愛知医科大学医学会雑誌 (J. Aichi Med. Univ. Assoc.)
Vol. 42, No. 1/2/3/4, pp. 1–10 (December 15, 2014)

Effects of Gonadotropin-releasing Hormone Agonist on Vascular Reactivity, Oxidative Stress, and Plasma Levels of Asymmetric Dimethylarginine, Inflammatory Markers, Glucose, and Lipids in Women with Endometriosis

RYUSUKE HARADA, SHOKO KINUGASA, KOICHI SHINOHARA and AKIHIKO WAKATSUKI

Department of Obstetrics and Gynecology, Aichi Medical University School of Medicine, Aichi, Japan (Chief professor: Akihiko Wakatsuki)

Objective: To assess the effects of gonadotropin-releasing hormone agonist (GnRHa) on endothelial function, and measured plasma levels of asymmetric dimethylarginine (ADMA), glucose and lipids, markers of systemic inflammation we oxidative stress that may affect vascular reactivity in women with endometriosis.

Methods: A total of 17 women with endometriosis were treated with GnRHa for 6 months. Plasma levels of estradiol (E2), lipids, glucose, insulin, inflammatory markers (e.g., high sensitivity C reactive protein (hs-CRP), serum amyloid protein A (SAA), and interleukin-6 (IL-6)), and cell adhesion molecules (e.g., intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1), and E-selectin) were measured before and 3 and 6 months after therapy. Levels of ADMA, reactive oxygen metabolites (d-ROMs; marker of oxygen free radicals), and biological antioxidant potential (BAP; a marker of antioxidants) were also measured. Vasodilatory responses were assessed by measuring flow-mediated vasodilation (FMD) with high-resolution ultrasonography.

Results: GnRHa therapy significantly decreased plasma E2 levels. FMD significantly decreased from 8.93±1.01% to 6.97±0.93% at 3 months, and to 6.35±0.97% at 6 month ($P=0.01$) after therapy. GnRHa significantly increased plasma levels of ADMA (380.6±16.2 pmol/L to 455.0±17.9 pmol/L at 6 months, $P=0.0001$) and low-density lipoprotein (LDL) cholesterol at 6 months after therapy. GnRHa also significantly increased plasma levels of ICAM-1, VCAM-1, and E-selectin, although it significantly decreased those of hs-CRP and IL-6, but not SAA. Plasma levels of d-ROMs, BAP, insulin, and glucose, as well as the homeostasis model assessment ratio (a marker of insulin resistance), did not change significantly after GnRHa therapy.

Conclusion: Although GnRHa has favorable effects by inhibiting systemic inflammation, estrogen deficiency-induced increases in plasma levels of ADMA, cell adhesion molecules, and LDL cholesterol may further impair endothelium-dependent vascular reactivity in women with endometriosis.

Key words: endometriosis, inflammation, flow-mediated vasodilation, asymmetric dimethylarginine, endothelial function

Received January 8, 2014; Accepted January 14, 2014

INTRODUCTION

Endometriosis, a common gynecological disorder characterized by growth of the endometrial gland and stroma outside the uterus, is associated with symptoms such as dysmenorrhea, hypermenorrhea, and chronic abdominal pain. Endometriosis has been noted in 20～50 % of patients undergoing gynecological laparotomies[1], is diagnosed in women of reproductive age, and is accompanied by infertility in 5～10 % of cases.

Endothelial dysfunction is one of the earliest events in the development of atherosclerosis[2]. Nitric oxide (NO), which is produced during arginine oxidation by NO synthase in endothelial cells, has anti-atherosclerotic effects. We previously demonstrated that endothelium-dependent vasodilation, which is mediated through the release of vasodilators such as NO[3], is impaired in women with endometriosis[4]. We also found that enhanced vascular inflammation and increased plasma levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthesis, may be associated with impaired endothelial function in such women[4]. This suggests that chronic inflammation and decreased vascular reactivity may lead to the development of atherosclerosis in women with endometriosis.

