined by a nitrite assay using 2, 3-diaminonaphthalene. Fluorescence of 1-(H)-naphthotriazole was measured by excitation and emission wavelengths of 365 and 450 nm. Standard curves were constructed with sodium nitrite. A stock solution of 1 mmol NO_3 in PBS (pH 7.4) was prepared and then standards (from 0 to 50 μM/L) were obtained by diluting the stock solution in PBS solution (pH 7.4). Two hundred and fifty micro liter standard, blank, control, and serum samples were diluted by adding 250 μL PBS to plastic vials. Each mixture was then thoroughly mixed and the serum centrifuged in spin filter columns for 60 min at room temperature. Subsequently, 100 μL Griess reagent, 1% sulphanilamide plus 0.1% naphthyl-ethylenediamine dihydrochloride prepared in 3.1% H_3PO_4 solution was added to each vial. Reagents were mixed for 1 min at 185 rpm on a shaker and plates incubated for 10 min at 37°C. Samples were placed in a spectrophotometer and the absorbance was read at 490 nm. Nonspecific fluorescence was determined in the presence of NG-monomethyl-L-arginine (3 mmol/L). All samples, kit controls and standards were analysed in duplicates.

Statistical analysis

Statistical analysis was performed using SPSS statistical package (Version 19.0 for Windows, SPSS Inc, Chicago, Illinois, USA). Data are presented as medians, interquartile ranges, percentages and coefficients. The Kolmogorov–Smirnov test was used to assess the normality of data distribution. According to this, continuous non parametric data were compared with the Mann–Whitney U test (for two independent samples) or the Kruskal–Wallis test (for various independent samples). Chi-square and Fisher's exact tests were used to compare percentages. Rho Spearman coefficients were calculated to determine correlations between NO and cytokine levels and various numeric variables. A p value of <0.05 was considered as statistically significant.

Results

Median age of all studied women (n = 88) was 54.4 years, 62.5% had abdominal obesity, 14.8% hyperglycemia, 59.1% high TG and 44.3% hypertension. Women with the METS (n = 44) displayed higher BMI values and higher rates of abdominal obesity, hyperglycemia, hypertriglyceridemia, hypertension and low HDL-C levels. Median NO and IL-6 levels were significantly higher in women with the METS as compared to controls (p < 0.05) (Table I).

NO and cytokine levels according to each diagnostic component are depicted on Table II. Independent of having the METS, analytes were higher in those displaying abdominal obesity (IL-6), hypertension (IL-6 and TNF-α), and more METS diagnostic criteria and abnormal HDL-C, TG and glucose levels (NO) (Table II).

Both cytokines positively correlated with the number of METS criteria, age and time since menopause, IL-6 positively correlating with waist circumference and TNF-α positively with blood pressure levels. NO levels inversely correlated with HDL-C values and positively with the number of METS criteria, glucose and TG levels; correlation with the latter being the highest (r^2 = 0.65, p = 0.0001) (Table III).

Discussion

The METS involves inflammatory activity and endothelial dysfunction. However, its pathophysiology is still a matter of debate. Indeed, studies exploring the exact role of inflammatory markers in the pathogenia of the METS and related adverse cardiovascular outcomes are scarce. Nevertheless, the hypothesis that chronic low-grade inflammation is associated to insulin resistance is widely accepted [21]. The main goal of the present research was to measure NO and cytokine levels in women with and without the METS and correlate levels with each of its components. It was found that NO levels were higher in women with the METS, situation correlated to altered serum HDL-C, TG and glucose levels. NO is endogenously synthesized from L-arginine and acts as a cell-to-cell mediator or signal. Its half-life is very short and is highly reactive [22]. Our data contrasts with that found in the literature indicating that individuals with the METS display lower NO levels [23]. The higher NO levels found in our series may well reflect a compensatory feed-back mechanism perhaps highlighting the presence of an earlier stage of the syndrome. Diverse results found in the literature could also be contrasting differences of the studied population (gender, age, hormonal and nutritional status) or the used NO measuring methodology. Conflictive

Table I. Baseline characteristics according to the presence or not of the METS.

Parameters	METS ($n = 44$)	non METS ($n = 44$)	p Value*
Age (years)	54.5 [9.0]	54.4 [9.0]	0.98
Time since menopause onset (years)	5.0 [8.0]	4.0 [8.0]	0.82
Sedentary (%)	20 (45.5)	20 (45.5)	0.99
Body mass index (kg/m^2)	31.2 [5.8]	27.6 [5.9]	0.001
Abdominal obesity >88 cm (%)	40 (90.9)	15 (34.1)	0.0001
Glycemia >110 mg/dl. (%)	13 (29.5)	0 (0.0)	0.0001
Triglycerides >150 mg/dl. (%)	36 (81.8)	16 (36.4)	0.0001
HDL-C <50 mg/dl. (%)	38 (86.4)	15 (34.1)	0.0001
Blood pressure ≥130/85 mmHg (%)	26 (59.1)	13 (29.5)	0.005
Total cholesterol ≥240 mg/dL (%)	13 (29.5)	10 (22.7)	0.46
LDL-C ≥160 mg/dL (%)	6 (13.6)	4 (9.1)	0.50
IL-6 (pg/mL)	3.1 [2.6]	2.0 [1.5]	0.02
TNF-α (pg/mL)	7.9 [3.1]	7.7 [2.4]	0.24
NO (mmol/L)	2.4 [1.2]	1.9 [1.3]	0.03

Data are presented as medians [interquartile range] or percentages n (%).
*p value obtained after comparing women with and without the METS using the Mann–Whitney U test, χ^2 test or Fisher's exact test when appropriate.
BMI, body mass index; IL 6, interleukin 6; HDL C, high density lipoprotein cholesterol; LDL C, low density lipoprotein cholesterol; NO, nitric oxide; TNF α, tumor necrosis factor α.

Table II. NO and cytokine levels according to the components of the METS.

Parameters	NO (mmol/L)	IL-6 (pg/mL)	TNF- α (pg/mL)
Abdominal obesity >88 cm			
Yes n 55	2.3 [1.2]	3.3 [2.4]	8.0 [3.3]
No n – 33	2.0 [1.4]	2.0 [1.0]	7.5 [2.3]
	[p – 0.31]	[p – 0.01]	[p – 0.48]
Glycemia >110 mg/dl.			
Yes n – 13	2.7 [1.1]	2.0 [1.7]	7.2 [4.3]
No n = 75	2.1 [1.3]	2.5 [2.2]	7.9 [3.1]
	[p = 0.04]	[p = 0.46]	[p = 0.67]
Triglyceride ≥150 mg/dL			
Yes n = 52	2.7 [1.3]	2.4 [2.1]	8.0 [3.3]
No n = 36	1.8 [0.9]	2.2 [2.2]	7.5 [2.4]
	[p = 0.001]	[p = 0.65]	[p = 0.321]
HDL-C <50 mg/dL			
Yes n – 53	2.3 [1.5]	2.5 [2.5]	7.9 [3.1]
No n – 35	2.1 [1.4]	2.0 [1.9]	8.1 [2.7]
	[p – 0.04]	[p – 0.32]	[p – 0.32]
Blood pressure >130/85 mmHg			
Yes n – 39	2.1 [0.9]	3.3 [2.6]	8.1 [2.8]
No n = 49	2.3 [1.5]	2.0 [1.5]	7.4 [3.0]
	[p = 0.15]	[p = 0.006]	[p = 0.04]
Number of METS diagnostic criteria			
3 n = 26	2.3 [1.2]	2.5 [2.3]	8.0 [3.1]
4 n = 15	2.4 [1.1]	4.1 [3.1]	7.9 [3.3]
5 n = 3	3.2 [1.6]	3.5 [1.7]	8.0 [3.0]
	[p = 0.01]	[p = 0.09]	[p = 0.39]

Data are presented as medians [interquartile range].
p values in square brackets were calculated with Mann–Whitney U test or Kruskal–Wallis test when appropriate.

data regarding NO levels may also be found in women with preeclampsia who display various degrees of disease severity and hence endothelial dysfunction. Indeed studies have reported higher [24] and also lower NO levels in preeclampsia [25], which may depend on genetic variants of the disease. Recent meta-analyses and prospective studies have confirmed the association between preeclampsia and the emergence of an unfavorable cardiovascular risk profile, in particular a 3.6-fold increased prevalence of the METS only 8 years after the index pregnancy [26–28]. Preeclampsia and the METS share several features, including obesity, hypertension, dyslipidemia, hypercoagulability, insulin resistance and most of all both entities display endothelial dysfunction [28]. As for studied cytokines only IL-6 was found higher among METS cases, situation that is in agreement with other studies [3,29]. It is important to bear in mind that categorizing the presence or not of the disease imposes difficulty among interpreting results. Hence we sought at also analysing cytokine and NO levels in relation to the presence or not of each component of the METS. In this regard, both cytokines were found significantly higher among women presenting higher blood pressure levels, with only IL-6 found higher in those displaying abdominal obesity.

Obesity is a frequent component of the METS (90.0% of our METS cases) which predisposes to insulin resistance. Our results suggest a close relation between abdominal obesity and serum IL 6 levels probably due to the high production of this cytokine by the adipocytes and macrophages in the adipose tissue. These increased IL-6 circulating levels may have negative effects on gene expression, TG, lipoprotein lipase activity, and insulin sensitivity [30] that may contribute to the development of the METS and its associated metabolic alterations. IL-6 levels did not correlate with glucose, TG and HDL-C levels. However, we must bear in mind that IL-6 may act as a circulating hormone or a local regulator of insulin action. Design of our study does not allow determining the role of IL-6 as a factor causing insulin resistance. Previous studies have reported that TNF-α is highly increased in obese subjects as compared to those lean (BMI < 25 kg/m^2) [31] fact that was not found in the present series. In addition to finding higher NO levels in women categorized as having the METS most interesting was determining a positive correlation between NO levels and number of METS criteria and glucose and TG levels; with an inverse relation with HDL-C levels. Although NO and IL-6 were found higher in METS cases these analytes did not correlate with each other. Contrary to this both cytokines displayed a positive and significant correlation. Significance of these findings will warrant further studies. All differences found upon bivariate analysis were confirmed during Spearman coefficient calculations.

Table III. Correlations between NO and cytokine levels and various numeric variables among all studied women.

Numeric parameter	NO (mmol/L)	IL-6 (pg/mL)	TNF-α (pg/mL)
Age (years)	0.14 (0.17)*	0.21 (0.04)	0.24 (0.02)
Time since menopause (years)	−0.15 (0.14)	0.23 (0.02)	0.22 (0.03)
Waist circumference (cm)	0.04 (0.66)	0.26 (0.01)	0.08 (0.44)
Body mass index (kg/m²)	−0.03 (0.75)	0.16 (0.12)	0.10 (0.72)
Glycemia (mg/dL)	0.22 (0.03)	−0.04 (0.69)	0.08 (0.43)
Triglyceride (mg/dL)	0.65 (0.0001)	0.01 (0.88)	0.05 (0.63)
HDL-C (mg/dL)	−0.27 (0.01)	−0.18 (0.09)	−0.12 (0.26)
SB pressure (mmHg)	0.02 (0.83)	0.16 (0.12)	0.22 (0.04)
DB pressure (mmHg)	−0.001 (0.99)	0.09 (0.39)	0.19 (0.04)
Number of METS criteria (0–5)	0.30 (0.004)	0.25 (0.01)	0.18 (0.03)
NO (mmol/L)	–	0.01 (0.89)	0.17 (0.11)
IL-6 (pg/mL)	0.01 (0.89)	–	0.27 (0.03)

*In parenthesis *p* values.
DB, diastolic blood; IL-6, interleukin 6; METS, metabolic syndrome; NO, nitric oxide; SB, systolic blood; TNF alpha, tumor necrosis factor α.

