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Endothelial dysfunction and cardiovascular outcomes in pre-menopausal women with high vs low breast fat accumulation: The role of TSP-1

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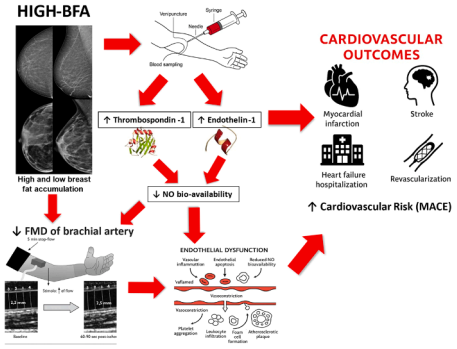
GRAPHICAL ABSTRACT



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ABSTRACT

Background: In premenopausal women, adipose tissue accumulation of the breast gland is an independent risk factor for cardiovascular diseases (CVDs) and major-adverse-cardiac-events (MACE).
Aims: We aimed to evaluate endothelial dysfunction (ED) via brachial artery flow-mediated dilation (FMD), and to assess serum endothelin-1 (ET-1), thrombospondin-1 (TSP-1), and MACE at 5-year follow-up among women with different degrees of breast fat accumulation (BFA).

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Methods: Women aged 40–55 years undergoing mammography were consecutively enrolled and classified into high-BFA and low-BFA groups; those with baseline ED were excluded. ED, inflammatory markers, ET-1, TSP-1, and MACE were reassessed at 5-year follow-up.
Results: At follow-up, ED occurred in 44 % of high-BFA vs. 28.3 % of low-BFA women, and MACE in 11.2 % vs. 2.5 % ($p < 0.05$). ED was independently predicted by pre-diabetes (HR 1.14, 95 % CI 1.03–1.68) and TSP-1 (HR 1.08, 95 % CI 1.01–1.12). Predictors of MACE included impaired FMD (HR 1.19, 95 % CI 1.06–1.46), reduced LVEF (HR 1.03, 95 % CI 1.01–1.06), elevated WBC (HR 1.61, 95 % CI 1.40–1.83), ET-1 (HR 1.46, 95 % CI 1.21–1.77), TSP-1 (HR 1.17, 95 % CI 1.01–1.32), and high BFA (HR 1.968, 95 % CI 1.96–2.01).
Conclusions: Women with High-BFA vs Low-BFA showed higher rates of ED and MACE, along with increased inflammatory/oxidative markers and elevated ET-1 and TSP-1 levels. TSP-1 independently predicted both ED (HR 1.081) and MACE (HR 1.170). ET-1 levels predicted greater risk of MACE (HR 1.459). High BFA (HR 1.65) and impaired FMD (HR 1.189) independently predicted MACE at 5 years.

1. Introduction

Adipose tissue accumulation of the breast gland is an independent risk factor for cardiovascular diseases (CVDs) in pre-menopausal women [1]. Indeed, pre-menopausal women with high (High-BFA) vs low-breast fat accumulation (Low-BFA) have worse clinical outcomes and higher rates of major adverse cardiac events (MACE) independently of CVDs risk factors [2]. These adverse outcomes may be explained by non-atherogenic mechanisms [3]. In this context, endothelial dysfunction (ED) is a key contributor to CVDs and MACE in women, even in the absence of atherosclerosis [4]. ED is characterized by impaired flow-mediated vasodilation (FMD), due to reduced endothelial nitric oxide synthase (eNOS) activity and decreased nitric oxide (NO) availability—molecules essential for vasodilation and vascular protection [5]. Excess adipose tissue exacerbates ED, promoting a pro-inflammatory and pro-thrombotic endothelial phenotype with leukocyte adhesion, platelet activation, and oxidative imbalance [5,6]. Among the molecular mediators, endothelin-1 (ET-1) plays a central role by inducing vasoconstriction and amplifying inflammatory signaling [6]. Notably, the ET-1 interacts with thrombospondin-1 (TSP-1), a glycoprotein released by endothelial cells in response to shear stress and hypoxia [7]. TSP-1 modulates vascular tone through pro-inflammatory and pro-thrombotic pathways, partly by reducing NO bioavailability [7]. Intriguingly, ET-1 and TSP-1 share similar expression profiles, and both impair endothelial-dependent vasodilation, suggesting a coordinated role in vascular regulation [8]. However, several prior studies have implicated ET-1 and TSP-1 in the development of ED and MACE [4-8]. Here, our study aims to extend prior knowledge by evaluating ET-1 and TSP-1 in a novel context, focusing on premenopausal women and linking breast fat accumulation to ED and MACE. We may hypothesize that worse cardiovascular outcomes in High-BFA vs Low-BFA women might be caused by ED, via over-inflammation/oxidative stress and enhanced expression of ET-1 and TSP-1. On the other hand, there is no conclusive data on the implications of the ET-1/TSP-1 pathways on ED and MACE in these patient cohorts. Moreover, to address this study's hypothesis, we compared well-matched pre-menopausal High-BFA vs Low-BFA women without a clinical diagnosis of ED at baseline and without atherosclerotic disease. In these patients, we evaluated the serum expression of molecular/cellular inflammatory markers, ET-1, and TSP-1 at baseline and at 5 years of follow-up, and the rate of ED and MACE at 5 years of follow-up. The ED was diagnosed by Doppler indexes and FMD values of the brachial artery, as currently indicated [6].