Gonadotropin-releasing hormone agonist (GnRHa), which induces hypoestrogenism by inhibiting pituitary gonadotropin secretion, is used in the treatment of estrogen-dependent diseases such as uterine myomas and endometriosis. GnRHa-induced reductions in plasma estrogen levels reported to be associated with bone loss[5], vasomotor symptoms, and memory complaints. Moreover, estrogen deficiency may increase the risk of cardiovascular disease since women become more susceptible to coronary heart disease after menopause. The correlation between serum estrogen levels, particularly those of estradiol, and vascular endothelial function support inhibition of endothelium-dependent vasodilation in postmenopausal women[6]. Accordingly, it is likely that GnRHa-induced hypoestrogenism may inhibit vascular reactivity in women with endometriosis with existing impairments in endothelial function.

In this study, we investigated the effects of GnRHa therapy on endothelium-dependent vasodilation in women with endometriosis and evaluated changes in plasma levels of ADMA, markers of inflammation, oxidative stress, cell adhesion molecules, glucose, insulin, and lipids that may affect endothelial function.

MATERIALS AND METHODS

1. Subjects

Subjects were 17 Japanese women with the American Society for Reproductive Medicine (ASRM) stage III to IV (mean age, 37.9 years; range, 24～54 years; mean body mass index, 20.0 kg/cm^2; range, 16.8～25.5 kg/cm^2) between April 1, 2011 and March 31, 2012. Surgeons completed operative records, which noted the presence or absence of endometriosis and the stage of endometriosis according to revised ASRM criteria. Endometriosis was diagnosed by laparoscopy and confirmed by histopathologic examination. Exclusion criteria were the presence of diseases such as diabetes mellitus, hypertension, cardiovascular disease, dyslipidemia, systemic lupus erythematosus, and infection. Women who were smokers and/or used any medication were also excluded. Subjects received a subcutaneous injection of 1.88 mg GnRHa (leuprolide acetate) monthly for 6 months. Before and 3 and 6 months after GnRHa treatment, venous blood samples were obtained between 8:00 and 10:00 AM following a 12-hour fast. Before GnRHa therapy, basal

body temperature was used to determine menstrual cycle phase, with all women showing a biphasic basal body temperature pattern. Blood samples were drawn at the mid-follicular phase (day 7～10) of the menstrual cycle.

Written informed consent was obtained from each subject prior to participation. The study was approved by the Ethics Committee of Aichi Medical University.

2. Laboratory analysis

Plasma levels of total cholesterol, triglycerides, and low-density lipoprotein (LDL)-cholesterol were measured using enzymatic methods as previously describe[7]. Levels of high-density lipoprotein (HDL)-cholesterol were determined using similar methods after precipitation of apolipoprotein B-containing lipoproteins with sodium phosphotungstate in the presence of magnesium chloride[8]. Levels of estradiol (E2) and follicle-stimulating hormone (FSH) were measured using enzyme immunoassay (Tosoh Corp, Japan). Plasma levels of CA-125, a marker for evaluating the severity of endometriosis, were measured by enzyme immunoassay (Fujirebio Corp, Japan). Plasma glucose and insulin levels were measured by the hexokinase technique and radioimmunoassay, respectively (Tosoh Corp, Japan), and the homeostasis model assessment ratio (HOMA-R), an estimate of insulin resistance, was calculated from fasting glucose and insulin levels.

Levels of high sensitive C reactive protein (hs-CRP) were analyzed using the Behring Latex-Enhanced CRP assay on the Behring Nephelometer Analyzer System (Dade, Behring USA). Serum amyloid A protein (SAA) levels were determined with a latex agglutination turbidimetric immunoassay (SRL, Japan). Levels of vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and E-selectin were analyzed with ELISA kits (SRL, Japan). Levels of interleukin 6 (IL-6) were measured with a chemiluminescent enzyme immunoassay (SRL, Japan).