Authors acknowledge sample size (convenience sample) as a limitation to the study. However our study presents an interesting scenario: pro-inflammation in the presence of high NO levels. We hypothesize that women with the METS or any of its components are still at an earlier stage of the disease in which NO production may be increased in order to protect against the adverse effects of chronic inflammation. Interesting was finding that age and time since the menopause positively correlated with both cytokine levels indicating that in time chronic inflammation will ensue. Our results support the "window of opportunity" theorem in which lower estrogen levels have still not produced irreversible endothelial damage. Hence, female lifestyle changes and preventive measures at this stage are crucial and should be highly encouraged. More research is warranted within this perspective. The METS is a complex entity and when it comes to assessing cardiovascular risk one must not consider the syndrome as a whole, yet analyse the adverse impact that each individual component could have on female health. Therefore case individualization in women with the METS should be favored in which cytokine and NO measurement could have an interesting potential role.

In conclusion, postmenopausal women with the METS displayed higher IL-6 and NO levels, with significant correlations found between studied analytes and some of the components of the syndrome.

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Angiogenesis, inflammation and endothelial function in postmenopausal women screened for the metabolic syndrome

Peter Chedraui[a,*], Gustavo S. Escobar[a], Faustino R. Pérez-López[b], Giulia Palla[c], Magdalena Montt-Guevara[c], Elena Cecchi[c], Andrea R. Genazzani[c], Tommaso Simoncini[c], Research Group for the Omega Women's Health Project

[a] Institute of Biomedicine, Research Area for Women's Health, Facultad de Ciencias Médicas, Universidad Católica de Santiago de Guayaquil, Guayaquil, Ecuador
[b] Department of Obstetrics and Gynecology, Facultad de Medicina, Lozano Blesa University Hospital, Universidad de Zaragoza, Spain
[c] Department of Clinical and Experimental Medicine, Division of Obstetrics and Gynecology, University of Pisa, Italy

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ABSTRACT

Background: Prevalence of the metabolic syndrome (METS) increases after the menopause; nevertheless, concomitant vascular, inflammatory and endothelial changes have not been completely elucidated.
Objective: To measure serum markers of angiogenesis, inflammation and endothelial function in postmenopausal women screened for the METS.
Methods: Serum of 100 postmenopausal women was analyzed for angiopoietin-2, interleukin-8 (IL-8), soluble FAS ligand (sFASL), interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α), soluble CD40 ligand (sCD40L), plasminogen activator inhibitor-1 (PAI-1), and urokinase-type plasminogen activator (uPA). Comparisons were made in accordance to the presence or not of the METS and each of its components. Modified Adult Treatment Panel III criteria were used to define the METS.
Results: Women with the METS ($n = 57$) had similar age and time since menopause as compared to those without the syndrome ($n = 43$). In general, women with the METS displayed a trend for higher levels of the analyzed markers. Nevertheless, only IL-6 levels were found to be significantly higher and uPA levels significantly lower among METS women as compared to those without the syndrome. When analyte levels were compared as to presenting or not each of the diagnostic features of the METS, it was found that IL-6 levels were higher among women with abdominal obesity, low HDL-C and high triglyceride levels. Women with low HDL C and high triglyceride levels presented significantly lower uPA levels and those with high glucose and low HDL-C displayed significantly higher sCD40L levels.
Conclusion: Postmenopausal women with the METS in this sample displayed higher IL-6 (inflammation) and lower uPA levels (endothelial dysfunction). These were mainly related to metabolic and lipid abnormalities. More research is warranted in this regard.

1. Introduction

The prevalence of the metabolic syndrome (METS) increases after the onset of the menopause. This syndrome is a cluster of variables closely related to obesity, inflammation, insulin resistance, prothrombosis and atherogenesis that increase cardiovascular disease, cancer and mortality risk [1–3]. It is a chronic condition related to lifestyle habits that affects a quarter of women and males worldwide [4]. The METS is associated with increased inflammation, endothelial dysfunction, oxidative stress and abnormalities in both the macro- and microvasculature [5]. Female menopausal transition and ageing affect metabolic and inflammatory pathways that relate to vascular dysfunction and increased metabolic and cardiovascular risk. The relative contribution to inflammation and vascular dysfunction related to the menopause or the METS remains to be elucidated. Hence, the aim of the present study was to measure serum markers of angiogenesis, inflammation and endothelial function in postmenopausal women with and without the METS and each of its components.

2. Methods

2.1. Participants and study design

A METS screening programme was carried out from December 2011 to June 2012 at the Institute of Biomedicine of the Medical

* Corresponding author at: Institute of Biomedicine, Research Area for Women's Health, Facultad de Ciencias Médicas, Universidad Católica de Santiago de Guayaquil, Ecuador, PO BOX 09-01-4671. Tel.: +593 4220 6958; fax: +593 4220 6958.
E-mail address: peter.chedraui@cu.ucsg.edu.ec (P. Chedraui).

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Faculty of the *Universidad Católica de Santiago de Guayaquil*, Guayaquil, Ecuador [6]. A total of 204 natural postmenopausal women (40–65 years) participated in the programme recruited through newspaper advertising. All women were non-hormone therapy (HT) users. Those taking phytoestrogens or drugs intended to decrease lipid levels were excluded from the study. Research protocol of the study was reviewed and approved by the Scientific Research Committee of the Institute of Biomedicine. Eligible women were asked to attend the Institute to be informed about the study, its purposes and provide written consent of participation. Those consenting and fulfilling the inclusion criteria were asked to return after an 8 h overnight fast, moment in which sociodemographic data, waist circumference, weight, height and blood pressure measurements were recorded. In addition, a 10–15 ml peripheral venous blood sample was obtained.

To fulfil the aim of the present study, serum of 100 participants of the original cohort was reassessed and analyzed for angiopoietin-2, interleukin-8 (IL-8), and soluble FAS ligand (sFASL) (angiogenesis); interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) (inflammation); and soluble CD40 ligand (sCD40L), plasminogen activator inhibitor-1 (PAI-1) and urokinase-type plasminogen activator (uPA) (endothelial function). Analyte levels were compared in accordance to the presence or not of the METS and each of its components.

2.2. Diagnostic criteria for the metabolic syndrome

The METS was defined using Adult Treatment Panel III diagnostic criteria modified by the American Heart Association and the National Heart, Lung, and Blood Institute [7]. This was the case if three or more of five criteria were encountered: abdominal obesity (waist circumference >88 cm), increased serum triglycerides (TG) (≥150 mg/dL), decreased high density lipoprotein cholesterol (HDL-C) (<50 mg/dL), high fasting glucose (≥100 mg/dL, or the use of hypoglycemic agents) and increased blood pressure (≥130/85 mmHg, or the use of antihypertensive medications) [7]. Method for assessing abdominal perimeter has previously been described [6].

2.3. Serum assays

Blood samples taken from each participant were centrifuged at 5 °C for 10 min at 3000 rpm. The obtained serum was treated accordingly to manufacturer instructions, decanted into 0.5 ml aliquots and then stored at −70 °C. Subsequently, and before proceeding with analysis, DDP-IV inhibitor and aprotinin (Sigma–Aldrich, St. Louis, MO, USA) were added to the samples at a final concentration of 100 μM and 0.013%, respectively.

2.3.1. Measurement of the different analytes

TG, HDL C and glucose levels were assayed using the enzymatic colorimetric method with a Hitachi 717 automatic photometric analyzer (Roche Diagnostics GmbH, Mannheim, Germany). The concentration of angiopoietin-2, IL-8 and sFASL, IL-6, TNF-α, sCD40L, PAI-1, and uPA were measured using Bio-Plex 200 System® (Bio-Rad Laboratories, Inc., CA, USA) at the Bioclarma srl, Turin, Italy [8].

2.3.2. Assay format

The Bio-Plex® multiplex assay employs a standard enzyme immunoassay formatted on magnetic beads using a 96-well plate format. However, rather than a flat surface, it uses differentially detectable bead sets as a substrate reacting with analytes in solution. After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin–phycoerythrin conjugate. Phycoerythrin serves as a fluorescent indicator or reporter. The use of differentially detectable beads enables the simultaneous identification and quantification of many analytes in the same sample (2 μl of serum sample) [9].

Data acquisition and analysis from the reactions are performed using a Bio-Plex system or similar Luminex-based reader. When a multiplex assay suspension is drawn into the Bio-Plex 200 reader, a red (635 nm) laser illuminates the fluorescent dyes within each bead to provide bead classification and thus assay identification. At the same time, a green (532 nm) laser excites phycoerythin to generate a reporter signal, which is detected by a photomultiplier tube. A high-speed digital processor manages data output, and the Bio-Plex Manager™ software (version 6.1, Bioclarma Research and Molecular Diagnostics, Torino, Italy) presents data as median fluorescence intensity (MFI) as well as concentration (pg/ml). The concentration of the analyte bound to each bead is proportional to the MFI of reporter signal [10].

2.4. Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA) and the Statistical Package for the Social Sciences (IBM SPSS, Armonk, NY, USA). Data are presented as mean ⊥ standard deviations, frequencies and percentages. The Mann–Whitney *U* test was used to analyze group comparison differences (continuous data) and the chi square test to analyze percentages. Spearman coefficients were calculated to determine correlations between studied analyte levels and the components of the METS expressed as numeric variables. Additionally, multiple linear regression analysis was performed adjusting for several confounding factors (female age, time since menopause onset, BMI, parity). A p value of <0.05 was considered as significant.

3. Results

A 57% (n = 57/100) of the analyzed serum samples were defined as METS and 43% (n = 43) as non METS (controls). A 29% and 10% of women indicated being hypertense and diabetic, respectively. Age and time since menopause onset were similar in both studied groups. Women with the METS presented a higher rate of modified ATP III diagnostic criteria and a non-significant trend for higher levels of angiopoietin-2, IL-8 and sFASL (Table 1). These molecules are involved in processes of angiogenesis. Markers of general inflammation, such as TNF-α, and of endothelial dysfunction, such as PAI-1 and sCD40L, were not different amongst groups. However, the inflammatory cytokine IL-6 was significantly higher in women with the METS, and uPA, a marker of endothelial function associated with activation of fibrinolysis, was instead significantly lower in METS women (Table 1 and Fig. 1). When analyte levels were compared as to presenting or not each of the METS components it was found that IL-6 levels were higher among women with abdominal obesity, low HDL-C and high TG levels. Women with low HDL-C and high TG levels presented significantly lower uPA levels and those with high glucose and low HDL-C displayed significantly higher sCD40L levels (Data not shown on Table). Upon bivariate Spearman analysis, IL-6 levels positively correlated with abdominal perimeter and TG levels (r^2 = 0.40 and 0.38, respectively, both p = 0.02), and inversely with HDL-C (r^2 = −0.37, p = 0.01). uPA levels directly correlated with HDL-C levels and inversely with TG levels (r^2 = 0.39 and −0.36, respectively, both p = 0.01). There was a positive correlation between sCD40L and glucose levels (r^2 = 0.42 and 0.37, respectively, both p = 0.01), and an inverse correlation with HDL-C levels (r^2 = −0.36, p = 0.01). These correlations were confirmed after multiple linear regression analysis controlling for several confounding factors.

Table 1
Socio-demographic data, METS components and measured analytes among studied women.