2. Methods

In a prospective multicenter study, we evaluated a cohort of 16,352 pre-menopausal women aged 40 to 55 years, who underwent screening digital mammography in accordance with Italian guidelines for breast disease prevention [1]. Patients were derived from the BRECARD clinical registry, a prospective multicenter study of pre-menopausal women >40 years without baseline cardiovascular disease or breast cancer, which demonstrated an association between lower breast density (higher adiposity) and increased long-term risk of MACE [1]. If women received >1 screening mammogram during the study period, we randomly selected 1 mammographic exam, as previously reported [1]. The study population met the following inclusion/exclusion criteria.

Inclusion criteria: indication to receive screening mammography in women aged >40 and <55 years; women without a previous history of cardiovascular or cerebrovascular adverse events (without previous MACE); women without any evidence of CVDs (echocardiography, electrocardiography, and electrocardiography stress test).

Exclusion criteria: diagnosis of menopause; mammographic evidence of breast artery calcifications; previous or current diagnosis of breast cancer, inflammatory chronic disease, or other neoplastic diseases; women with previous MACE, and diagnosis of ED; women who did not provide informed consent to participate in the study.

Therefore, from the evaluated cohort of 16,352 pre-menopausal women we excluded 3204 (19.6 %) women with mammographic evidence of breast cancer diagnosis, 1108 (6.8 %) women with mammographic evidence of breast artery calcifications, 588 (3.6 %) women with rheumatoid arthritis, 24 (0.15 %) women with rheumatic/chronic inflammatory diseases. Fig. 1. Finally, we excluded the 364 (3 %) women with a diagnosis of ED at baseline. Fig. 1. Furthermore, we had an included study population of 11,064 patients, then divided into 3208 (29 %) pre-menopausal High-BFA women vs 7856 (71 %) Low-BFA women, according to diagnostic mammographic criteria as previously reported [3]. Fig. 1. Mammography was repeated each year of follow-up in the study cohorts, and, for multiple exams, the authors randomly selected 1 exam. The women with the lowest breast density (A grade of BIRADS classification) were classified as High-BFA women [3]. Other breast density classes (BIRADS grades B, C, and D) were classified as Low-BFA [3]. BFA was assessed using standardized imaging protocols and mammographic projections. The description of the digital mammography technique and the diagnosis of breast fat composition are fully reported in the **supplementary files**. The ED was diagnosed by derived-echo color Doppler values of FMD of the brachial artery [9,10]. Fig. 1. All examinations were performed by trained physicians under standardized conditions, with measurements synchronized to the cardiac cycle and conducted in the morning in fasting and resting state. To ensure reproducibility of FMD measurements, intra- and inter-observer validation was performed. Two experienced sonographers independently repeated FMD assessments under standardized fasting and resting conditions. A detailed description of image acquisition and technical procedures is provided in the **Supplementary Material**.

From the initial cohort of 11,064 patients, we then divided them into 3208 (29 %) pre-menopausal High-BFA women vs 7856 (71 %) Low-BFA women. After propensity score matching (PSM), we had a study cohort of 1410 pre-menopausal High-BFA women vs 1366 Low-BFA women. PSM analysis was built to assess the probability of breast density, as assessed by mammography. As reported for the Brecard study [1], we used the PSM analysis to equate the two groups with respect to measured baseline covariates and to achieve a comparison between the study

cohorts with reduced selection bias. The PSM was used to mimic randomization and overcome the selection bias that plagues non-experimental methods. See the supplementary files for the full description of PSM.