Plasma levels of reactive oxygen metabolites and derivatives (d-ROMs) were measured spectrophotometrically with the d-ROMs Test (Diacron, Grosseto, Italy). This test is based on the ability of transition metals to catalyze, in the presence of peroxides, the formation of free radicals, which are then trapped by an alkylamine. The ensuing reaction forms a colored radical detectable at 505 nm through a kinetic reaction that is linear up to 500 U CARR (Carratelli units). Normal values for this test range from 250 to 300 U CARR. In the biological antioxidant potential (BAP) test (Diacron, Grosseto, Italy), plasma is dissolved in a colored solution, which has been previously obtained by mixing a source of ferric ions with a special chromogenic substrate. The solution becomes discolored after incubation. The amount of reduced ferric ions is evaluated by assessing the extent of discoloration, and is used as a readout for antioxidant potentia[9].

Plasma asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) levels were measured by high-performance liquid chromatography, using precolumn derivatization with o-phthalaldehyde (OPA). Plasma samples and internal standards were extracted and incubated with the OPA reagent (5.4 mg/mL OPA in borate buffer, pH 8.5, containing 0.4% mercaptoethanol). OPA derivatives of ADMA and SDMA were separated on a C6H5 column (Macherey and Nagel) with the fluorescence monitor set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm[10].

3. Endothelial function

Patients rested in the supine position for 10 minutes before initiating the examinations. High-resolution Doppler ultrasonography

equipment (Sonovista-Color model MEU-1582, Mochida) with a 10-MHz transducer was used to image the right brachial artery, and vasodilatory responses were measured. A non-tortuous segment of the brachial artery was scanned longitudinally 4 to 5 cm above the elbow, where the clearest image could be obtained. When an adequate transducer position was determined, the skin was marked and the arm was kept in a constant position throughout the study. After baseline images of the brachial artery were obtained and arterial flow velocity determined, a blood pressure cuff encircling the proximal portion of the arm was inflated to 250 mm Hg for 5 minutes, and then suddenly deflated. Increased blood flow after sudden cuff deflation, termed reactive hyperemia, results in flow-mediated vasodilation (FMD)[11]. Flow velocity in the artery was determined again, and 1 minute after cuff deflation, the brachial artery was imaged. Blood pressure and heart rate were recorded during the investigation. The diameter of the brachial artery was measured from the anterior to posterior interface between the media and adventitia ("m" line) at the end of diastole, incident with the R wave on a continuously recorded electrocardiogram. Diameters for four cardiac cycles were determined from the images and averaged. All scans were recorded for later analysis. FMD was calculated as the percent increase in arterial diameter during hyperemia and was used as an index of endothelium-dependent vasodilation[11]. Blood flow was calculated by multiplying the time velocity integral of the angle-corrected Doppler flow signals by the heart rate and the mean cross-sectional vessel area. Intra-observer and inter-observer variability for repeated measurements were 0.03±0.02 and 0.05±0.03 mm, respectively. Variability for FMD performed on 2 separate days was 2.1±0.9 %.

4. Statistical Analysis

Data are expressed as mean±standard error (SE). Changes in parameters measured in this study were analyzed by one-way analysis of variance (ANOVA). When a significant difference was observed, Scheffe's multiple comparison procedure was used to determine which groups were significantly different. $P<0.05$ was considered significant.

RESULTS

As shown in Table 1 GnRHa therapy significantly decreased plasma levels of E2 and FSH at 3 months, plasma levels of CA125 tended to reduce at 3 months. Plasma concentrations of total and LDL cholesterol significantly increased at 3 and 6 months, respectively, while concentrations of HDL cholesterol and triglycerides did not change significantly by therapy. No significant changes were observed in levels of glucose, insulin, and HOMA-R.

Table 2 shows that systolic and diastolic BP, heart rate, brachial artery diameter, blood flow, and the percent increase in blood flow induced by reactive hyperemia did not change significantly with GnRHa therapy. However, FMD significantly decreased from 8.93±1.01 to 6.97±0.93% at 3 months, and to 6.35±0.97% at 6 months ($P=0.01$) after therapy. Figure 1 indicated that GnRHa therapy was inhibited endothelial function in women with endometriosis.