Parameters	METS, $n = 57$	Non METS, $n = 43$	p value*
General data			
Age (years)	56.7 ± 4.8	54.8 ± 5.4	NS
Time since menopause (years)	9.5 ± 7.5	8.4 ± 6.2	NS
Parity	3.2 ± 1.8	2.8 ± 1.5	NS
Education (years)	11.8 ± 5.3	13.2 ± 5.0	NS
METS components			
Abdominal obesity (waist >88 cm)	41 (71.9%)	14 (32.6%)	0.0001
Serum triglycerides (≥150 mg/dl)	37 (64.9%)	3 (6.9%)	0.0001
Serum HDL-C (<50 mg/dL)	50 (87.7%)	12 (27.9%)	0.0001
Fasting glucose (>100 mg/dL)	30 (52.6%)	7 (15.3%)	0.03
Blood pressure (≥130/85 mmHg)	41 (71.9%)	18 (41.9%)	0.002
Analytes Angiogenesis			
Angiopoietin-2 (pg/ml)	386.8 ± 249.7	292.8 ± 145.5	NS
IL-8 (pg/ml)	10.1 ± 4.3	9.8 ± 3.7	NS
sFASL (pg/ml)	77.6 ± 27.6	72.8 ± 28.9	NS
Inflammation			
IL-6 (pg/ml)	6.9 ± 2.1	5.8 ± 2.8	0.006
TNF-α (pg/ml)	6.0 ± 2.1	5.6 ± 2.0	NS
Endothelial function			
sCD40L (pg/ml)	1202 ± 702	1023 ± 567.8	NS
PAI-1 (pg/ml)	66,347.0 ± 4922.0	62,839 ± 8991.0	NS
uPA (pg/ml)	685.5 ± 327.1	948.9 ± 550.8	0.01

Data are presented as mean ± standard deviations or n (%); *p value when comparing METS and non METS women as determined with the Mann–Whitney test or the chi-square test; NS, non significant ($p > 0.05$); uPA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor; sFASL, soluble FAS ligand; IL-8, interleukin 8; IL-6, interleukin 6; TNF-α, tumour necrosis factor alpha; sCD40L, soluble CD40 ligand; HDL-C, high density lipoprotein cholesterol.

4. Discussion

The present study found that postmenopausal women with the METS displayed higher IL-6 and lower uPA levels, markers of inflammation and endothelial dysfunction respectively. These changes may contribute to the development and progression of clinically silent atherosclerosis [11]. The present data is in accordance with our previous observations [12] and that of others [13] in terms of higher IL-6 levels found among postmenopausal women with the METS. Adipocytes and adipose-tissue macrophages are involved in the production of IL-6, which is one of the main mediators of chronic inflammation [14]. Indeed, when analyte levels were determined per each component of the METS (independent of having or not the METS) the trend for higher IL-6 levels were observed among women who had abdominal obesity, low HDL-C and high TG levels. This finding indicates that more important than categorizing women as having or not the METS, should be analyzing cytokine levels per diagnostic METS feature. In this sense, one should bear in mind that not all obese women will fall into the category of having the METS and still have dyslipidemia and increased pro-inflammatory adipose secretion. Hence, IL-6 secretion from adipose tissue macrophages seems to be a general one and may vary depending on different circumstances and through several mechanisms [15]. One study found that IL-6 levels in postmenopausal women were significantly associated with TG levels and other variables included in the METS [16]. Our data seems to support these findings. Another study found that in patients with coronary heart disease, inflammation (high IL-6 levels) attenuates the protective levels of HDL-C (inverse correlation with cardiac pathology) [16].

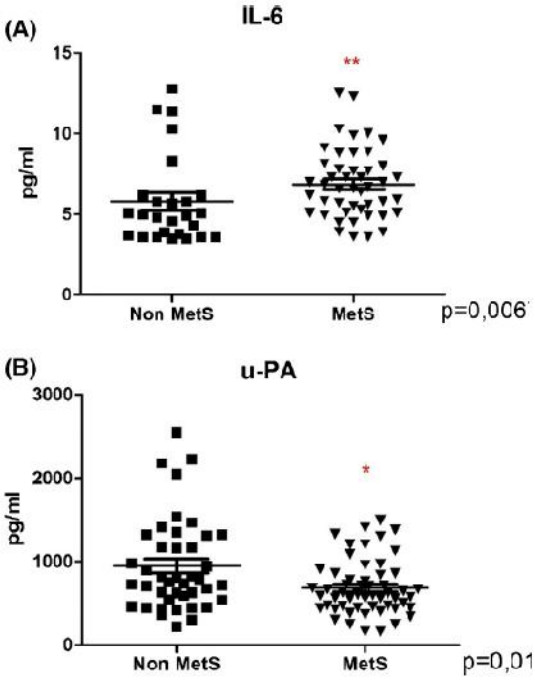

Fig. 1. Significant higher IL-6 levels among postmenopausal women with the METS (A); significant lower levels of u-PA among METS women (B).

Elevated IL-6 is an established risk factor for cardiovascular events in women after the menopause; thus it is interesting to find that the presence of METS, rather than the menopause itself, was related to increased levels of this inflammatory marker. IL-6 serum levels are associated with visceral adipose tissue and can influence insulin levels [17]. METS women of the present study displayed significantly higher insulin levels in direct correlation with IL-6 (data not shown). The importance of genetics in the pathogenesis of the METS and related cardiovascular risk is currently being the focus of intense research. For instance, it has been reported that IL-6 polymorphisms may play a role in the pathogenesis of the METS through the modulation of IL-6 levels [18].

In relation to endothelial function, we analyzed three serum biomarkers: PAI-1, uPA and sCD40L. Our METS women displayed significant lower uPA levels and a non-significant trend for higher PAI-1. Interestingly, upon analyzing uPA levels per METS items, lower uPA levels were observed among women with low HDL-C and high TG levels. Urokinase-type plasminogen activator (uPA) is

a serine protease that activates plasminogen to plasmin. It is synthesized as an inactive precursor that undergoes a rapid proteolytic activation and binds to a specific receptor (uPAR) localized at the cell surface [19]. Both uPA and its receptor are expressed by inflammatory cells, including neutrophils, monocytes, macrophages, and activated T lymphocytes. In these cells, uPA and the binding to its receptor play important roles in cell activation, adhesion, proliferation and migration [20–23]. In addition, the uPA–uPAR complex may also be involved in many other processes such as wound healing, angiogenesis, invasion, immune response, vascular remodelling and cancer.

uPA and sCD40 are involved with the plasminogen–plasmin system and also tissue remodelling. PAI-1 has influence on cell adhesion and migration with variations depending on cell type, conformation and concentration [24–26]. Therefore, lower amounts of uPA, along with the observed trend towards higher amounts of PAI-1 may be responsible for the higher rate of thromboembolic events observed in postmenopausal women with the METS. uPA is under the regulation of PAI-1. Under normal conditions, uPA and its receptor levels are higher in the postmenopausal state while PAI-1 levels are higher in the premenopausal state [27]. The increase in PAI-1 levels throughout the menopausal transition has been well characterized and is thought to play a role in increasing cardiovascular risk through female ageing. Although not reaching significance, our data shows a trend for higher PAI-1 levels among METS women and per each diagnostic feature, supporting the fact that inflammatory and vascular changes are present at an early stage of the menopause and perhaps the METS (mean age of our METS women: 56 years), time when oestrogen therapy could be of benefit. Indeed, clinical trials have suggested that oestrogen administration to postmenopausal women may result into lowered PAI-1 levels [28,29]. While endothelial cells are sensitive to endocrine changes that determine modified production of PAI-1 [30], it is even more interesting finding that after the menopause the presence of the METS enhances endocrine changes and determine further endothelial dysfunction. Our data seems to support further research in the evaluation of the PAI-1/uPA system as a useful marker for future metabolic and cardiovascular risk assessment in menopausal women.

Soluble CD40L is expressed on antigen-presenting cells (B-cells, macrophages, dendritic cells, thymic cells) and is involved in the atherosclerotic genesis contributing to inflammatory and thrombotic processes [31,32]. Reports indicate that individuals with the METS present higher circulating levels of sVCAM and sCD40L, suggesting endothelial activation [33,34], and that higher sCD40L levels correlate with adverse cardiovascular outcomes linked to atherothrombotic complications [34,35]. Although our METS women displayed a non-significant trend for higher sCD40L levels, these levels were found significantly higher among those with high glucose and low HDL-C levels. Again, this finding highlights the importance of assessing each diagnostic feature individually instead of considering the METS as a category. Our data supports the findings of Lim et al. [36], who found higher SCD40L and IL-6 levels among diabetic patients.

Epidemiological studies suggest that gender differences may be involved in atherogenesis. For instance, in women, C-reactive protein is a stonger inflammatory marker for insulin resistance and metabolic changes as compared to men [37,38]. In peri- and postmenopausal women the hypertriglyceridemic phenotype has been related to the METS and to excess of inflammatory markers [39]. The pro-inflammatory status associated to the METS induces dysfunction of the anti-inflammatory and atheroprotective properties of the apolipoprotein A-I and HDL particles, increasing the risk of diabetes and cardiovascular disease [38]. Our results may well support the latter, in that higher IL-6 levels found among METS women lead to abnormal lipid profile (low HDL-C and high TG levels) and also endothelial dysfunction (lower uPA levels).

Finally, as for the limitations of the present study one can mention sample size and lacking analyte determination among premenopausal women, for comparison purposes, independent of having or not the METS. Our programme did not include a detailed analysis of diet content which may influence caloric intake and the development of the various components of the METS [40,41]. We did however assess women who were or were not sedentary; yet analyte levels did not differ in either case. Future studies should include longitudinal design in order to demonstrate the evolution of our analyzed biomarkers, from pre to the postmenopausal stage, and adjust for various covariates including age and the presence of the METS.

Despite the aforementioned limitations, the present study found that postmenopausal women with the METS displayed higher IL-6 (inflammation) and lower uPA levels (endothelial dysfunction). These were mainly related to metabolic and lipid abnormalities. More research is warranted in this regard.

Contributors

Peter Chedraui, Faustino R. Pérez-López, Tommaso Simoncini and Andrea Genazzani were involved in the conception and design of the study. Gustavo Escobar, Giulia Palla, Magdalena Monitt-Guevara, Elena Cecchi performed biochemical assays. Giulia Palla, Elena Cecchi and Peter Chedraui performed statistical analysis. Faustino R. Pérez-López, Peter Chedraui and Tommaso Simoncini performed drafting of the manuscript. All authors were involved in critically revising the manuscript for its intellectual content and approving the final version.

Competing interest

None declared.

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Chapter 6

Possible role of endothelial dysfunction during reproductive years in the genesis of the metabolic syndrome after the menopause

***Chedraui P**, Solis EJ, Bocci G, Gopal S, Russo E, Escobar GS, Hidalgo L, Pérez-López FR, Genazzani AR, Mannella P, Simoncini T.*

Feto-placental nitric oxide, asymmetric dimethylarginine and vascular endothelial growth factor (VEGF) levels and VEGF gene polymorphisms in severe preeclampsia.