Among this matched cohort, a subgroup of 120 well-matched High-BFA and 120 Low-BFA women was randomly selected for serum biomarker analysis of ET-1 and TSP-1, based on biobank availability and predefined feasibility criteria.

The study was approved by the Institutional Review Board, and all procedures were conducted in accordance with the Declaration of Helsinki. The Ethical Committee of the University of Campania "Luigi Vanvitelli" approved the study protocol with the number 332. All patients were informed about the nature of the study and gave signed informed consent to participate, and all personal data were anonymized. Data collection and handling complied with all relevant data protection regulations, including the General Data Protection Regulation (GDPR). Enrollment began on January 10, 2010, and ended on January 30, 2017, with a 5-year duration (the follow-up of the last enrolled patient ended on February 1, 2022; the mean follow-up duration was 65 ± 3 months). Follow-up was completed on February 2, 2022, to evaluate the primary (ED rate) and secondary study endpoints (MACE, inflammatory burden, ET-1, and TSP-1 values).

2.1. Study endpoints

We evaluated, as the primary study endpoint, the number of women with ED at 5 years of follow-up. As secondary study endpoints, we evaluated the rate of MACE, the serum inflammatory molecular/cellular burden, and the serum expression of ET-1 and TSP-1 as markers of ED at follow-up [9]. To avoid the effects of adipose tissue activity on the ED, we evaluated leptin serum levels in the study cohorts at baseline and at the follow-up end. The ED was diagnosed using the FMD cut-off values [9]. The FMD values and measurements were calculated from echo-color Doppler of the brachial artery, a non-invasive technique [9]. The FMD of the brachial artery is a measure of endothelium-dependent function [9]. According to the FMD values of the brachial artery, with an established cut-off at 7.1 %, we found that women with ED vs. those without ED at follow-up [9]. The MACE was a composite study endpoint, indicating any CVDs events, hospital admissions for heart failure, and ischemic cardiovascular events [1]. The CVDs diagnosis included ischemic heart disease, peripheral arterial disease, stroke/transitory ischemic attack, or revascularization procedures [1]. The CVDs were diagnosed according to the International Classification Codes of Diseases-10 [1]. The ED and MACE were diagnosed based on data collected during clinical visits, patient interviews, and visual inspection of hospital discharge