Table 3 indicated that GnRHa therapy significantly increased plasma levels of AMDA and SDMA by 6 months, but plasma d-ROM and BAP levels did not change significantly. Table 4 shows that levels of ICAM-1, VCAM-1, and E-selectin significantly increased at 3 months, while those of hs-CRP and IL-6, but not SAA, significantly decreased, after therapy.

Table 1. Changes in plasma levels of lipids, glucose, insulin, hormones, and CA-125 by GnRHa therapy

	Baseline	3 months	6 months	P
Total cholesterol (mg/dL)	170.6±5.5	184.1±8.2	192.9±14.3[cd]	0.0006
LDL cholesterol (mg/dL)	96.7±5.2	108.2±6.4[b]	113.3±9.6[c]	<0.0001
HDL cholesterol (mg/dL)	62.1±2.0	65.1±2.1	61.2±4.9	0.12
Triglycerid (mg/dL)	80.2±10.2	88.8±11.9	78.6±8.4	0.69
Insuline (IU/mL)	8.7±1.9	6.3±1.4	14.5±6.8	0.59
Glucose (mg/dL)	91.1±2.4	91.1±2.1	91.3±6.1	0.97
HOMA-R	2.1±0.5	1.4±0.3	4.4±2.7	0.52
Estradiol (pg/mL)	66.9±10.9	15.3±2.5[c]	12.1±1.5[c]	<0.0001
FSH (mIU/mL)	9.4±1.0	6.5±0.8[c]	7.1±0.9	0.004
CA-125 (U/mL)	60.9±8.6	34.3±5.9[a]	28.8±6.1[a]	0.04

Data are expressed as mean±standard error.
LDL, low-density lipoprotein: HDL, high-density lipoprotein; HOMA-R, homeostasis model assessment ratio; FSH, follicle-stimulating hormone
[a] P<0.1; [b] P<0.05; [c] P<0.01 vs. Baseline
[d] P<0.05 vs. 3 months

Table 2. Changes in blood pressure, heart rate, brachial artery diameter, and blood flow by GnRHa therapy

	Baseline	3 months	6 months	P
Systolic BP (mmHg)	114±3	113±3	114±3	0.95
Diastolic BP (mmHg)	73±2	73±2	74±2	0.82
Heart rate (betas/min)	69±2	68±2	69±3	0.84
Baseline diameter (mm)	3.23±0.12	3.26±0.11	3.27±0.07	0.32
Baseline flow (ml/min)	198.7±39.6	169.6±15.4	169.6±33.0	0.99
Hyperemic flow (%)	292.3±41.8	294.6±34.2	247.0±21.1	0.71