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Feto-placental nitric oxide, asymmetric dimethylarginine and vascular endothelial growth factor (VEGF) levels and VEGF gene polymorphisms in severe preeclampsia

Peter Chedraui[1,2], Emilio J. Solis[1], Guido Bocci[3], Santhosh Gopal[4], Eleonora Russo[4], Gustavo S. Escobar[2], Luis Hidalgo[1], Faustino R. Pérez-López[5], Andrea R. Genazzani[4], Paolo Mannella[4] & Tommaso Simoncini[4]

[1]*High Risk Pregnancy Labor and Delivery Unit, Enrique C. Sotomayor Obstetrics and Gynecology Hospital Guayaquil, Ecuador,* [2]*Institute of Biomedicine, Facultad de Ciencias Médicas, Universidad Católica de Guayaquil, Ecuador,* [3]*Division of Pharmacology, Department of Internal Medicine, University of Pisa, Italy,* [4]*Molecular and Cellular Gynecological Endocrinology Laboratory, Division of Obstetrics and Gynecology, Department of Reproductive Medicine and Child Development, University of Pisa, Italy, and* [5]*Department of Obstetrics and Gynecology, Universidad de Zaragoza, Lozano Blesa University Hospital, Zaragoza, Spain*

Objective: **To measure plasma nitric oxide (NO), asymmetric dimethylarginine (ADMA) and vascular endothelial growth factor (VEGF) levels and VEGF gene polymorphisms in fetal circulation in severe preeclampsia.** ***Methods:*** **Cord vessels of singleton gestations complicated with severe preeclampsia 36 weeks or more (n = 31) and controls were sampled upon delivery for analyte measuring. Additionally, DNA was extracted from umbilical vein whole blood to determine the frequency of VEGF gene single nucleotide polymorphisms (SNPs): −2578 A/C, −1498 C/T, −1154 A/G, −634 C/G and +936 C/T. Coefficient correlations between analyte levels and placental and neonatal weight were calculated.** ***Results:*** **NO plasma levels in umbilical vessels (artery and vein) were significantly higher in preeclampsia cases as compared to controls (4.67 ± 3.0 vs. 0.82 ± 0.90; 4.46 ± 3.0 vs. 0.82 ± 0.99 mmol/L, respectively, $p = 0.0001$ both). ADMA levels displayed a similar increased trend in both fetal vessels, but this did not reach statistical significance (2.57 ± 1.03 vs. 2.34 ± 0.57; 2.74 ± 0.94 vs. 2.42 ± 0.59 mmol/L, respectively, $p > 0.05$). VEGF was significantly lower in artery but not in vein in preeclampsia cases (200.48 ± 225.62 vs. 338.61 ± 287.03 pg/mL, $p = 0.04$). A significant positive correlation was found between NO and ADMA levels (artery and vein) among preeclampsia cases. Overall, the frequency of the studied VEGF gene SNPs did not differ among pre-eclamptic cases and controls; nevertheless, a significant trend toward lower umbilical vein VEGF levels was observed in pre-eclampsia cases in the presence of -2578 CC and −1154 AG genotypes.** ***Conclusion:*** **Near term gestations complicated with severe preeclampsia presented higher NO levels in fetal circulation, which correlated to ADMA and lower artery VEGF values. More research is warranted to confirm that selected VEGF SNPs may be associated with lower umbilical vein VEGF.**

Keywords: Asymmetric dimethylarginine, nitric oxide, preeclampsia, umbilical vessels, vascular endothelial growth factor

Introduction

Despite attempts at intervention, pre-eclampsia is still a leading cause of maternal and fetal morbidity and mortality [1]. Reduced placental perfusion at early stages of pregnancy is a key event in its development [2], in which defective trophoblastic invasion of the uterine spiral arteries and arterioles leads to incomplete vascular remodeling and impaired utero-placental blood flow. Although the intrinsic involved mechanisms are still unclear, vascular dysfunction found in pre-eclampsia is likely to be a consequence of reduced maternal circulating angiogenic factors [3] and increased levels of placental debris [4], reactive oxygen species [5], pro inflammatory cytokines [6], and anti-angiogenic factors [7]. Nitric oxide (NO) synthesis is involved in some of these proposed pathological mechanisms. However, how NO exerts its effects over vascular development and placental function in preeclampsia is still unclear [8]. In pre-eclampsia, increased maternal and fetal serum NO levels may be a compensatory protective mechanism to maintain blood flow and reduce platelet aggregation in the fetal and maternal circulation. In addition, NO production is directly related to pre-eclampsia severity [9]. Asymmetric dimethylarginine (ADMA) is an aminoacid that circulates in plasma, is excreted in urine, and is found in various tissues and cells. It inhibits NO synthase (NOS) and has been proposed as a marker of endothelial dysfunction. ADMA is increased in women with pre-eclampsia, even before clinical manifestation [10,11]. On the other hand, vascular endothelial growth factor (VEGF) mediates important signaling pathways during fetal growth and in the maternal circulatory system. VEGF concentration is deranged in gestations complicated with preeclampsia [12].

Genetic background may also influence preeclampsia development and the concomitant endothelial dysfunction. Hypertension and angiogenesis are linked, since microcirculatory vasoconstriction is in part due to defective angiogenic processes [13]. Single nucleotide polymorphisms (SNPs) of the promoter region of the VEGF gene are related to higher or lower VEGF production and to altered risk of developing diseases characterized by deranged angiogenesis [14]. In particular, the VEGF -2578 CC genotype has been associated with higher VEGF expression than the AA genotype, which is consistent for a protective effect of VEGF in atherosclerosis development [15]. Recent studies suggest

Correspondence: Peter Chedraui MD, MSc, High Risk Pregnancy Labor and Delivery Unit, Enrique C. Sotomayor Obstetrics and Gynecology Hospital, Pedro Pablo Gómez s/n y 6 de Marzo, 196 Guayaquil – Ecuador, PO BOX 09-01-4671. Tel: + (5934) 241-3300 extension: 3050 – 3079. Fax: + (5934) 220-6958. E-mail: pchedraui@jbgye.org.ec

that some VEGF genotypes are more common in women with pre-eclampsia [16]. However, it is currently unknown whether such genetic asset is related to abnormal circulating amounts of VEGF during pre-eclampsia.

The aim of the present study was to measure plasma NO, ADMA, and VEGF levels in fetal circulation in gestations complicated with severe preeclampsia. Additionally, the frequency of common SNPs of the VEGF gene and their relation to circulating umbilical vein VEGF levels were also analyzed.

Materials and methods

Study design and population

The present pilot research collaboration was carried out at the Enrique C. Sotomayor Obstetrics and Gynecology Hospital, Guayaquil, Ecuador. Sotomayor Hospital is a major referral center that provides maternal and neonatal healthcare to the low-income population of Guayaquil, Ecuador. It has the highest delivery rate in Latin America (>30 000 per year) in addition to increased rates of high risk pregnancies [17].

For this study, singleton gestations fulfilling severe preeclampsia criteria admitted for delivery to the High Risk Pregnancy Labor and Delivery Unit were recruited. Normal pregnant women delivering in the low risk Unit served as controls which were matched for maternal and gestational age, parity, and laboring status. All participants were recruited after signing informed consent. Women with known medical disorders and on any medication before hospital admission (particularly non-steroidal anti-inflammatory drugs) were excluded from the study.

ACOG criteria was used to define severe preeclampsia as a blood pressure ≥ 160/110 mmHg on two occasions at least 6 hours apart and proteinuria +++ or more on at least two random samples 4 hours apart [18].

Maternal and neonatal information was recorded on a data sheet and included: maternal age, blood pressure and dipstick scores, neonatal gestational age and birth weight and route of delivery. The Institutional Review Board of the Enrique C. Sotomayor Hospital approved the research study protocol.

Biochemical assays

Blood samples

Upon infant delivery a 25 cm fetal cord segment was double clamped from which a 5 ml blood sample was taken from each cord vessel (artery and vein). Tubes were immediately centrifuged at 3,000 rpm at 6°C for 20 minutes. The plasma and cell layers were aliquoted into several micro-centrifuge tubes, which were frozen and stored at −70°C until further analysis.

Nitrite assay

Nitric oxide production was determined by a nitrite assay using 2, 3-diaminonaphthalene. Fluorescence of 1-(H)-naphthotriazole was measured by excitation and emission wavelengths of 365 and 450 nm. Standard curves were constructed with sodium nitrite. A stock solution of 1 mmol NO3 in PBS (pH 7.4) was prepared and then standards (from 0 to 50 μM/L) were obtained by diluting the stock solution in PBS solution (pH 7.4). Plasma, blank, standard, and control samples (250 μl each) were diluted by adding 250 μl PBS to plastic vials. Each mixture was then thoroughly mixed and the plasma centrifuged in spin filter columns for 60 minutes at room temperature. Subsequently, 100 μl Griess reagent, 1% sulphanilamide + 0.1% naphthylethylenediamine dihydrochloride prepared in 3.1% H_3PO_4 solution was added to each vial. Reagents were mixed on a shaker for one minute at 185 rpm and plates incubated for 10 minutes at 37°C. Samples were placed in a spectrophotometer and the absorbance was read at 490 nm. Non-specific fluorescence was determined in the presence of NG-monomethyl-L-arginine (3 mmol/L). All samples, kit controls and standards were analyzed in duplicates.

Enzyme-linked immunosorbent assay

Acylation was conducted in the 96-well reaction plate supplied with the kit according to the instructions of the manufacturer. Standards, kit controls and samples (20 μl) were mixed with 25 μl acylation buffer and 25 μl equalizing reagent. Subsequently, 25 μl of acylation reagent was added and the reaction plate incubated for 30 minutes at room temperature on an orbital shaker. Diluted equalizing reagent (100 μl) was added and incubation continued for 45 minutes. After incubation, samples were ready for the enzyme-linked immunosorbent assay (ELISA) analysis.

The ADMA-ELISA kit (Biovendor, Cat. No.: REA201/96) consists of a split-type reaction plate (12×8) coated with ADMA, six standards (0–5 μM), rabbit anti-ADMA antiserum, goat anti-rabbit-IgG-peroxidase conjugate, TMB substrate solution, stop solution, and wash buffer. Aliquots (50 μl) of the acylated standards, kit controls or samples were processed according to the instructions of the kit manufacturer. Absorbances were measured with a microplate reader using a wavelength of 450 nm (reference wavelength 620 nm). All samples, kit controls, and standards were analyzed in duplicates.

The VEGF-A-ELISA kit (Antigenix America, Cat. No. RH88820CKC) consists of a split-type reaction plate (12×8) coated with VEGF-A polyclonal antibodies, goat anti-VEGF-A antiserum, anti-goat- Biotin-Conjugate, TMB substrate solution, stop solution, streptavidin-HRP, and wash buffer. Absorbances were measured with a microplate reader using a wavelength of 450 nm (reference wavelength 620 nm). All samples, kit controls, and standards were analyzed in duplicates.

VEGF genotyping

DNA was extracted from whole blood of the umbilical vein using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). Genotyping was performed using the Taqman platform using specific and validated primers (Applied Biosystems, Carlsbad, CA, USA) with an ABI PRISM 7900HT Sequence Detection System. The investigated SNPs were the following: −2578 A/C (rs699947; TaqMan SNP genotyping assay C_8311602_10), −1498 C/T (rs833061; C_1647381_10), −1154 A/G (rs1570360; C_1647379_10), −634 C/G (rs2010963; C_8311614_10), +936 C/T (rs3025039; C_16198794_10). PCR reaction was carried out according to the protocol of the manufacturer. Allelic distribution for VEGF SNPs was in Hardy–Weinberg equilibrium.

Statistical analysis

Statistical analysis was performed using SPSS statistical package (Version 19.0 for Windows, SPSS Inc, Chicago, IL, USA). Data are presented as means, standard deviations, medians, interquartile ranges, coefficients, and percentages. The Kolmogorov Smirnov test was used to assess the normality of data distribution. According to this, continuous data were compared with student's *t*-test (parametric), the Mann–Whitney test (non-parametric) or the Kruskal–Wallis test (various non-parametric samples). Chi-square, Yates' corrected chi-square, and Fisher's exact tests were used to compare percentages (including SNP frequency comparisons between cases and controls). Rho Spearman coefficients were calculated to determine correlations between analyte

levels (NO, ADMA, and VEGF) and placental and neonatal weight. A p value of < 0.05 was considered as statistically significant.