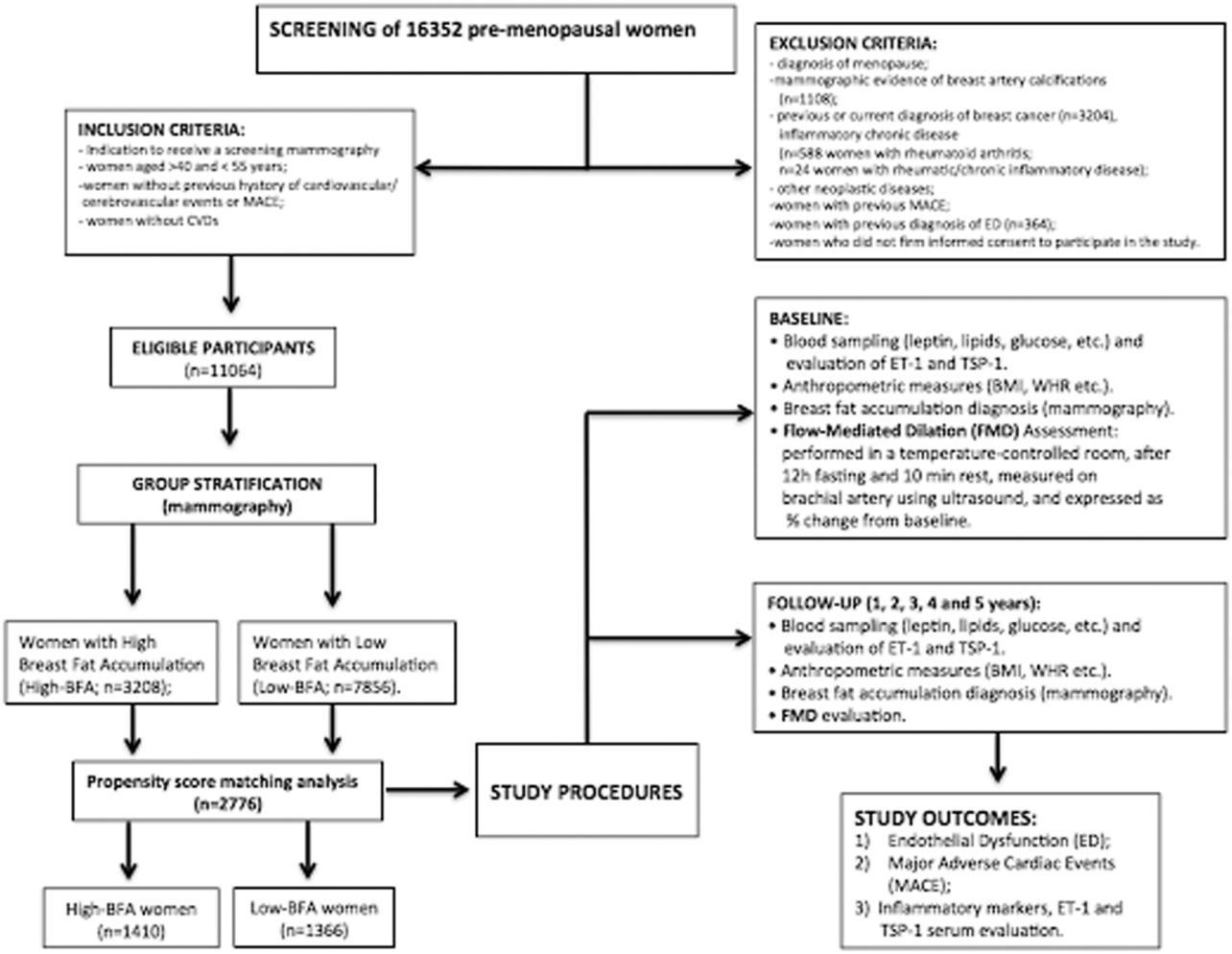


Fig. 1. In this figure, the study flow chart with the screening population, the inclusion and exclusion criteria, propensity score matching and the study cohorts of the women with High (High-BFA) vs Low-Breast fat accumulation (Low-BFA). We reported the methodology used for the diagnosis of High vs Low-Breast fat accumulation (mammography), and the flow-mediated vasodilation (FMD) of brachial artery (and the methodology used to assess the FMD). The FMD investigated at baseline and follow-up the rate of patients with endothelial dysfunction (ED). We have reported the exams used at baseline and follow-up. Finally, we reported the study outcomes: the ED, the Major adverse cardiac events and the serum inflammatory markers, the endothelin-1 (ET-1) and thrombospondin-1 (TSP-1). ET-1 and TSP-1 are markers of ED. BMI: body mass index; WHR: waist to hip ratio.

schedules.

2.2. Anthropometrics parameters, clinical data and analysis of blood samples

Clinical evaluation included anthropometric parameters, such as body mass index (BMI) and the waist-to-hip ratio (WHR), an index of central obesity [5]. We evaluated the metabolic profile by insulin, glycemia, and homeostasis model assessment of insulin resistance (HOMA-IR) [10,11], and lipid levels (total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL), and non-HDL cholesterol) [12]. Serum samples were collected after overnight fasting, stored at −80 °C, and analyzed for inflammatory/oxidative stress markers. We assayed serum cytokine levels —tumor necrosis factor alpha (TNFα), interleukin 1 and 6 (IL1, IL6), and Nitrotyrosine—in duplicate using a highly sensitive quantitative sandwich enzyme-linked immunosorbent assay (ELISA, Quantikine HS; R&D Systems, Minneapolis, MN). The current investigation was carried out on a sample of 120 patients per study group because the serum was stored in the biobank. The serum was donated by the patient, who signed a consent form for future studies. Full methodological details are reported in the **Supplementary Material.** Notably, the **hormonal status and menstrual phase** could modulate endothelial function and circulating biomarkers. Because menstrual cycle timing was not standardized for all premenopausal women, some degree of intra-group variability may persist. To explore potential hormonal influences, FMD, ET-1, and TSP-1 values were compared between these phases using independent-samples *t*-tests.