Data are expressed as mean±standard error.
BP, blood pressure

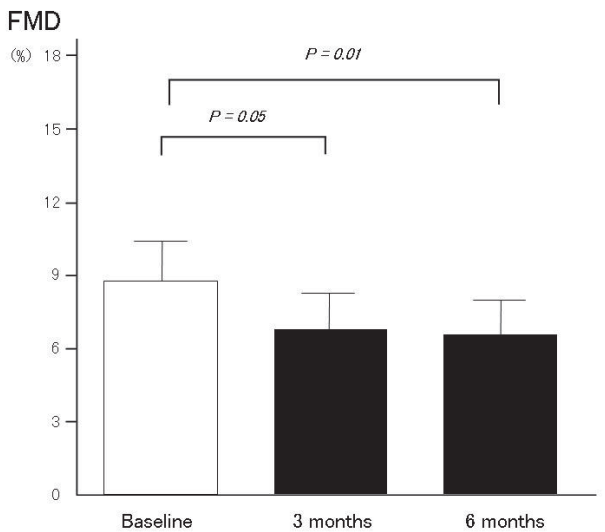

Fig 1. Changes in flow-mediated vasodilation after GnRHa therapy

DISCUSSION

In this study, we measured the effects of GnRHa therapy on endothelium-dependent vasodilation, which is associated with the development of atherosclerosis in women with endometriosis. Nitric oxide (NO), an endothelium-derived relaxing factor, is released in response to increased blood flow during reactive hyperemia. Given that several NO synthase inhibitors suppress endothelium-dependent vasodilation[12], FMD appears to represent a vasodilation-dependent effect mediated by endothelium-derived NO. In addition, FMD in the brachial artery has been reported to correlate with the severity and extent of coronary atherosclerosis[13]. FMD has provided valuable insights

Table 3. Changes in plasma levels of ADMA, SDMA, and markers of oxidative stress by GnRHa therapy

	Baseline	3 months	6 months	P
ADMA (pmol/L)	380.6±12.9	411.2±16.9	455.0±17.9[ab]	0.0001
SDMA (pmol/L)	360.0±16.2	400.6±18.2	434.6±20.8[a]	0.0004
d-ROM (CARR U)	352.7±42.7	351.6±21.3	298.2±26.9	0.51
BAP (μmol/L)	2517.5±135.5	2793.1±100.1	2700.1±230.3	0.59

Data are expressed as mean±standard error.
ADMA, asymmetrical dimethylarginine; SDMA, symmetrical dimethylarginine; d-ROMs, reactive oxygen metabolite; BAP, biological antioxidant potential.
[a] P<0.01 vs. baseline
[b] P<0.05 vs. 3 months

Table 4. Changes in plasma levels of inflammatory markers and cell adhesion molecules by GnRHa therapy

	Baseline	3 months	6 months	P
hs-CRP (ng/mL)	2241.5±1092.5	360.6±102.3[a]	415.3±177.1[a]	0.04
SAA (μg/mL)	7.13±2.07	4.62±1.07	4.67±1.09	0.26
IL-6 (pg/mL)	2.6±0.7	1.5±0.3[a]	1.2±0.2[a]	0.01
VCAM-1 (ng/mL)	637.0±38.0	711.3±33.4[b]	693.7±48.5[b]	0.0005
ICAM-1 (ng/mL)	172.5±13.4	225.1±35.3[a]	201.1±15.3[a]	0.01
E-selection (ng/mL)	26.8±2.4	30.6±2.8[a]	32.3±2.8[a]	0.005

Data are expressed as mean±standard error.
hs-CRP, high sensitivie C-reactive protein; SAA, serum amyloid protein A; IL-6, interleukin 6; VCAM-1, vascular cell adhesion molecule; VCAM-1, intercellular adhesion molecule
[a] P<0.05; [b] P<0.01 vs. baseline

into early atherosclerosis and the potential reversibility of endothelial dysfunction. We previously demonstrated that FMD in the brachial artery was reduced in women with endometriosis[4]. We also showed that increased plasma levels of ADMA and enhanced inflammation may be associated with endothelial dysfunction in this population[4]. Based on these findings, we speculated that women with endometriosis may be at increased risk of cardiovascular disease. In the present study, FMD significantly decreased to the postmenopausal level during the course of GnRHa therapy in women with endometriosis. This suggests that the decreased vascular reactivity in these women may be further impaired by GnRHa therapy. Since endothelial function in the brachial artery may change in parallel with that in the coronary artery, GnRHa-induced impairment of endothelial vascular reactivity may be a greater risk factor for cardiovascular disease in women with endometriosis. Similar to our findings, Yim et al. demonstrated that endothelium-dependent vascular reactivity was inhibited after 6 months of GnRHa therapy, while GnRHa therapy combined with estrogen/progestogen reversed the adverse effects of GnRHa[14]. Lieberman et al. demonstrated that decreased plasma estrogen levels are accompanied by the inhibition of endothelial function, while estrogen replacement improves endothelium-dependent vasodilation[15]. Accordingly, plasma estrogen may play a major role in regulating vascular reactivity in women. Because GnRHa significantly reduced plasma E2 levels in this study, the hypoestrogenism it induces

may be associated with impaired endothelial dysfunction in women with endometriosis whose endothelial function was already compromised.