Results

During the study period a total of 62 women were recruited. These included 31 women with severe preeclampsia and 31 controls matched for maternal and gestational age, parity, and laboring status. General characteristics of studied women are depicted on Table I. Arm circumference (cm) as well as blood pressure levels were significantly higher among women with pre-eclampsia. No significant differences were found for the other studied parameters. None of the pre-eclampsia cases presented clinical complications (i.e. oliguria, renal insufficiency, pulmonary edema, or neurological problems). As compared to controls, at birth, placental weight and neonatal weight and ponderal index were lower in women complicated with severe preeclampsia (Table II). Equally the percentage of neonates with low birth weight (< 2500 g) was higher in the pre-eclampsia group.

NO plasma levels in umbilical vessels (artery and vein) were significantly higher in preeclampsia cases than in controls ($p = 0.0001$). There was a non-significant trend toward higher ADMA levels in both umbilical vessels among women with pre-eclampsia. VEGF levels were found to be significantly lower only in umbilical artery of preeclampsia cases (Table III; Figure 1a, b, and c). Artery and vein NO levels displayed a positive and significant correlation with ADMA levels. No other significant correlation was found (Table IV).

There was no statistical difference between cases and controls in the distribution of the five investigated SNPs of the VEGF gene assessed from whole blood of the umbilical venous circulation (Table V). Interestingly, lower umbilical vein VEGF levels were found in pre-eclampsia cases in the presence of the VEGF −1154 AG and −2578 CC genotypes (Table VI). No differences were observed in the distribution of any of the analyzed SNPs or VEGF levels when pre-eclamptic women with small for gestational age fetuses were compared to those only with pre-eclampsia.

Discussion

Endothelial dysfunction is a feature of several disorders of pregnancy. Overt maternal and feto-placental endothelial dysfunction characterizes pre-eclampsia [19], which is associated to a higher risk for adverse maternal fetal outcomes [1,20]. Pre-eclampsia is thought to derive from shallow extravillous trophoblast invasion of the decidua and incomplete remodeling of spiral arteries and arterioles [21]. Defective angiogenesis and the related feto-placental vascular dysfunction are therefore considered key steps. However, the signs and symptoms of pre-eclampsia generally become apparent in the third trimester of pregnancy [1], which

Table I. Basal characteristics of studied women.

Parameters	All (n = 62)	Cases (n = 31)	Controls (n = 31)	p Value*
Age (years)	25.7 + 6.4 [26.0]	25.9 + 6.4 [26.0]	25.4 + 6.5 [26.0]	0.78[a]
Parity	1.4 ± 1.6 [1.0]	1.4 ± 1.6 [1.0]	1.4 ± 1.6 [1.0]	1.0[b]
Residency (%)				
Urban	11 (17.7)	6 (19.4)	5 (16.1)	0.73[c]
Urban-marginal	35 (56.5)	19 (61.3)	16 (51.6)	0.44[c]
Rural	16 (25.8)	6 (19.4)	10 (32.3)	0.24[c]
Patient was in labor upon recruitment (%)	20 (32.3)	10 (32.3)	10 (32.3)	1.0[c]
Educational level (years)	10.5 ± 3.4 [11.0]	10.8 ± 3.5 [12.0]	10.2 ± 3.4 [10.0]	0.49[b]
Number of antenatal visits	5.4 ± 2.2 [6.0]	5.1 ± 2.0 [5.0]	5.7 ± 2.3 [6.0]	0.15[b]
Arm circumference (cm)	28.4 ± 2.1 [28.0]	29.2 ± 2.0 [29.0]	27.6 ± 1.9 [28.0]	0.004[b]
Systolic blood pressure (mmHg)	132.9 + 27.3 [135.0]	156.2 + 17.2 [150.0]	109.5 + 9.7 [110.0]	0.0001[b]
Diastolic blood pressure (mmHg)	83.6 ± 16.2 [85.0]	97.7 ± 7.9 [100.0]	69.5 ± 8.0 [70.0]	0.0001[b]

Data are presented as mean + standard deviations [medians] or percentages n (%).
*p Value after comparing cases and controls as determined with Student t test[a], the Mann–Whitney test[b] or the chi-square test[c].

Table II. Neonatal outcome of studied women.

Parameters	Cases (n = 31)	Controls (n = 31)	p Value*
Gestational age (weeks)	37.3 ± 1.9 [38.0]	37.9 ± 1.5 [38.0]	0.14[a]
Neonatal weight (g)	2669.8 ± 623.9 [2873.0]	2945.1 ± 496.8 [2995.0]	0.02[b]
Ponderal index (g/cm^3)	2.5 ± 0.3 [2.6]	2.8 ± 0.7 [2.8]	0.02[a]
Low birth weight < 2500 g (%)	11 (35.5)	5 (16.2)	0.04[c]
Preterm (%)	9 (29.0)	5 (16.2)	0.22[c]
Small-for-gestational age (%)	5 (16.1)	1 (3.2)	0.19[d]
Placental weight (g)	667.3 ± 57.2 [650.0]	685.6 ± 38.4 [690.0]	0.02[b]
Apgar score < 7 at first minute (%)	7 (22.6)	2 (6.4)	0.14[d]
Apgar score < 7 at 5 minutes (%)	2 (6.4)	0 (0)	0.49[e]
Neonatal death (%)	0 (0)	0 (0)	0.90[e]
Admittance to NICU (%)	0 (0)	0 (0)	0.90[e]

Data are presented as mean + standard deviations [medians] or percentages n (%). *p Value after comparing cases and controls as determined with the Mann–Whitney test[a], the student t-test[b], the chi-square test[c], Yates' corrected chi-square test[d] or Fisher's exact test[e]. NICU, neonatal intensive care unit.

Table III. NO, ADMA, and VEGF levels in umbilical vessels: cases and controls.

Parameter	Cases	Controls	p Value *
Nitric oxide (mmol/L)			
Artery	4.67 ± 3.00	0.82 ± 0.90	0.0001
Vein	4.46 ± 3.00	0.82 + 0.99	0.0001
ADMA (mmol/L)			
Artery	2.57 ± 1.03	2.34 ± 0.57	0.79
Vein	2.74 ± 0.94	2.42 ± 0.59	0.21
VEGF (pg/mL)			
Artery	200.48 ± 225.62	338.61 ± 287.03	0.04
Vein	144.49 ± 351.95	130.51 ± 235.17	0.88

*p Value after comparing cases in controls with the Mann Whitney test. ADMA, asymmetric dimethylarginine; VEGF, vascular endothelial growth factor.

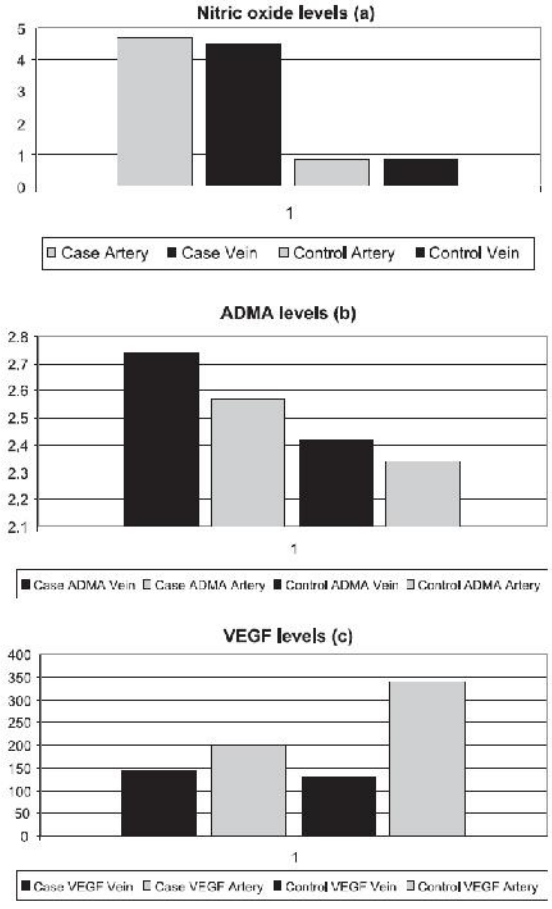

Figure 1. Levels in fetal circulation of NO (a), ADMA (b), and VEGF (c).

calls for the identification of early biomarkers capable of screening women at higher risk for pre-eclampsia. Several studies have observed a correlation between the onset of vascular dysfunction and pre-eclampsia with a reduction in circulating levels of angiogenic factors, such as placental growth factor (PLGF) and VEGF [3,22], and increased levels of anti-angiogenic factors [23]. Trofoblast cells isolated from pre-eclampsia placentas when cultured under regular oxygen condition produce more soluble endoglin, soluble fms-like tyrosine kinase receptor-1, and PLGF as compared to normal trofoblast cells [23]. These changes may contribute to systemic vascular damage, possibly determining some of the modifications found in the kidney that lead to hypertension and proteinuria typical of pre-eclampsia [1].

Increased NO together with the lower VEGF umbilical plasma levels observed in the present pre-eclamptic Ecuadorian series is in agreement with what is found in the literature for other populations, however, mostly reported from maternal circulation, consistent with the general anti-angiogenic state found in pre-eclampsia [4]. To note is the fact that not many studies can be found in the literature that report fetal umbilical cord circulating levels of the analytes measured in our research. Consistent with our findings, Braekke et al. [24] have reported higher umbilical vein ADMA levels among preeclampsia cases as compared controls. Interestingly, ADMA levels were found to be three times higher in fetal circulation than in the maternal circulation, suggesting that the placenta is the primary source of ADMA [24]. Another study failed to demonstrate higher ADMA levels in fetal circulation in women with pre-eclampsia yet higher levels related to prematurity and low birth weight [25]. Of interest was finding in this study [25] that ADMA levels were 4 fold higher in controls as compared to those of lactating women and healthy children and adults, implicating a physiological role for higher ADMA levels in maintaining vascular tone and blood redistribution to vital organs during birth, thereby favoring the circulatory transition from fetal to neonatal life [25]. The present study cannot answer whether the higher rate of small for gestational age found in pre-eclamptic pregnancies is related to altered amounts of feto-placental growth factors. Specifically, while lower umbilical concentrations of VEGF were observed in the present and other pre-eclampsia studies, such concentrations did not significantly correlate to placental or neonatal weight.

Elevated umbilical plasma NO levels may represent an adaptive response of the fetus and placenta to maintain adequate blood supply in face of increased uterine and systemic vascular resistances and to the alleged defective angiogenesis affecting placental circulation. Whether this change is cause or consequence of pre-eclampsia is not known, but may also be seen in other pathological conditions related to pregnancy, such as fetal growth restriction, suggesting that it may rather represent a fetal response to adverse conditions imposed to pregnancy [26]. One of the biochemical changes that may elicit a feto-placental activation of the NO system may well be the increased amount of ADMA (the endogenous NOS inhibitor) which also displayed an elevated trend in umbilical blood. Indeed, previous studies have found that higher ADMA levels early during pregnancy may be associated to later development of pre-eclampsia [27].