2.3. Patients monitoring and follow-up duration

Enrolled women were regularly followed by the treating physician with clinical visits at hospital discharge and for the 5 years of follow-up. Mammography was performed at baseline and follow-up as indicated [1,5]. The clinical evaluations included physical examinations, vital signs, and a review of adverse events. We performed fasting blood tests (at least 12 h after the last meal) to evaluate glycemia and lipid profile, including total cholesterol, triglycerides, HDL, and LDL at every visit. We collected the study endpoints and other clinical events during patients' interviews, visits, and hospital discharge schedules.

2.4. Statistical analysis

We prospectively collected data from electronic medical records (EMR) in the clinical setting at participants' Institutions, using electronic systems for data capture, collection, and monitoring, with onsite and real-time data entry. An experienced physician in statistical analysis (G.S) then analyzed the data. We reported quantitative variables as means ± standard deviations and categorical variables as numbers and percentages. Statistical analysis was performed by using Student's T-test (comparing the 2 groups, as High-BFA vs Low-BFA women) for the continuous variables, and Cochran's Q test for categorical variables. For the PSM analysis, we used a 1:1 nearest-neighbor PSM without replacement to minimize confounding and ensure comparability between groups. Thus, the matching algorithm incorporated a comprehensive set of baseline covariates selected for their known or plausible associations with both the exposure and outcomes of interest. See **supplementary files** for the full description.

Again, to verify the representativeness of the biomarker sub-cohort ($n = 240$; 120 High-BFA and 120 Low-BFA) compared with the entire propensity score–matched population ($n = 2776$), we performed a comparative analysis of baseline demographic, clinical, and metabolic variables. See **supplementary files** for the full description. Finally, to evaluate the effects of **hormonal status and menstrual phase** on endothelial function and circulating biomarkers, and because menstrual cycle timing was not standardized for all premenopausal women, FMD, ET-1, and TSP-1 values were compared between these phases using independent-samples *t*-tests. A Cox regression model analysis for primary (ED) and secondary (MACE) study endpoints at 5 years of follow-up, and corrected for age, BMI, smoking, dyslipidemia, hypertension, pre-diabetes, FMD, non-dense area percentage, glycemia, cholesterol, WBC, platelets, fibrinogen, high-sensitivity C-reactive protein (hs-CRP), ET1, TSP-1, IL1, LVEF, statin, and anti-hypertensive therapy was also performed. For interpretability, continuous biomarkers were also expressed per 1 SD and by quartiles (Q4 vs Q1); quartile contrasts were approximated from per-SD effects assuming normality of the biomarker distribution (details in Supplementary Methods). For continuous biomarkers (ET-1, TSP-1, etc.), effect sizes were estimated per 1 unit and additionally per 1 standard deviation (SD); we also present quartile contrasts (Q4 vs Q1) to enhance clinical interpretability. In detail, we calculated a univariate analysis to examine the association between a single risk factor and the 12-month study outcome. Then, we build a multivariate model using all variables with p-values < 0.1 from the univariate analysis. In the multivariate model, a p-value of <0.05 was considered statistically significant. 95 % confidence intervals (CI) were calculated for all independent predictors. Statistical significance was established at $p < 0.05$ for all other analyses.

Although multiple biomarker and outcome comparisons were performed, these analyses were predefined and hypothesis driven. Therefore, no formal correction for multiple testing was applied. The results should be interpreted as exploratory and may be subject to an increased risk of Type I error. Nonetheless, key findings remained consistent in multivariable Cox regression models adjusted for major confounders, reducing the likelihood that the observed associations are due to chance. We calculated the sample size based on the number of patients who reached the primary study endpoint, defined as the ED at follow-up. The sample-size/power calculation was specified for the post-PSM comparison. The sample size was calculated based on an expected effect size of 10 %, a significance level (α) of 0.05, and a statistical power of 80 %. Estimates for effect size and variance were derived from previous literature and preliminary data. To account for potential dropouts or non-responses, the calculated sample size increased by 15 %. Using these parameters, we determined that at least 386 participants per group were needed to detect a statistically significant difference between groups. See supplementary file for full description. Statistical analysis was performed using the SPSS software package for Windows 22.0 (SPSS Inc., Chicago, Illinois).

