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Endothelial dysfunction but not increased carotid intima-media thickness in young European women with endometriosis

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BACKGROUND: Atherosclerosis is a chronic and degenerative disease developing typically in the elderly; nonetheless, a condition of accelerated atherosclerosis can be observed precociously in the presence of some diseases. Endometriosis, a chronic benign gynecological disorder, shows some characteristics, such as oxidative stress, systemic inflammation and a pro-atherogenic lipid profile, which could increase the risk of developing accelerated atherosclerosis. The aim of our study was to evaluate markers of subclinical atherosclerosis in young European women with endometriosis.

METHODS: This cross-sectional study included 37 women with endometriosis and 31 control subjects. The presence of subclinical atherosclerosis was investigated by ultrasound evaluation of common carotid intima-media thickness (ccIMT) and flow-mediated dilation (FMD); in addition, serum levels of lipids, inflammatory and coagulation parameters, as well as markers of endothelial inflammation and activation, were determined.

RESULTS: Women with endometriosis showed significantly lower values of FMD compared with controls [mean difference: −4.62, 95% confidence interval (CI): −6.52, −2.73; $P < 0.001$], whereas no significant differences in ccIMT values were found between the two groups. As regards markers of endothelial inflammation and activation, women with endometriosis had significantly higher values of inter-cellular adhesion molecule 1 ($P < 0.001$), vascular cell adhesion molecule 1 ($P < 0.001$), E-selectin ($P < 0.001$), von Willebrand factor ($P = 0.004$) and ristocetin cofactor ($P = 0.001$) compared with controls.

CONCLUSIONS: Our study suggests that women with endometriosis have more subclinical atherosclerosis, resulting in a higher risk of developing cardiovascular disorders. Moreover, our findings demonstrate that endothelial dysfunction can occur in the absence of structural atherosclerotic changes; its evaluation might be helpful in young women with endometriosis.

Key words: atherosclerosis / endometriosis / endothelial dysfunction

Introduction

Cardiovascular diseases (CDs) represent, nowadays, the leading cause of death in developed countries (Lloyd-Jones *et al.*, 2009). Atherosclerosis, the underlying pathogenic process of most CD, is a chronic and degenerative disease that starts slowly during infancy and becomes clinically manifested generally with old age (Lusis, 2000). Nevertheless, several lines of evidence suggest that a condition of 'accelerated atherosclerosis' can be observed precociously in the presence of some diseases, such as rheumatoid arthritis and systemic lupus erythematosus (Wallberg-Jonsson *et al.*, 1999; Goodson and Symmons, 2002; Asanuma *et al.*, 2003; Hahn, 2003; Roman *et al.*, 2003; Schattner, 2003; Zinger *et al.*, 2010). In these conditions, it has been hypothesized that chronic inflammation could play a crucial role, as early atherosclerosis cannot be explained exclusively by traditional cardiovascular risk factors (Del Rincón *et al.*, 2007; Libby, 2008).

Endometriosis is a chronic gynecological disorder that affects ~5–10% of women of reproductive age (Giudice, 2010). Some

characteristics, such as oxidative stress, a systemic inflammatory state and the presence of a pro-atherogenic lipid profile, have led to the hypothesis that patients with endometriosis could show an increased risk of developing accelerated atherosclerosis. Moreover, in recent years a body of molecular evidence has indicated that altered responses to progesterone, including vascular ones, can occur in women with endometriosis, describing a condition of 'progesterone resistance' (Al-Sabbagh *et al.*, in press). Nevertheless, only two studies assessing markers of subclinical atherosclerosis in women with endometriosis have been reported in the literature, and these reach conflicting conclusions (Pretta *et al.*, 2007; Kinugasa *et al.*, 2011).

The aim of our study was to determine the presence of subclinical atherosclerosis in young European women with endometriosis, by comparing endothelial function and common carotid intima-media thickness (ccIMT) of these patients with that of control subjects, and correlating these instrumental parameters with laboratory markers of endothelial inflammation and activation.