Genetic background affects the likelihood of developing gestational hypertension and preeclampsia. Moreover, studies have associated pre-eclampsia to the development of subsequent cardiovascular risk [28]. Hence distribution of common SNPs of the VEGF gene was analyzed in the present research. These SNPs have also been related to cardiovascular disease [14]. Even if selected trends were noted, the limited sample size did not allow us to identify significant differences in the distribution of SNPs among analyzed groups. However, a potential strength of our study was finding a significant trend toward lower umbilical vein VEGF levels in pre-eclampsia cases in the presence of −2578 CC and −1154 AG genotypes. This is in agreement with the findings of other groups [16,29]. Contrary to this, Garza-Veloz et al. [30] have failed to demonstrate an association between pre-eclampsia

Table IV. Rho Spearman coefficients between artery and vein analyte levels (cases and controls) and placental and neonatal weight.

	Cases					
	NO artery	NO vein	ADMA artery	ADMA vein	VEGF artery	VEGF vein
ADMA	0.39 (0.04)*	0.47 (0.01)	-	-	−0.17 (0.43)	−0.07 (0.77)
VEGF	0.05 (0.79)	−0.04 (0.86)	−0.17 (0.43)	−0.07 (0.77)	-	-
Placental weight (g)	0.10 (0.63)	0.22 (0.28)	−0.26 (0.20)	−0.02 (0.90)	−0.05 (0.81)	−0.03 (0.90)
Neonatal weight (g)	−0.17 (0.40)	−0.28 (0.17)	−0.08 (0.70)	0.09 (0.65)	0.33 (0.10)	0.09 (0.65)
	Controls					
	NO artery	NO vein	ADMA artery	ADMA vein	VEGF artery	VEGF vein
ADMA	0.14 (0.48)	0.05 (0.80)	-	-	0.05 (0.80)	−0.27 (0.18)
VEGF	0.11 (0.58)	0.32 (0.11)	0.05 (0.80)	0.27 (0.18)	-	-
Placental weight (g)	−0.07 (0.73)	0.08 (0.67)	0.30 (0.14)	−0.14 (0.50)	0.17 (0.39)	0.04 (0.83)
Neonatal weight (g)	−0.08 (0.68)	0.04 (0.85)	−0.11 (0.60)	−0.08 (0.71)	0.32 (0.11)	−0.11 (0.59)

*p Values in parenthesis.

Table V. Single nucleotide polymorphisms of the promoter region of the VEGF gene among studied women.

SNP position and genotype	Preeclampsia		Controls		p Value*
	Genotype frequency	Allelic frequency	Genotype frequency	Allelic frequency	
VEGF (−2578 A/C)					
AA	3 (9.7%)	A = 35.5%	3 (9.7%)	A = 35.5%	0.66
AC	16 (51.6%)		16 (51.6%)		1.0
CC	12 (38.7%)	C = 64.5%	12 (38.7%)	C = 64.5%	1.0
VEGF (1498 C/T)					
CC	3 (9.7%)	C = 38.7%	4 (12.9%)	C = 38.7%	1.0
CT	18 (58.0%)		16 (51.6%)		0.60
TT	10 (32.3%)	T = 61.3%	11 (35.5%)	T = 61.3%	0.78
VEGF (−1154 A/G)					
GG	0 (0%)	G = 12.9%	1 (3.2%)	G = 16.1%	1.0
AG	8 (25.8%)		8 (25.8%)		1.0
AA	23 (74.2%)	A = 87.1%	22 (71.0%)	A = 83.9%	0.77
VEGF (−634 C/G)					
GG	11 (35.5%)	G 64.5%	17 (54.8%)	G 74.2%	0.12
CG	18 (58.0%)		12 (38.7%)		0.12
CC	2 (6.5%)	C = 35.5%	2 (6.5%)	C = 25.8%	1.0
VEGF (+936 C/T)					
CC	15 (48.4%)	C = 67.7%	17 (54.8%)	C = 74.2%	0.61
CT	12 (38.7%)		12 (38.7%)		1.0
TT	4 (12.9%)	T 32.3%	2 (6.5%)	T 25.8%	0.66

*p Value as determined by the chi-square or the Fisher's exact test when comparing genotype frequency of cases vs. controls.

and VEGF allele, genotype, or haplotype frequencies. Lower cord VEGF levels found in our *in vivo* model seem to fit well with previous *in vitro* findings reporting that in pre-eclampsia endogenous VEGF release is reduced at the placental site [31]. Although women with the 1154 allele have an increased risk for recurrent pregnancy losses [32], correlation between lower cord VEGF levels and the VEGF 1154 AG genotype is lacking in the literature.

Another interesting feature of our study was enrolling near term severe pre-eclamptic patients. About 75% of women delivering at Sotomayor Hospital have inadequate prenatal care due to low social and economic status [17]. This leads to delayed identification of pre-eclampsia and the development of overt disease, which is often diagnosed at the time of hospital admission for spontaneous labor. While most pre-eclampsia studies drawn from developed countries report data of women at lower gestational ages, our analysis shows that altered feto-placental levels of NO, ADMA, and VEGF were observed in pre-eclampsia near term.

As for the limitations of the present study one can mention the small sample size which does not allow drawing definitive conclusions in reference to the analyzed polymorphisms. Unfortunately, due to resource limitations, NOS polymorphisms were not explored, which may also be seen as a potential limitation. Nevertheless, exploring VEGF, NO, and ADMA levels and the VEGF polymorphisms on the fetal/placental side may be seen as strengths, providing insights for future research, which should include more cases and a broader range of analyzed polymorphisms (including NOS).

In summary, this study found elevated levels of NO and ADMA in the feto-placental circulation of pre-eclamptic women, along with lower VEGF levels. This biochemical pattern may suggest a combination of a defective angiogenesis coupled with a feto-placental endothelial adaptive response aimed at maintaining an adequate blood flow to the fetus. A genetic predisposition toward lower umbilical vein VEGF levels is also suggested by the association with the VEGF −2578 CC and −1154 AG genotypes. These findings, despite the aforementioned limitations, contribute to

Table VI. Umbilical vein VEGF levels according to SNP genotype of the promoter region of the VEGF gene among studied women.

Position and genotype	VEGF levels (pg/mL) all women	VEGF levels (pg/mL) pre-eclampsia	VEGF levels (pg/mL) controls	*p* Value*
VEGF (−2578 A/C)				
AA	40.9 [79.2]	52.5 [171.7]	29.2 [-]	0.70
AC	34.9 [130.3]	34.5 [151.6]	54.1 [121.6]	0.98
CC	57.6 [127.7]	33.8 [84.8]	101.1 [193.2]	0.04
p Value**	0.96	0.43	0.14	
VEGF (−1498 C/T)				
CC	52.5 [101.0]	52.5 [171.7]	50.5 [86.5]	0.85
CT	34.4 [139.5]	26.3 [144.6]	54.1 [145.1]	0.60
TT	66.7 [106.9]	50.9 [95.1]	93.8 [130.2]	0.19
p Value	0.67	0.77	0.40	
VEGF (1154 A/G)				
GG	10.2	-	10.2 [-]	-
AG	10.6 [24.1]	8.5 [20.5]	23.5 [118.5]	0.23
AA	86.4 [128.0]	75.8 [151.0]	93.8 [123.2]	0.54
p Value	0.003	0.02	0.04	
VEGF (634 C/G)				
GG	35.0 [95.8]	23.5 [193.8]	41.3 [75.1]	0.46
CG	81.1 [134.7]	50.9 [114.3]	136.7 [186.2]	0.19
CC	63.9 [186.9]	43.9 [-]	136.7 [-]	0.66
p Value	0.58	0.78	0.16	
VEGF (+936 C/T)				
CC	44.1 [129.8]	52.5 [165.2]	36.3 [117.1]	0.84
CT	51.3 [126.3]	16.3 [91.5]	110.4 [114.8]	0.10
TT	63.9 [146.9]	55.7 [101.8]	175.0 [-]	0.53
p Value	0.94	0.63	0.35	

Data are presented as medians [interquartile ranges]. *p Value as determined by the Mann–Whitney test when comparing cases vs. controls.**p Value obtained using the Kruskal–Wallis test after intragroup comparison.

the characterization of the biological basis of pre-eclampsia, and may help toward the future development of new strategies for the screening and treatment of pregnant women with this severe condition.

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Chapter 7

Cardiovascular disease during mid-life: biological mechanisms

Vassalle C, Simoncini T, ***Chedraui P,*** *Pérez-López FR.*

Why sex matters: the biological mechanisms of cardiovascular disease.

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SEX AND CARDIOVASCULAR DISEASE

Why sex matters: the biological mechanisms of cardiovascular disease

Cristina Vassalle[1], Tommaso Simoncini[2], Peter Chedraui[3] & Faustino R. Pérez-López[4]

[1]*G. Monasterio Foundation of the Toscana Region and Institute of Clinical Physiology-CNR, Pisa, Italy,* [2]*Department of Reproductive Medicine and Child Development, University of Pisa, Pisa, Italy,* [3]*Institute of Biomedicine, Catholic University of Guayaquil, Guayaquil, Ecuador, and* [4]*Department of Obstetrics and Gynecology, University of Zaragoza and Lozano Blesa Hospital, Zaragoza, Spain*

Cardiovascular disease (CVD) is the leading determinant of mortality and morbidity in women. However, a full understanding of the basic and clinical aspects of CVD in women is far from being accomplished. Sexual dimorphism in CVD has been reported both in humans and experimental animals. Menopause is a risk factor for CVD due to the reduction of endogenous estrogen, although the mechanisms underlying are poorly understood. Estrogens act through binding to vascular estrogen receptors and by non-genomic mechanisms. Advances in this field are essential to improve CVD diagnostic and clinical strategies in women, and to develop sex-specific prevention plans as much as female-oriented treatment algorithms. This paper reviews pathophysiology of CVD in women and its potential clinical implications. Particular emphasis is given to biochemical markers and to indicators of cardiovascular dysfunction and damage. Estimation of these parameters, central to cardiovascular pathophysiology, could represent a particularly relevant tool in female patients. More research is needed to identify women who will profit most of early intervention.

Keywords: Cardiovascular risk, sex, gender, menopause, steroid hormones

Introduction

Cardiovascular disease (CVD) is the leading cause of death and morbidity in the developed countries. There are marked gender differences in its pathogenesis, symptoms, responses to therapies and, ultimate outcomes. Within the past few years, a number of International Organizations have initiated extensive research and educational programs on CVD in women, aimed to facilitate the development of specific management strategies for their health [1,2]. At the basis of this ongoing effort is the need to understand the gender-related characteristics in the pathophysiology and pharmacology of CVD [3,4].

This review focuses on the pathophysiology of CVD in women. Particular emphasis is given to biochemical markers, and to indicators of cardiovascular dysfunction and damage. Estimation of these parameters, central to cardiovascular pathophysiology, could represent a particularly relevant tool in female patients.

Steroid hormones and the cardiovascular system

Steroid hormones intimately regulate the fundamental cardiovascular functions including blood pressure, blood flow, vasodilatation and vasoconstriction, vascular inflammation and remodeling atherosclerosis [5]. Vascular endothelial cells, vascular smooth muscle cells as well as cardiomyocytes are all well equipped with sex steroid hormone receptors as well as with the steroid metabolizing enzyme aromatase [6]. One obvious difference between genders is represented by the remarkably complex changes in sex steroids observed in women between puberty and menopause. The equilibrium of estrogen or progesterone concentrations changes is associated with significant modifications of cardiovascular function in women.

The mechanistic actions of sex steroid hormones in cultured cardiovascular cells have been investigated in detail and are thought to explain a large part of the gender specificities in the cardiovascular system [7]. Sex steroids act by binding deputed receptors. This leads to structural changes in the tertiary and quaternary structure of the receptor, favoring (or decreasing) the interaction with scaffolds and other interacting proteins, known as co-activators or co-repressors, and facilitating the nuclear localization of these modified complexes. Sex steroid receptors are herein able to identify specific DNA response elements on regulatory areas of regulated genes, and by doing so they guide the regulatory elements they interact with to the final targets. This modifies the expression of a large set of proteins, enzymes and receptors, thus changing the functional status of the cell [8].