Factors other than estrogen also regulate endothelial function. For example, insulin resistance and hypertension are known to be associated with impaired endothelial function. The fact that HOMA-R, a marker of insulin resistance, and blood pressure did not change with GnRHa therapy in this study suggests that these factors may not affect endothelial function in women with endometriosis. Oxidative stress, particularly the oxidation of LDL, is also a potent inhibitor of endothelium-dependent vascular relaxation[16]. Oxidized LDL interrupts G protein-dependent stimulation of NO release, and lipid peroxidation products directly block the physiologic action of NO[17]. Plasma levels of BAP, an antioxidant marker, and d-ROMs, a marker of oxygen free radicals, did not change with GnRHa therapy either, suggesting that oxidative stress may not be related to vascular reactivity in these women.

GnRHa therapy increased plasma LDL cholesterol levels. We previously reported that a decrease in plasma estrogen levels enhances lipoprotein lipase activity, which may increase plasma LDL levels, in postmenopausal and oophorectomized women[18]. Additionally, Arca et al. suggested that hypercholesterolemia in postmenopausal women results from impaired LDL receptor activit[19]. Accordingly, hypoestrogenism induced by GnRHa may be associated with increased plasma LDL cholesterol levels. This, in turn, may directly impair endothelial function[20], whereas reductions of plasma cholesterol rapidly improve endothelial function[21]. Thus, the accumulation of LDL cholesterol in plasma may adversely affect on endothelium-dependent vasodilation in women with endometriosis.

ADMA suppresses vascular NO production and impairs vascular reactivity, leading to endothelial dysfunction and vasoconstriction. In our previous study, ADMA levels in women with endometriosis were elevated and inversely associated with FMD, suggesting that increased plasma ADMA levels may be associated with impaired endothelial dysfunction[4]. In this study, levels of ADMA and SDMA, which lacks NO synthase inhibitory activity, increased in response to GnRHa therapy. ADMA is metabolized to citrulline by dimethylarginine dimethylaminohydrolase (DDAH), which is present in many tissues. ADMA levels in plasma can increase through a number of mechanisms, for example, increased production, impaired metabolic degradation, or reduced clearance. Although dimethylarginine is excreted via kidneys and accumulates in patients with chronic renal failure, none of our subjects showed signs of renal failure. Estrogen reportedly decreases plasma ADMA levels by stimulating DDAH activity[22]. Thus, hypoestrogenism induced by GnRHa may inhibit DDAH activity, resulting in elevated plasma ADMA levels, which in turn may adversely affect vascular reactivity in women with endometriosis.

Cell adhesion molecules, once expressed on the surfaces of endothelial cells or leukocytes following cytokine stimulation, are shed from the surface within 24 hours[23]. Plasma levels of cell adhesion molecules are associated with the extent of atherosclerosis and the occurrence of coronary events[24]. In the present study, GnRHa elevated the levels of ICAM-1, VCAM-1, and E-selectin. Since the levels of cell adhesion molecules increase after menopause[25], hypoestrogenism induced by GnRHa may stimulate the production of cell adhesion molecules involved

in endothelial vasodilatory function[26]. Inflammatory responses may also be associated with decreased endothelium-derived NO[27], which promotes leukocyte adhesion and thrombosis formation, leading to atherosclerosis. Inflammation activates macrophages that induce IL-6 secretion. IL-6, in turn, stimulates hepatic CRP production. Importantly, previous studies have reported that CRP levels negatively correlate with endothelial function[28][29]. For example, Vita et al. reported that systemic inflammation may contribute to impaired vasomotor function in microvessels in the Framingham Offspring Study, which involved a large subject population[30]. Since SAA is synthesized in the liver in response to infection and inflammation, as in the case of CRP, it is also considered a sensitive inflammation marker. We previously demonstrated that inflammatory markers such as hs-CRP, SAA, and IL-6 were all elevated in women with endometriosis, suggesting an enhanced inflammatory response in these women. Moreover, we found that hs-CRP levels correlated negatively with FMD, indicating that increased CRP production may be associated with impaired vascular reactivity in women with endometriosis[4]. In contrast, GnRHa therapy reduced plasma hs-CRP and IL-6 levels, suggesting that GnRHa has a favorable effect of reducing inflammation. Although this inhibitory effect appears to improve vascular reactivity, the effect may be overcome by GnRHa-induced hypoestrogenism and increase in levels of plasma LDL cholesterol, ADMA, and cell adhesion molecules, resulting in impaired endothelial function in women with endometriosis.