Materials and Methods

Patients

All patients referring to our Institute of Obstetrics and Gynecology between 1 July 2010 and 30 June 2011 underwent laparoscopy or laparotomy for a suspected benign gynecological disorder (i.e. ovarian cysts, infertility, endometriosis and uterine myomas) were approached for the study. Exclusion criteria were age <18 years, body mass index (BMI) >30 kg/m^2, use of vasoactive drugs, hormonal therapy within the previous 3 months or presence of diseases impairing endothelial function (i.e. diabetes, arterial hypertension, metabolic syndrome, hyperlipidemia, chronic inflammatory disorders, hepatic cyrrhosis, polycystic ovary syndrome, etc.). Arterial hypertension was defined as systolic blood pressure >140 mmHg and diastolic blood pressure >90 mmHg (Mancia *et al.*, 2007). Hyperlipidemia was defined as low-density lipoprotein cholesterol (LDL-C) >130 mg/dl, total cholesterol level >200 mg/dl and triglycerides >150 mg/dl, whereas low levels of high-density lipoprotein cholesterol (HDL-C) were defined as values <50 mg/dl [National Cholesterol Education Program (NCEP) Expert Panel, 2002].

Written informed consent was obtained from all the participants, before entry into the study. The study was performed in accordance with the Declaration of Helsinki and was approved by the ethics committee of the Catholic University of Rome (ethic committee reference number: A.1285/C.E./2010).

Physical and laboratory examinations

Thorough examinations including weight, height and blood pressure were performed for each patient and body mass index (BMI) was calculated as weight (kg) divided by height (m^2).

After overnight fasting, at 8:00 a.m. of the day planned for the surgical procedure, blood samples were taken from each patient, for assessment of serum glucose, creatinine, total cholesterol, LDL-C, HDL-C, triglycerides, transaminases, homocysteine, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), full blood count, estradiol, luteinizing hormone, follicle-stimulating hormone, progesterone, Ca-125 and coagulation parameters including von Willebrand factor (vWF) antigen levels and ristocetin cofactor. For CRP serum levels, the cut-off value of 3 mg/l was used, according to international recommendations (Pearson *et al.*, 2003). Estimation of renal function was performed using the CKD-EPI equation, which is considered the gold standard for glomerular filtration rate assessment (Levey *et al.*, 2009). Patient blood was centrifuged, and serum samples separated, aliquoted and kept frozen at -80°C. Serum levels of interleukin (IL)-6, IL-8, IL-10, tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), inter-cellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin were determined by enzyme-linked immunosorbent assays (ELISAs), according to the manufacturer's instructions (Thermo Scientific, Rockford, IL, USA for IL-6, IL-8, IL-10, TNF-α and VEGF, and R&D Systems, Minneapolis, MN, USA for ICAM-1, VCAM-1 and E-selectin). All assays were done in duplicates and laboratory staff were blinded to the clinical data.

All patients were also asked to complete a questionnaire where they recorded life habits (e.g. smoking and alcohol consumption), family history of CD and day of menstrual cycle. The latter was used together with sex hormones serum levels to establish the menstrual cycle phase.

Ultrasound imaging

A non-invasive ultrasound examination for ccIMT measurement and endothelial function assessment was performed on the morning of the surgical procedure, soon after the blood sampling, using a high-resolution Philips iU22 sonograph (Philips Medical Systems, Monza, Italy) and a linear 17-5 MHz transducer.

For the study of ccIMT, the B-mode ultrasound imaging technique was used. Briefly, patients were placed in the supine position, with the head rotated to one side. Longitudinal two-dimensional ultrasound images were focused on the posterior (far) wall of carotid arteries on each side. ccIMT was defined as the distance from the leading edge of the lumen–intima interface to the leading edge of the media–adventitia interface of the far wall. A minimum of three measurements of the IMT on the common carotid far wall were taken as 5, 10 and 15 mm proximal to bifurcation on each side to derive the mean ccIMT. Results were expressed in millimeters, with values ≥ 0.9 mm corresponding to increased ccIMT.