On top of this, sex steroid receptors also regulate vascular cells through rapid actions elicited at the membrane or within the cytoplasm. These effects require interactions between steroid receptors and signaling intermediates, such as G proteins or small guanosine triphosphate (GTP)-ases and lead to rapid changes of intracellular ion (particularly potassium and calcium) concentrations and of the phosphorylation status of kinases (such as mitogen-activate protein kinases) [9]. These extra-nuclear mechanisms of actions turn into faster changes of functional status of cells as compared to the nuclear ones, and are particularly important in the vascular system. Whenever changes in circulating estrogen or progesterone ensue, such as after menopause, modifications in the activity of all the above-mentioned signaling activities can be identified in the cardiovascular system, and these changes relate to discrete functional modifications.

Human arteries and veins express aromatase, which locally converts testosterone (T) into estradiol. In men, aromatase deficiency accelerates atherogenesis [10]. In a large population of women, T (total and free), dehydroepiandrosterone sulfate (DHEAS), and androstenedione (A) levels decrease with aging [11]. Although androgens appear to be correlated with different cardiovascular risk factors, an inverse relationship has been observed between T and A and carotid atherosclerosis in both

Correspondence: Faustino R. Pérez-López, Department of Obstetrics and Gynecology, Universidad de Zaragoza Facultad de Medicina, Hospital Clínico de Zaragoza, Domingo Miral s/n, Zaragoza 50009, Spain. Tel: +34 976 76 1734. Fax: +34 976 76 1735. E-mail: faustino.perez@unizar.es

premenopausal and postmenopausal women [12,13]. Thus, the relationship between androgens and CVD in women remains controversial.

Endothelial cells

Endothelium represents an elective cellular target for estrogens. Chronic estrogen administration enhances endothelial function in a number of vascular beds. Conversely, women exhibit an age-related impairment of flow-mediated vasodilation as men, but this reduction is particularly marked after menopause, suggesting a protective role of estrogens. Estrogen receptors (ERs) are expressed in endothelial cells and have an atheroprotective effect. Through the recruitment of ERs, estradiol increases endothelial nitric oxide (NO) and prostacyclin synthesis, thus slowing early atheroma formation. Estradiol also decreases synthesis of pro-inflammatory cytokines by circulating or resident immune cells. In addition, estradiol facilitates endothelial vascular healing and neo-angiogenesis. While many of these effects are regulated by either ER-α or ER-β, ER-α is found to be dominant at the vascular level [14] and in animal models this receptor sub-type mediates protection of coronary endothelial dysfunction after ischemia [15]. Emerging evidence suggests that estradiol also exerts vascular actions through other receptors, and particularly through the recently-identified G-protein-coupled receptor dubbed GPR30. GPR30 immunoreactivity has been demonstrated in endothelial and vascular smooth muscle cells of carotid arteries from both rat genders. In addition, GPR30 agonists elicit endothelial-derived NO-dependent relaxation of the carotid artery in male and female rats [16].

Protective effects exerted by estrogens on endothelium include multiple cellular mechanisms, as evidenced by a number of experimental and clinical data. Estrogen has been demonstrated to activate calcium-dependent potassium channels and induce a rapid increase in NO release [17]. These non-genomic effects of estrogens on NO production are paralleled by their genomic actions exerted by activation of endothelial NO synthase (eNOS) through a receptor-mediated system [18]. Estrogen has anti-oxidant and anti-inflammatory properties, acting through multiple effects. Among them, estrogen may upregulate prostacyclin synthase and the expression of vascular endothelial growth factor. Conversely, it inhibits endothelin-1 release, and modulates adhesion-molecule and tumor necrosis factor-α (TNF-α) expression and endothelial cell apoptosis [19]. Moreover, estrogen can act by upregulating superoxide dismutase in the vascular district, which contributes to increased superoxide ion clearance [20]. In addition to this genomic effect, estrogen can detoxify superoxide ions through binding with the proton in the hydroxyl group of its aromatic ring [21].

Most of experimental studies suggest the protective role of estrogen in terms of the oxidative stress status that may improve the oxidative balance in the vascular sites, improving local NO bioavailability and consequently enhancing endothelium-dependent dilatation. Estrogen may also influence the redox balance through modulation of mitochondrial enzyme activity. Thus, the antioxidant effects are regarded as one of the main mechanisms by which hormones protect women during their fertile life, when they are at lower risk of cardiovascular events respect to men [22]. In fact, oxidative stress is generally higher in men compared to premenopausal women [23]. After the menopause, when hormonal levels markedly fall, the risk of experiencing cardiovascular events rapidly rise in women, in parallel to a rapidly increase of oxidative stress biomarker levels [24]. However, although some studies evidenced that women have higher levels of oxidative stress than do men, other authors found higher oxidative stress biomarkers in men, and others no gender-related differences [23,25,26]. Nonetheless, in old populations of healthy and coronary artery disease subjects, women appear more susceptible to oxidative damage [24]. Thus, the estimation of oxidative stress could represent a promising biomarker for cardiovascular risk estimation particularly relevant in female patients. Moreover, it is likely that women might benefit more than men from antioxidant vitamin supplementation.

Low levels of androgen are associated with endothelial dysfunction, adverse lipid profiles, and inflammatory responses. In recently postmenopausal women, T levels have proatherogenic effects, including an association of T with C-reactive protein (CRP) and endothelin-1, waist circumference and blood pressure [27]. Physiological levels of T and dihydrotestosterone (DHT) increase endothelial synthesis of NO, whereas at supra-physiological doses of T or DHT the induction of NO synthesis is lost. Testosterone may activate both androgen and estrogen receptors (by local aromatase conversion to estradiol) in cardiovascular tissues, thus providing important atheroprotective effects through estrogen-dependent mechanisms [13,28]. Therefore, T and DHT act on endothelial cells through androgen receptors (ARs) or via conversion to estradiol. In fact, conversion of T to estrogen by aromatase may help maintain normal vascular tone as is supported by data in healthy human males showing that aromatase inhibitors negatively affect vascular relaxation, while aromatase knockout mice demonstrate abnormal vascular relaxation [24]. In cell culture studies, T inhibits expression of adhesion molecules, such as vascular cell adhesion molecule (VCAM) 1, and this effect, being reversed by an aromatase inhibitor, seems essentially due to conversion of T into estrogen [29].

DHEAS is an acute pig coronary artery vasodilator, although less potent than T. Its action might be mediated through androgen receptors and may involve ATP-sensible potassium channels [19]. T does not affect vascular proliferation, whereas DHEA, in particular, increases endothelial proliferation and angiogenesis, probably by increasing endothelial NO production [30]. Accordingly, DHEA is metabolized in the endothelial cell to other biologically active steroids, including estradiol. However, DHEA also increases endothelin-1 secretion, exerting vasoconstrictive effects [13].

Progesterone and progestin effects on adhesion molecule and endothelial cells are even more unclear, depending of progestin type and concentration [31]. However, progesterone has inhibitory effects on different endothelial cell types [6]. In general, there is evidence that the molecular actions of progesterone receptor (PR) agonists in endothelial cells can be quite different, likely due to the induction of specific tertiary structures upon binding to the ligand binding pocket of the receptor [32]. The progestin drospirenone is a unique compound that acts on both mineralcorticoid and progesterone receptors. Drospirenone induces rapid activation and expression of eNOS, in addition, it also antagonizes the detrimental effect of aldosterone on NO secretion [33]. Aldosterone antagonism is limited to drospirenone, since progesterone or medroxyprogesterone do not produce this effect [34].

Vascular smooth muscle cells

Female vascular smooth muscle cells (VSMCs) have greater expression of ER-α and ER-β than males, while having similar quantity of GPR30. The lower contractile activity of female VSMCs has been related to this gender-related difference in the expression of ER-α and ER-β [35]. An important effect of estrogen on VSMC related to atherosclerosis is the inhibition of their proliferation following vascular injury, an opposite effect

respect to the induction of proliferation in endothelial cells. The divergence of the estrogen effects on different cellular types may depend upon the modulation of transcriptional factors involved in the regulation of the proliferative mediators. Specifically, treatment with estradiol increased both insulin growth factor (IGF) I and cyclooxygenase-2 mRNA expression in human umbilical venous endothelial cells, with an opposite effect on the transcription of these genes in human aortic smooth muscle cells [36].

In cultured VSMCs from young rats pre-treated with estradiol, induced premature senescence was suppressed in a dose-dependent manner, and these senescent-inhibiting effects of estradiol could be blocked by the estrogen receptor antagonist ICI 182,780. On the contrary, in VSMCs from old rats the senescent-inhibiting of estradiol is not present and even some senescent-promoting effect was demonstrated [37]. These experimental findings support the "time window theory" of menopause hormone treatment. In addition, it seems that estradiol may have opposite actions, under some circumstances it has proliferative actions and under others has pro-senescent vascular effects.

Most available data suggest that progesterone and progestins in general inhibit, while androgens increase VSMC proliferation [6]. Progesterone also increases oxidative stress in VSMC and antagonizes the vasoprotective effect of estrogen on antioxidant enzyme expression and function [38]. Recent experimental results using rat aortic VSMC cultures have shown that progesterone enhances cell proliferation, migration and apoptosis [39]. In experimental setting, acute administration of T induces arterial vasodilatation, through an endothelium-independent mechanism, rather involving ATP sensitive potassium channels on smooth muscle cells [40]. Accordingly, ATP-sensitive K+ channels appear to play a role in the vasodilatory effect of T in an in vivo model [41].

Cardiomyocites

Sex differences exist in normal heart function, as cardiac contractility results greater in women than in men, and myocardial mass appears better preserved in women with aging [42]. These effects appear due to multiple mechanisms including sex differences in mRNA expression of functional and structural cardiac proteins. Sex differences also exist in cardiac electrophysiological function and in characteristics of both inherited and acquired forms of heart disease. In particular, specific familial hypertrophic cardiomyopathies result more severe in male than in female subjects, while women with aortic stenosis retain hypertrophic hearts with a better contractile function respect to those of men with the same disease [43].

Treatment with T may improve exercise capacity, ventilator function, muscle strength, and insulin sensitivity in men [44]. Since postmenopausal women have relative low T levels, androgen supplementation could produce similar effects in women to those reported in men. A recent study has reported that T supplementation improves functional capacity, insulin sensitivity and muscle strength in a small number of older women with congestive heart failure, and the mechanism of action seems to be peripheral without action on the left ventricular function [45].

Natriuretic peptides

In healthy subjects, brain natriuretic peptide has been found to vary by age and sex, with higher levels seen in women than in men [46]. On the other hand, in cycling women estrogens exert a stimulating effect on cardiac endocrine function by increasing the secretion of natriuretic peptides from cardiomyocytes, whereas hormone replacement therapy also induces a rise in the levels of cardiac natriuretic hormones, suggesting that atrial natriuretic peptides may play an important role in mediating the cardioprotective effects of female steroid sex hormones throughout female life. Cardiac natriuretic hormones have several important physiological actions, in terms of decreasing blood pressure, increasing natriuresis and diuresis, inhibiting the sympathetic nervous system and releasing several hormones, including aldosterone, angiotensin II, endothelins, renin, and vasopressin [47]. Conversely, in subjects with heart failure brain natriuretic peptide levels rise, paradoxically, to a lesser degree in women than in men, even in cases of comparable functional impairment [48].