According to our previous study, women with endometriosis may be at increased risk for cardiovascular disease, as evidenced by the impaired endothelial function resulting from enhanced inflammation and increased plasma ADMA levels. Although GnRHa favorably affects endometriosis-related symptoms such as dysmenorrhea and abdominal pain, the resulting hypoestrogenism, mediated by increased ADMA, cell adhesion molecules, and LDL cholesterol levels, may further impair vascular reactivity in women with endometriosis. Long-term use of GnRHa during reproductive years may lead to an enhanced risk of atherosclerosis. Additional studies are needed to evaluate whether GnRHa administration to women with endometriosis further increases the risk of future cardiovascular disease.

REFERENCES

1) Matorras R, Rodíquez F, Pijoan JI, Ramón O, Gutierrez de Terán G, Rodríguez-Escudero F. Epidemiology of endometriosis in infertile women. Fertil Steril 1995; 63: 34–8.
2) Gilligan DM, Quyyumi AA, Cannon RO 3rd. Effects of physiological levels of estrogen on coronary vasomotor function in postmenopausal women. Circulation 1994; 89: 2545–51.
3) Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987; 327: 524–6.
4) Kinugasa S, Shinohara K, Wakatsuki A. Increased asymmetric dimethylarginine and enhanced inflammation are associated with impaired vascular reactivity in women with endometriosis. Atherosclerosis 2011; 219: 784–8.
5) Friedman AJ, Juneau-Norcross M, Rein MS. Adverse effects of leuprolide acetate depot treatment. Fertil Steril 1993; 59: 448–50.
6) Hashimoto M, Akishita M, Eto M, Ishikawa M, Kozaki K, Toba K, et al. Modulation of endothelium-dependent flow-mediated dilatation of the brachial artery by sex and menstrual cycle. Circulation 1995; 92: 3431–5.
7) Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. Clin Chem 1974; 20: 470–5.