For the study of endothelial function, we evaluated the brachial artery reactivity, which has emerged as the most well-established technique used in adults. The technique assesses flow-mediated dilation (FMD) which measures the nitric oxide-mediated vasodilation produced by increased flow after a period of ischemia (endothelium-dependent vasodilation); non-endothelium-dependent dilation measures the arterial changes induced by external administration of a sublingual dose of nitroglycerin which reflects predominantly the smooth muscle response (NMD). This evaluation was conducted according to actual guidelines (Corretti *et al.*, 2002; Deanfield *et al.*, 2005). Briefly, after a 15- to 20-min supine rest period, the right brachial artery was scanned over a longitudinal section of 5–7 cm above the antecubital fossa and its diameter was measured from the tunica intima of both anterior and posterior walls; moreover, a pulse Doppler velocity signal was recorded. After the basal measurements, blood pressure cuff around the forearm distal to the target area was inflated to a pressure of 250 mmHg for 5 min and then abruptly deflated, after which a second scan was performed continuously for 90 s, to measure diameter changes after reactive hyperemia. A pulse Doppler velocity signal was also obtained no more than 15 s after deflation to measure the maximal hyperemic velocity. After a further period of 20 min of rest, a new scan was performed over the same area to measure the brachial artery diameter and pulse Doppler velocity signal at rest. Then, a sublingual dose of nitroglycerin (300 μg) was administered and the same measurements were again recorded during the following 90 s, to assess the NMD. Both FMD and NMD data were expressed as percentage increases relative to baseline diameters.

All ultrasound scans were performed in a quiet, temperature-controlled room at the same morning hour (8.00 a.m.), also to avoid the reported circadian variation of endothelial function of premenopausal women (Walters *et al.*, 2006), by a single skilled examiner blinded to the subject's details. All patients had refrained from exercise and from ingesting foods

and all vasoactive substances (i.e. drugs, tobacco, coffee) for at least 12 h before the examination.

Surgical procedures

After blood sampling and ultrasound imaging evaluations, subjects underwent surgical procedure and were classified as patients with and without endometriosis, and surgical diagnosis was confirmed by histological examination. Patients affected by endometriosis were then staged according to the American Society for Reproductive Medicine 1996 (1997) criteria.

Statistical analysis

The distribution of continuous variables was evaluated with a combination of visual inspection of histograms, normality plots, skewness, kurtosis and the Shapiro–Wilk test for normality. NMD, BMI, platelet count, ESR, serum triglycerides, vWF, IL-10 and TNF-α had a right-skewed distribution and were log-transformed. VEGF, IL-6, IL-8, homocysteine and ccIMT, were not normally distributed and the distribution did not improve after log-transformation; they were reported as median with interquartile range. All the other parameters were normally distributed.

The null hypothesis of no difference of mean values of continuous variables between subjects with and without endometriosis was tested with the *t*-test; equality of variances was evaluated with *F*-test and the Satterthwaite version was chosen for FMD, log-transformed ESR, fibrinogen, log-transformed Ca-125 and ristocetin cofactor.

Differences in mean ranks for ccIMT, VEGF and IL-8 between subjects with and without endometriosis were analyzed with the Wilcoxon rank-sum test.

Bivariate correlations between continuous variables were analyzed with the Pearson or Spearman correlation, as appropriate.

The distribution of discrete variables among subjects with and without endometriosis was reported as crude number and proportions; the null hypothesis of no difference in the distribution of discrete variables among subjects with and without endometriosis was tested with the Fisher exact test.

The main hypothesis was that FMD was significantly different between subjects with and without endometriosis. The measure of effect reported for the difference in FMD was the mean difference with a 95% confidence interval (CI). The adjusted difference was then obtained with a linear multivariable model with FMD as the dependent variable, presence of endometriosis as the independent variable and age, BMI, smoking, familial history of CD, serum LDL-C, HDL-C, triglycerides and creatinine as covariates.

The linear relationship between FMD and severity of endometriosis was analyzed with a trend ANOVA with severity of endometriosis treated as a continuous variable.

The association between the phase of menstrual cycle and FMD was analyzed with an ANOVA model.

All statistical tests were performed with SAS version 9.1 (Cary, NC, USA). The accepted level of statistical significance was $P < 0.05$.

Results

During the whole study period, 84 females were eligible for the study after considering the exclusion criteria, 16 subjects refused to participate and 68 subjects were finally enrolled. Among these, 37 (54.4%) patients had a definitive diagnosis of endometriosis, while the other 31 (45.6%) subjects were enrolled as controls. Among patients with endometriosis, the severity class was 1 for 8 subjects (21.6%), 2 for 12 subjects (32.4%), 3 for 11 subjects (29.7%) and 4 for 6 subjects (16.2%), according to the American Society for Reproductive Medicine 1996 (1997) criteria.