Vascular inflammation and oxidative stress

Vascular inflammation and oxidative stress represent crucial basic mechanisms which take part in all the steps from the onset, to development and progression of CVD. The complexity of effects exerted by estrogen is evidenced by controversial data obtained in this field, being the estrogen either anti- or pro-inflammatory depending on diverse factors such as the target cell type and organ, timing and levels of estrogen. The pro-inflammatory role of estrogens and their impact in the modulation of B-cell and T-cell immunity seems underlined by sex-related differences in the occurrence of inflammatory immunological diseases, including rheumatoid arthritis and systemic lupus erythematosus, and from the estrogenic modulation of their symptoms associated with the different phases of female life. As puberty, pregnancy and menopause [49].

Estrogen effects on myogenic- and shear stress-dependent mechanisms of arterioles significantly contribute to the control of local blood flow and peripheral resistance, also through modulation of endothelial mediators, such as NO and prostaglandins [50]. Consequently, postmenopausal women presented higher systolic and diastolic blood pressure values than premenopausal women and even higher than in men of the same age [10].

Accordingly, elevated levels of pro-inflammatory cytokines and increasesd expression of iNOS are induced by chronic administration of estradiol in animal studies, whereas estradiol increases pro-inflammatory cytokine production from activated peritoneal macrophages, all these effects through mediated ER-α. Conversely, estrogens reduced levels of pro-inflammatory cytokines, such as TNF-α, interleukin (IL)-1β, IL-6, monocyte chemotactic protein-1, and metalloproteinases, and increase those of anti-inflammatory cytokines, as IL-4, IL-10, transforming growth factor (TGF) β and tissue inhibitor of metalloproteinases, again mediated by ER-α [6]. In further support of the anti-inflammatory effects of estrogen, women with higher estrogen status have significantly lower levels of monocyte chemo-attractant protein-1 (a chemokine responsible for the recruitment of monocytes to sites of inflammation) than those with lower estrogen [51]. Conversely, pro-inflammatory cytokines and expression of cellular surface adhesion molecules (E-selectin, P-selectin, VCAM-1 and intercellular adhesion molecule-1) increase in parallel to the decrease of estrogens after the menopause, and replacement hormonal therapy is able to restore levels to those of pre-menopause [6].

Among cytokines, the IL-6, that stimulates hepatocytes to synthesize acute phase response proteins such as CRP and fibrinogen, has been the most studied in relation to cardiovascular risk. Data from the British Women's Heart Study evidenced the association of IL-6 levels and coronary heart disease (CHD) risk factors. However, after adjusting for components of the metabolic

syndrome, the association lost significance [52]. Conversely, a previous analysis of 17 studies found an association between IL-6 and CHD, which remained significant also after adjustment for established CHD risk factors [53].

Endothelial microparticles and thrombogenicity

Vascular injury induces a progressive endothelial loss of function and integrity, until the detachment of whole endothelial cells or endothelial microvesicles or microparticles (MP) derived from activated or apoptotic cells [54]. These endothelial MPs are important in the CVD pathophysiology and transport specific biochemical messengers which, in turn, may initiate or accelerate inflammatory response and vascular dysfunction in other territories [6]. They disturb coagulation, angiogenesis and vascular homeostasis contributing to atherosclerosis [55]. In vitro study of isolated arteries exposed to endothelial MP from patients diagnosed with metabolic syndrome, acute coronary syndrome, severe renal failure or preeclampsia produced endothelial dysfunction as compared to endothelial MP from healthy individuals [56,57].

Healthy women show menstrual cycle-specific differences in platelet-cell-derived and endothelial MP which differ significantly from age-matched men. Number of these cells and their thrombogenic capacity were significantly greater in recent postmenopausal women with high coronary artery calcium who would not be evidenced by considering traditional screening cardiovascular risk parameters, such as BMI, lipid profile and glucose [58]. Moreover, estrogen regulates cell-specific endothelial MP, being the number of endothelium-, platelet-, monocyte- and granulocyte-derived MP greater when estrogen is low [59].

Endothelial progenitor cells

Endothelial progenitor cells (EPCs) are bone marrow-derived cells actively involved in cardiovascular homeostasis, providing a circulating pool of cells that repair endothelial damage in physiological conditions. These cells are mobilized from bone marrow and other organs to peripheral blood towards the damaged sites by ischemic insult to promote angiogenesis [60]. EPCs repair endothelial structure and enhance activity of eNOS, restoring the functional status of the endothelium. Contrary to mature endothelial cells which have limited regenerative capacity, EPCs reside at sites of endothelial injury and ischemia proliferating and integrating into the endothelium. In addition, they produce vascular growth factors [60,61]. EPCs are higher in fertile women than men, their number fell after menopause, and their cyclic EPCs mobilization changes with menstrual/hormonal cycle and may be related to endometrial regeneration [62]. EPC subpopulations in premenopausal women may partially explain the lower prevalence of cardiovascular events in menstruating women as compared to men [63,64]. EPC number is reduced in castrated mice and enhanced in hyperstimulated ovaries from women included in in vitro fertilization protocols [65,66]. These cell number and functions are also influenced by lifestyle, adiposity, weight changes and exercise [60]. Despite this, it seems that EPC are better predicitive factor for vascular health than conventional risk factors, such as lipid profile, hypertension and diabetes. The evaluation of EPC number and function might reflect the degree of cardiovascular protection of the female population. However, improvement of available methods for ECP quantification is expected, in order to allow a better characterization of different cellular phenotypes and functional properties.

Final remarks

Available evidence clearly indicates that being male or female is a variable that may profoundly affect the course of CVD. The burden of CVD in middle-aged women relative to men is increasing [67]. Sex represents an important modifier of the cardiovascular system and should be recognized as an important factor in both basic science and clinical research involved in the pathophysiology of CVD. Currently, research on sex-related differences in pathophysiology of CVD has begun, but sex in all its complexity is only starting to be recognized as a scientific category in the medical practice. Until now, no risk factor has been recognized as acting on one sex but not on the other. This finding implies that general mechanisms involved in the pathogenesis of CHD are evidently very similar for men and women. Thus, it is unlikely to image specific biomarkers or additional testing only targeted for women. However, the influence of sexual hormones is critical in determining the weight of cardiovascular risk factors between men and women. In this sense, the effect of the menopause on both cardiovascular risk factors and CHD is unique for women, which represent a distinct subpopulation within CVD patients.

New tests are needed to define risk and guide treatment according to sex issues also in laboratory medicine, because sex-related differences may have a great impact on patient stratification and outcome. These aspects are not generally taken into account when using biochemical biomarkers as discriminating parameters in clinical studies. Consequently, a significant part of the commercially available assays lack these data. Thus, it would be desirable the availability of gender-related reference values for relevant biochemical parameters, and even differential for the pre or post-menopausal status.

The areas related to recently proposed biomarkers, such as inflammatory and oxidative stress parameters as well as EPC, endothelial MP and genetic factors appear promising in providing new basic information and development in the clinical practice, also from a sex point of view. However, we yet need to understand the entity of sex-related differences in CVD and whether multiple biomarkers contribute to improved CHD risk prediction when compared with assessment using traditional risk factors in post-menopausal women. This knowledge will allow to identify more important sex-associated biomarkers or panels to optimize diagnostic and therapeutic strategies targeted for men and women when appropriate.

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CHAPTER 8

Summary of conclusions

Summary

1. The prevalence of the METS and factors relating to each of its composing factors were high and associated to metabolic and lipid abnormalities. Abdominal obesity was related to lower education; hence there is a need to educate high risk populations and increase awareness of the problem.
2. Components of the syndrome were significantly related to an increase in the severity of menopausal symptoms. Indeed, abdominal obesity related to hot flushes, depression and muscle and joint pain.
3. As determined by the The Athens Insomnia Scale, the METS or its components did not significantly affect sleep (insomnia). Other psycho-somatic female and partner issues correlated to insomnia in postmenopausal women.
4. Postmenopausal women with the METS displayed higher IL-6 and NO levels as compared to those without the syndrome, with significant correlations found between studied analytes and some of the METS components. The study found an interesting scenario: pro-inflammation in the presence of high NO levels. We hypothesized that analyzed women with the METS or any of its components were still at an earlier stage of the disease in which NO production may be increased in order to protect against the adverse effects of chronic inflammation when instead lower NO levels would ensue as a consequence of endothelial dysfunction. Interesting was finding that age and time since the menopause positively correlated with both cytokine levels indicating that in time chronic inflammation will ensue. Our results support the “window of opportunity” theorem in which lower estrogen levels have still not produced irreversible endothelial damage. Hence, female lifestyle changes and preventive measures at this stage are crucial and should be highly encouraged.
5. Postmenopausal women with the METS displayed higher IL-6 (marker of inflammation) and lower uPA (marker of endothelial dysfunction) levels. These were mainly related to metabolic and lipid abnormalities.

6. Women with severe preeclampsia displayed higher NO and ADMA fetal circulating levels (vein and artery) and lower VEGF umbilical vein levels. There was a significant trend of finding lower VEGF levels in the presence of -2578 CC and -1154 AG genotypes. Results of this study provides insights for new study designs using preeclampsia as a study model of endothelial dysfunction that will aim at finding a biomolecular marker capable of establishing the link between endothelial changes found in preeclampsia as well as in postmenopausal METS.
7. The analysis of inflammatory and oxidative stress biomarkers and genetic factors appear promising in providing new basic information and development in the clinical practice. Nevertheless, we yet need to understand the entity of sex-related differences in cardiovascular disease and whether multiple biomarkers contribute to improved coronary heart disease risk prediction when compared to assessment using traditional risk factors in postmenopausal women. This knowledge will allow to identify more important sex-associated biomarkers or panels to optimize diagnostic and therapeutic strategies targeted for men and women when appropriate.

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To my children, for the time I have subtracted from their lives in order to complete this scientific achievement, I will always be in debit with them.

And finally to the cornerstone of all my life achievements, my mother, for all she has given me, her sacrificed life has always been my inspiration.

Curriculum Vitae

Peter Chedraui was born on August 3rd, 1965, in New York City. He grew up in Astoria Queens until the age of 10, when he moved up to Guayaquil, Ecuador to complete primary and secondary school. After this he obtained his medical and master degree in science at the Universidad Católica de Santiago de Guayaquil. Subsequently he completed an OB GYN residency program at Enrique C. Sotomayor Hospital of Guayaquil.

After completing his residency he was awarded a Fellowship sponsored by FIGO and ACOG in Maternal Fetal Medicine at NYU Medical Center under the tutorship of Dr. Charles J. Lockwood. Since 2005 has been Chief of the High Risk Pregnancy Labor and Delivery Unit at Sotomayor Hospital. Since 2002 he has been Director of the Institute of Biomedicine, of the Universidad Católica and Adjunct professor at the same University.

He is a member of several National and International Scientific Societies, with more than 120 peer-reviewed publications supporting his scientific research career.

He is currently President of the Ecuadorian Climacteric and Menopause Society, Vice-President of the Latin American Gynecological Endocrinology Association, Re-Elected Board Member of the International Menopause Society and Editorial member of various international peer reviewed journal devoted to women's health.

He has garnished multiple grants from the Universidad Católica, the pharmaceutical industry and various private foundations. To date he is actively serving as a research collaborator for several international joint efforts.

His primary research interests are genetic aspects of preeclampsia and preterm birth and their impact of adult female health such as the metabolic syndrome and cardiovascular risk. In addition is interested in the epidemiology of the menopause and other female healthcare issues of mid-life.

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