3. Results

3.1. Clinical characteristics of study cohorts at baseline

We reported the clinical characteristics of study cohorts as the overall population (n 2766 patients) and pre-menopausal High-BFA (n 1410) vs Low-BFA (n 1366) women. The High-BFA vs Low-BFA women had higher hs-CRP levels in serum ($p < 0.05$). Table 1. We did not find a significant difference regarding the general characteristics, risk factors, biochemical measurements, non-HDL cholesterol serum values, (other) serum inflammatory markers, echocolor Doppler parameters, and medications at baseline ($p > 0.05$). The number of parities was 1.8 ± 1 vs. 1.8 ± 1 in the High-BFA vs Low-BFA women ($p > 0.05$). Regarding the mammographic measures, comparing High-BFA vs Low-BFA women, we observed significant differences in percentage density, dense area, and non-dense area ($p < 0.05$). Table 1.

3.2. Serum expression of inflammatory cytokines, ET-1 and TSP-1 at baseline

At baseline, we did not find significant differences about serum expression of inflammatory cytokines, ET1, and TSP1 values comparing High-BFA vs Low-BFA women ($p > 0.05$). Fig. 1.

Table 1
Clinical characteristics of study population at baseline and at 1 year of follow-up.

Clinical variables	BASELINE			1 YEAR OF FOLLOW-UP		
	High-BFA women (n 1410)	Low-BFA women (n 1366)	P value	High-BFA women (n 1410)	Low-BFA women (n 1366)	P value
General characteristics						
Age (years)	45.1 ± 3.3	45.3 ± 3.2	0.105	46.1 ± 3.3	46.3 ± 3.4	0.116
BMI (kg/h2)	26.63 ± 2.40	26.39 ± 2.16	0.36	26.90 ± 2.43	26.71 ± 2.12	0.028
Waist/hip	1.023 ± 0.004	1.029 ± 0.003	0.50	1.026 ± 0.007	1.030 ± 0.006	0.086
Systolic blood pressure (mmHg)	126.5 ± 10.9	127.6 ± 9.8	0.49	125.8 ± 11.2	126.8 ± 10.0	0.130
Diastolic blood pressure (mmHg)	78.9 ± 6.6	79.4 ± 6.9	0.051	73.7 ± 6.7	74.2 ± 7.0	0.155
Heart rate	72.6 ± 9.9	72.8 ± 6.9	0.536	72.1 ± 5.6	71.8 ± 6.1	0.178
Risk factors						
Family history of ischemic heart disease, n (%)	118 (8.4)	104 (7.6)	0.507	/	/	/
Hypertension, n (%)	116 (8.2)	104 (7.6)	0.598	140 (9.9)	124 (9.1)	0.484
Dyslipidemia, n (%)	294 (20.8)	284 (20.8)	1.000	316 (22.4)	300 (22.0)	0.811
Prediabetes (%)	204 (14.5)	190 (13.9)	0.713	230 (16.3)	212 (15.5)	0.604
Type 2 diabetes, n (%)	24 (1.7)	16 (1.2)	0.311	27 (1.9)	18 (1.3)	0.273
Current smoking, n (%)	136 (9.6)	152 (11.1)	0.223	130 (9.2)	134 (9.8)	0.642
Mammography measures						
Density, %	20.6 ± 0.6	63.1 ± 0.9*	0.001	19.7 ± 0.5	58.8 ± 0.7*	0.001
Dense Area, cm2	27.3 ± 0.3	102.0 ± 18.2*	0.001	26.9 ± 0.3	100.8 ± 18.6*	0.001
Breast area, cm2	156.2 ± 9.0	155.6 ± 9.5	0.088	157.8 ± 9.4	158.3 ± 9.1	0.155
Non dense Area, cm2	125.5 ± 5.1	61.8 ± 4.4*	0.001	127.9 ± 5.6	62.3 ± 5.0*	0.001