Demographic and clinical data of study population are reported in Table I.

Laboratory parameters were similar in the two groups as shown in Table II, except for serum values of creatinine, HDL-C and Ca-125, which were significantly higher in patients with endometriosis.

As regards instrumental markers of subclinical atherosclerosis, women with endometriosis had significantly lower values of FMD compared with controls (mean difference: −4.62, 95% CI: −6.52, −2.73; $P < 0.001$), whereas values of NMD were not significantly different ($P = 0.200$) (Fig. 1). This difference was significant even after multivariable adjustment for age, BMI, smoking habit, familial history of CD, serum LDL-C, HDL-C, triglycerides and creatinine (adjusted mean difference: −4.04, 95% CI: −6.44, −1.64; $P = 0.002$). In the evaluation of ccIMT, we found that one patient with endometriosis showed bilateral increased ccIMT and one control subject showed monolateral increased ccIMT. However there were no significant differences between the two groups when considering the absolute values (Fig. 1).

No linear association between FMD and severity of endometriosis was observed ($P = 0.138$).

Table I Demographic and clinical data of the study population.

	Patients	Controls	*P*-value
Age (years)	32.9 ± 5.8	35.3 ± 5.4	0.086
BMI (kg/m^2)	21.8 (20.7, 22.7)	22.0 (21.1, 22.9)	0.770
Smoking habit (*n*, %)	13 (35.1)	10 (32.3)	>0.999
Alcohol consumption (*n*, %)	9 (24.3)	6 (19.4)	0.771
History of CD (*n*, %)	12 (32.4)	9 (29.0)	0.798
Phase of menstrual cycle (*n*, %)			0.364
Follicular	16 (44.4)	14 (48.3)	
Ovulatory	7 (19.4)	2 (6.9)	
Luteal	13 (36.1)	13 (44.8)	

BMI, body mass index; CD, cardiovascular diseases.

Table II Laboratory parameters of the study population.

	Patients	Controls	*P*-value
Platelet count ($\times 10^3/mm^3$)	257 (233, 281)	255 (230, 276)	0.787
ESR (mm/h)	6.8 (5.5, 8.2)	6.2 (4.6, 8.4)	0.636
Fibrinogen (mg/dl)	255 ± 43	268 ± 59	0.340
Glucose (mg/dl)	88.6 ± 8.3	88.5 ± 6.6	0.940
Total cholesterol (mg/dl)	171 ± 31	168 ± 25	0.676
LDL-C (mg/dl)	96.1 ± 26.0	97.2 ± 22.6	0.872
HDL-C (mg/dl)	58.4 ± 12.2	52.1 ± 10.3	0.045
Triglycerides (mg/dl)	75.9 (66.7, 86.5)	74.4 (66.7, 83.9)	0.847
Creatinine (mg/dl)	0.87 ± 0.11	0.78 ± 0.14	0.006
CRP >3 mg/l (*n*, %)	4 (15.4)	4 (12.1)	0.722
Ca-125 (UI/ml)	27.3 (44.7)	12.9 (11.8)	<0.001
Homocysteine (μmol/l)	9.5 (4.9)	8.9 (3.7)	0.366

ESR, erythrocyte sedimentation rate; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; CRP, C-reactive protein.

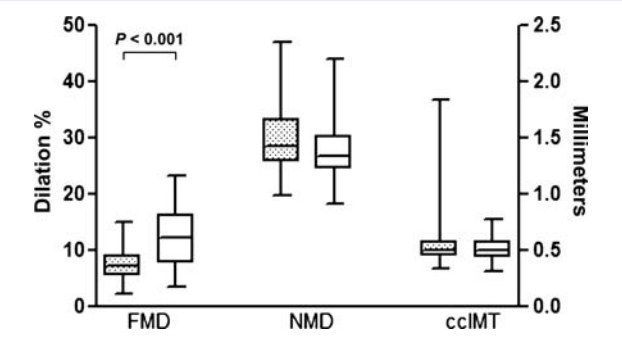

Figure 1 Differences in FMD, NMD and ccIMT between subjects with and without endometriosis. Grayed out boxes, subjects with endometriosis; white boxes, controls. ccIMT, common carotid intima-media thickness; FMD, flow-mediated dilation; NMD, nitroglycerin-mediated dilation.