8) Ikeda T, Shibuya Y, Senba U, Sugiuchi H, Araki S, Uji Y, et al. Automated immunoturbidimetric analysis of six plasma apolipoproteins: correlation with radial immunodiffusion assays. J Clin Lab Anal 1991; 5: 90–5.
9) Rubanyi GM, Romero JC, Vanhoutte PM. Flow-induced release of endothelium-derived relaxing factor. Am J Physiol 1986; 250: H1145–9.
10) Kielstein JT, Bode-Böger SM, Frölich JC, Ritz E, Haller H, Fliser D. Asymmetric dimethylarginine, blood pressure, and renal perfusion in elderly subjects. Circulation 2003; 107: 1891–5.
11) Rubanyi GM, Romero JC, Vanhoutte PM. Flow-induced release of endothelium-derived relaxing factor. Am J Physiol 1986; 250: H1145–9.
12) Joannides R, Haefeli WE, Linder L, Richard V, Bakkali EH, Thuillez C, et al. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. Circulation 1995; 91: 1314–9.
13) Neunteufl T, Katzenschlager R, Hassan A, Klaar U, Schwarzacher S, Glogar D, et al. Systemic endothelial dysfunction is related to the extent and severity of coronary artery disease. Atherosclerosis 1997; 129: 111–8.
14) Yim SF, Lau TK, Sahota DS, Chung TK, Chang AM, Haines CJ. Prospective randomized study of the effect of "add-back" hormone replacement on vascular function during treatment with gonadotropin-releasing hormone agonists. Circulation 1998; 98: 1631–5.
15) Lieberman EH, Gerhard MD, Uehata A, Walsh BW, Selwyn AP, Ganz P, et al. Estrogen improves endothelium-dependent, flow-mediated vasodilation in postmenopausal women. Ann Intern Med 1994; 121: 936–41.
16) Tanner FC, Noll G, Boulanger CM, Lüscher TF. Oxidized low density lipoproteins inhibit relaxations of porcine coronary arteries. Role of scavenger receptor and endothelium-derived nitric oxide. Circulation 1991; 83: 2012–20.
17) Chin JH, Azhar S, Hoffman BB. Inactivation of endothelial derived relaxing factor by oxidized lipoproteins. J Clin Invest 1992; 89: 10–8.
18) Wakatsuki A, Sagara Y. Lipoprotein metabolism in postmenopausal and oophorectomized women. Obstet Gynecol 1995; 85: 523–8.
19) Arca M, Vega GL, Grundy SM. Hypercholesterolemia in postmenopausal women. Metabolic defects and response to low-dose lovastatin. JAMA 1994; 271: 45345–9.
20) Vita JA, Treasure CB, Nabel EG, McLenachan JM, Fish RD, Yeung AC, et al. The coronary vasomotor response to acetylcholine relates to risk factors for coronary artery disease. Circulation 1990; 81: 491–7.
21) Dupuis J, Tardif JC, Cernacek P, Théroux P. Cholesterol reduction improves endothelial function after acute coronary syndromes. The RECIFE (Reduction of cholesterol in ischemic and function of the endothelium) Trial. Circulation 1999; 99: 3227–33.
22) Holden DP, Cartwright JE, Nussey SS, Whitley GS. Estrogen stimulates dimethylarginine dimethylaminohydrolase activity and the metabolism of asymmetric dimethylarginine. Circulation 2003; 108: 1575–80.
23) Newman W, Beall LD, Carson CW, Hunder GG, Graben N, Randhawa ZI, et al. Soluble E-electin is found in supernatants of activated endothelial cells and is elevated in the serum of patients with septic shock. J Immunol 1993; 150: 644–54.
24) Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM Jr, et al. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. Circulation 1997; 96: 4219–25.
25) Van Baal WM, Emeis JJ, Kenemans P, Kessel H, Peters-Muller ER, Schalkwijk CG, et al. Short-term hormone replacement therapy: reduced plasma levels of soluble adhesion molecules. Eur J Clin Invest 1999; 29: 913–21.
26) Holmlund A, Hulthe J, Millgård J, Sarabi M, Kahan T, Lind L. Soluble intercellular adhesion molecule-1 is related to endothelial vasodilatory function in healthy individuals. Atherosclerosis 2002; 165: 271–6.
27) Widlansky ME, Gokce N, Keaney JF Jr, Vita JA. The clinical implications of endothelial dysfunction. J Am Coll Cardiol 2003; 42: 1149–60.
28) Tan KC, Chow WS, Tam SC, Ai VH, Lam CH, Lam KS. Atorvastatin lowers C-reactive protein

and improves endothelium-dependent vasodilation in type 2 diabetes mellitus. J Clin Endocrinol Metab 2002; 87: 563–8.
29) Brevetti G, Silvestro A, Di Giacomo S, Bucur R, Di Donato A, Schiano V, et al. Endothelial dysfunction in peripheral arterial disease is related to increase in plasma markers of inflammation and severity of peripheral circulatory impairment but not to classic risk factors and atherosclerotic burden. J Vasc Surg 2003; 38: 374–9.
30) Vita JA, Keaney JF Jr, Larson MG, Keyes MJ, Massaro JM, Lipinska I, et al. Brachial artery vasodilator function and systemic inflammation in the Framingham Offspring Study. Circulation 2004; 110: 3604–9.