Breast Area for BMIx10^{-1}, cm	0.59 ± 0.6	0.58 ± 0.6	0.661	0.59 ± 0.1	0.58 ± 0.7	0.601
Biochemical measurements						
Glucose (mg/L)	88.6 ± 9.3	89.7 ± 9.4	0.102	90.1 ± 10.6	91.2 ± 11.5	0.109
HbA1c (%)	5.4 ± 0.21	5.4 ± 0.32	0.989	5.3 ± 0.76	5.3 ± 0.81	0.896
Insulin (μU/mL)	8.83 ± 2.51	8.79 ± 2.54	0.677	9.45 ± 2.65	9.41 ± 2.72	0.695
HOMA-IR	19.1 ± 1.6	19.4 ± 1.5	0.102	21.0 ± 1.8	20.8 ± 1.9	0.104
Cholesterol (mg/L)	166.88 ± 29.92	169.96 ± 33.49	0.810	160.32 ± 28.82	162.24 ± 30.62	0.089
HDL (mg/L)	52.65 ± 14.53	51.63 ± 14.73	0.066	51.87 ± 14.63	50.84 ± 14.60	0.064
LDL (mg/L)	107.46 ± 21.49	106.51 ± 25.28	0.287	108.66 ± 25.48	106.91 ± 22.44	0.059
Non-HDL cholesterol (mg/L)	96.2 ± 35.5	98.3 ± 37.3	0.129	96.1 ± 34.7	99.2 ± 33.6	0.087
Triglycerides (mg/L)	151.38 ± 34.13	148.20 ± 26.60	0.106	163.30 ± 39.69	160.70 ± 30.80	0.054
Creatinine (μmol/L)	93.55 ± 15.88	91.88 ± 18.62	0.091	94.71 ± 16.01	93.45 ± 18.49	0.055
Estradiol (pg/mL)	58.93 ± 23.09	60.23 ± 10.61	0.056	54.53 ± 10.18	57.42 ± 10.23	0.068
FSH (mIU/mL)	10.31 ± 5.91	10.27 ± 6.79	0.869	12.91 ± 9.83	13.11 ± 8.24	0.561
Leptin (ng/mL)	7.95 ± 2.25	7.92 ± 2.28	0.727	8.90 ± 2.52	8.86 ± 2.58	0.680
White blood cells (10^9/L)	6.44 ± 0.36	6.43 ± 0.65	0.618	6.68 ± 0.41	6.69 ± 0.76‡	0.668
Granulocytes (10^9/L)	3.81 ± 0.58	3.85 ± 0.64	0.085	4.25 ± 0.68	4.32 ± 0.71‡	0.108
Platelets (10^9/L)	278 ± 27	276 ± 21	0.129	289 ± 30	282 ± 23*,‡	0.001
Fibrinogen (mg/dL)	323 ± 38	324 ± 33	0.459	359 ± 42	356 ± 36‡	0.063
hs-CRP (mg/dL)	1.10 ± 0.31	1.06 ± 0.29*	0.001	1.33 ± 0.36	1.27 ± 0.39*, ‡	0.001
Echocolor Doppler parameters						
LVEDd, mm	50.8 ± 3.67	51.1 ± 4.41	0.052	50.9 ± 3.70	51.0 ± 4.45	0.520
LVESd, mm	33.6 ± 4.22	33.8 ± 4.23	0.213	33.7 ± 4.20	33.6 ± 4.26	0.534
LAD, mm	39.5 ± 7.4	40.1 ± 5.5	0.015	39.7 ± 6.9	39.9 ± 5.4	0.394
Septum, mm	10.8 ± 2.1	10.7 ± 1.9	0.188	10.8 ± 2.6	10.8 ± 2.1	0.982
Posterior wall, mm	9.2 ± 1.7	9.1 ± 2.0	0.156	9.1 ± 1.6	9.0 ± 1.9	0.134
IMT, mm	9.5 ± 1.6	9.4 ± 1.6	0.100	9.6 ± 1.3	9.5 ± 1.5	0.061
LV mass, g	192.3 ± 54.6	194.1 ± 63.2	0.423	193.2 ± 45.7	194.2 ± 63.02	0.633
LVEF, %	56.5 ± 7.7	55.8 ± 6.2	0.082	56.0 ± 7.0	55.5 ± 6.0	0.063
FMD	6.34 ± 0.45	6.32 ± 0.44	0.037	6.41 ± 0.48	6.45 ± 0.55*	0.041
Medications						
Oral contraceptives	246 (17.4)	240 (17.6)	0.972	274 (19.4)	282 (20.6)	0.453
Anti-hypertensive drugs	104 (7.4)	98 (7.2)	0.895	114 (8.1)	117 (8.6)	0.697
Statins (%)	266 (18.9)	264 (19.3)	0.794	296 [21]	292 (21.4)	0.841
Aspirin (%)	210 (14.9)	206 (15.1)	0.932	226 (16.0)	218 (15.9)	0.920
Hypoglycemic drugs (%)	120 (8.5)	112 (8.2)	0.820	136 (9.6)	126 (9.2)	0.753
Insulin	/	/	/	18 (1.3)	17 (1.2)	0.784