There was no also association between FMD and the phase of menstrual cycle ($P = 0.334$).

As regards markers of endothelial inflammation and activation, women with endometriosis had significantly higher values of ICAM-1, VCAM-1, E-selectin, ristocetin cofactor and vWF with respect to controls, whereas serum levels of IL-6, IL-8, IL-10, TNF-α and VEGF were similar between the two groups (Table III). In the study sample, the analysis of correlation among FMD, NMD and ccIMT with markers of endothelial inflammation and activation showed an inverse linear relationship between values of FMD and serum levels of vWF ($r = -0.32$, $P = 0.007$), ristocetin cofactor ($r = -0.29$, $P = 0.01$), VCAM-1 ($r = -0.62$, $P < 0.001$), ICAM-1 ($r = -0.85$, $P < 0.001$) and E-selectin ($r = -0.34$, $P = 0.005$); also a linear correlation was found between ccIMT and ristocetin cofactor ($r = 0.28$, $P = 0.022$). All the other correlations were not statistically significant.

Discussion

Our study demonstrated that young women with endometriosis show significantly impaired FMD compared with control subjects, whereas ccIMT did not differ between the two groups. These findings suggest that young women with endometriosis, although not yet presenting structural vascular changes, have a functional endothelial damage.

Endothelial dysfunction is due to reduced nitric oxide production by endothelial cells, and is considered an early event of atherosclerosis that precedes structural atherosclerotic changes in the vascular wall (Ross, 1999). Several studies have demonstrated that impaired FMD is predictive of cardiovascular events by showing a positive correlation with traditional cardiovascular risk factors, such as hypertension, dyslipidemia, diabetes mellitus, smoking habit, sedentary behaviour and inflammation (Salonen and Salonen, 1991; O'Leary *et al.*, 1999; Simons *et al.*, 1999; Li *et al.*, 2000; Lee *et al.*, 2001; Felmeden *et al.*, 2003; Quyyumi, 2003). Furthermore, it has been demonstrated that impaired FMD predicts cardiovascular morbidity and mortality independently of traditional cardiovascular risk factors and the Framingham risk score (Chan *et al.*, 2003; Grewal *et al.*, 2003). In the light of these considerations, our results suggest that young women with endometriosis have more subclinical atherosclerosis and are at higher risk for future cardiovascular disorders compared with the general population.

In the first study reported in the literature evaluating the possible association of atherosclerosis with endometriosis, the authors assessed only ccIMT values, finding that women with endometriosis did not have more subclinical atherosclerosis compared with control subjects (Pretta *et al.*, 2007). They suggested to confirm their preliminary observations with further studies including patients aged >50 years, so hypothesizing that in older subjects traditional cardiovascular risk factors could play a crucial role. On the other hand, very recently, Kinugasa *et al.* (2011) have reported, for Japanese women with endometriosis, an inhibition of endothelial function associated with inflammation, without exploring other instrumental parameters of subclinical atherosclerosis.

Table III Serum markers of endothelial inflammation and activation of the study population.

	Patients	Controls	*P*-value
VCAM-1 (ng/ml)	446 ± 52	389 ± 47	<0.001
ICAM-1 (ng/ml)	236 ± 32	168 ± 27	<0.001
E-selectine (ng/ml)	50.7 ± 15.1	40.2 ± 17.0	<0.001
VEGF(pg/ml)	112 (124)	112 (100)	0.620
IL-6 (pg/ml)	59.7 (88.9)	15.4 (138.6)	0.637
IL-8 (pg/ml)	6.1 (2.5)	6.9 (3.2)	0.334
IL-10 (pg/ml)	27.1 (15.7, 46.5)	25.2 (16.1, 39.4)	0.833
TNF-α (pg/ml)	2.07 (3.12, 4.71)	3.22 (2.09, 4.95)	0.916
vWF (%)	115.6 (106.7, 125.2)	97.5 (90.9, 105.6)	0.004
Ristocetinic cofactor (%)	107.5 ± 26.2	89.5 ± 16.2	0.001

VCAM-1, vascular cell adhesion molecule 1; ICAM-1, inter-cellular adhesion molecule 1; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor-α; vWF, von Willebrand factor antigen levels.

In our study, we have investigated for the first time both structural (ccIMT) and functional (FMD) parameters of subclinical atherosclerosis, thus overcoming previous limitations. We found that in young women with endometriosis, even if a structural alteration is confirmed to be absent, endothelial dysfunction has already occurred. These results are consistent with the fact, that in a young population, an increase in ccIMT reflects a structural vascular damage that takes a longer time to realize.

The results of our study are independent of the presence of identified potential confounding factors, as the presence of diseases impairing endothelial function (such as diabetes, hypertension and obesity) was excluded, and the other factors known to play a role in atherosclerosis development (such as age, BMI, lipid profile and smoking habits) were similar between the two study groups. Furthermore, to reduce confounding, a multivariable analysis was performed, and confirmed the unadjusted findings. Hence, it can be postulated that endometriosis itself can influence the development of subclinical atherosclerosis through other mechanisms; in particular, a chronic inflammatory state could play a crucial role, considering that inflammation has become well established over the past decade as a key pathogenetic mechanism in atherosclerosis (Ross, 1999). Moreover, endothelial dysfunction itself, although often reported only as a loss of the vasodilator capacity, describes a condition in which all the endothelial properties in controlling vascular tone, coagulation and inflammatory responses, are compromised. Notably, in women with endometriosis, an inflammatory state has been reported not only in the peritoneal cavity, but also at a systemic level (Darai *et al.*, 2003; Kondera-Anasz *et al.*, 2005). In this regard, we found that serum levels of VCAM-1, ICAM-1, E-selectin, ristocetin cofactor and vWF in women with endometriosis were statistically higher compared with that in control subjects, reflecting inflammation and activation of endothelial cells as an early step of the atherosclerotic process of these patients. It is well known, in fact, that peripheral levels of these molecules are a surrogate of their expression on the vascular wall, and so an increase in their serum values can be considered a marker of endothelial damage and dysfunction (Pannekoek and Voorberg, 1989; Gearing *et al.*, 1992; Blann, 1993; Newman *et al.*, 1993; Pearson, 1993). Surprisingly, other biochemical markers and cytokines reflecting inflammation were not statistically different in women with endometriosis compared with control subjects; to explain these findings we have hypothesized that some inflammatory markers may be not associated with a condition of chronic low-grade inflammation such as that observed in endometriosis. In the light of this consideration, a possible criticism to these results can be made about our assessment of CRP levels that was not performed with a high-sensitivity assay, using only the recommended cut-off of 3 mg/l (Pearson, 2003).

The entity of FMD impairment directly correlates with endothelial inflammation and activation status, as emerged by univariate analysis among FMD and VCAM-1, ICAM-1, E-selectin, vWF and ristocetin cofactor; other correlations involving traditional markers of inflammation (such as CRP, IL-6 and TNF-α) were not statistically significant. Similar findings have been already reported in other studies assessing the relationship between FMD and inflammatory markers (Van Haelst *et al.*, 2003; Makino *et al.*, 2008).

In our study we have also evaluated the possible influence of menstrual cycle on interpretation of data on endothelial function. It is well known that female sex hormones have several actions on the cardiovascular system, including effects on plasma lipid levels and on vessel wall physiology; interestingly, it has been demonstrated that FMD increases from the menstrual to the late follicular phase, decreases in early luteal phase and increases again in the late luteal phase (Williams *et al.*, 2001). In this regard, we found no association between FMD and the phase of menstrual cycle; moreover, no significant differences in menstrual phases between the two study groups were observed.

In our patients with endometriosis, NMD was preserved; this finding indicates that endometriosis may have no effect on the vascular smooth muscle function.

In conclusion, our study suggests that women with endometriosis have more subclinical atherosclerosis compared with the general population, and hence are at higher risk for future cardiovascular disorders. Moreover, our findings show that endothelial dysfunction can occur in the absence of structural atherosclerotic changes in the vascular wall. Its evaluation might be helpful in discriminating individuals at risk of atherosclerosis, especially in studies involving young people, considering the potential reversibility of this condition.

Authors' roles

L.S. planned and executed the study, and wrote the manuscript. F.D'.O. contributed in the execution of the study; S.C. and V.C. contributed to the study design; P.M.F. contributed to the statistical analysis and manuscript drafting; P.T. and A.F. contributed in drafting the manuscript; and A.G. and A.S. contributed to critical discussion.

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Conflict of interest

None declared.

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