BMI: body mass index; Hb1Ac: hemoglobin A1c; FMD: flow mediated dilatation; FSH: follicular stimulating hormone; HOMA-IR: homeostasis model assessment of insulin resistance; hs-CRP: high-sensitivity C-reactive protein. HDL: high density lipoprotein; LDL: low density lipoprotein; LVEDd; left ventricle end diastolic diameter; LVESd: left ventricle end systolic diameter; LVEF: left ventricle ejection fraction; LAV: left atrium diameter; LV: left ventricle; BSA: body surface area; MPI: myocardial performance index; IMT: intima media thickness; ‡Difference in square-root breast area adjusted for BMI. * p value <0.05 vs women with low-breast fat accumulation; ǂ p value <0.05 follow-up vs. baseline in any cohort.

3.3. Clinical characteristics of study cohorts at follow-up

At follow-up (1st, 2nd, 3rd, 4th, and 5th year), we noted higher values of platelets, hs-CRP, white blood cells, granulocytes, platelets, fibrinogen and hs-CRP comparing the High-BFA vs Low-BFA women ($p < 0.05$). Table 1, 2, 3. At 4th and 5th year of follow-up, the High-BFA vs Low-BFA women had a higher rate of pre-diabetes, and values of serum glucose, HOMA-IR ($p < 0.05$), Table 3; at 5 years of follow-up, the High-BFA vs Low-BFA women had a higher rate of diabetes mellitus, and values of serum insulin ($p < 0.05$). Table 3. At follow-up, we did not find significant differences in non-HDL cholesterol serum levels between High-BFA and Low-BFA women ($p > 0.05$). Tables 1, 2 and 3. The mean

Table 2
Clinical characteristics of study population at 2nd and 3rd year of follow-up.

Clinical variables	2 YEARS OF FOLLOW-UP			3 YEARS OF FOLLOW-UP		
	High-BFA women (n 1410)	Low-BFA women (n 1366)	P value	High-BFA women (n 1410)	Low-BFA women (n 1366)	P value
General characteristics						
Age (years)	47.1 ± 3.3	47.3 ± 3.4	0.116	48.1 ± 3.3	48.3 ± 3.4	0.116
BMI (kg/h2)	27.30 ± 2.51	27.14 ± 2.22	0.075	27.49 ± 2.48	27.33 ± 2.20	0.072
Waist/hip	1.091 ± 0.006	1.092 ± 0.001	0.122	1.099 ± 0.006	1.098 ± 0.011	0.003
Systolic blood pressure (mmHg)	126.3 ± 10.9	126.1 ± 10.1	0.616	125.7 ± 11.0	125.9 ± 10.2	0.619
Diastolic blood pressure (mmHg)	80.2 ± 6.7	80.6 ± 7.9	0.151	77.7 ± 6.8	78.2 ± 7.9	0.074
Heart rate	67.9 ± 10.4	67.4 ± 8.9	0.173	69.5 ± 9.6	68.8 ± 8.2	0.139
Risk factors						
Family history of ischemic heart disease, (%)	118 (8.4)	104 (7.6)	0.507	/	/	/
Hypertension (%)	164 (11.6)	141 (10.3)	0.297	180 (12.8)	155 (11.3)	0.276
Dyslipidemia (%)	334 (23.7)	315 (23.1)	0.729	351 (24.9)	328 (24.0)	0.620
Prediabetes (%)	254 (18.0)	231 (16.9)	0.474	275 (19.5)	244 (17.9)	0.289
Type 2 diabetes(%)	47 (3.3)	32 (2.3)	0.146	61 (4.3)	44 (3.2)	0.154
Current smoking (%)	127 (9.0)	129 (9.4)	0.740	123 (8.7)	126 (9.2)	0.693
Mammography measures						
Density, %	19.2 ± 0.3	57.9 ± 0.6*	0.001	18.7 ± 0.4	56.3 ± 0.7*	0.001
Dense Area, cm2	26.1 ± 0.4	99.3 ± 17.8*	0.001	25.5 ± 0.5	97.8 ± 16.9*	0.001
Breast area, cm2	159.3 ± 9.4	160.0 ± 9.6	0.052	160.5 ± 9.7	162.3 ± 10.1	0.156
Non dense Area, cm2	129.1 ± 6.2	63.2 ± 5.4*	0.001	131.3 ± 6.8	63.9 ± 6.2*	0.001
Breast Area for BMIx10^{-1}, cm	0.58 ± 0.6	0.58 ± 0.3	0.882	0.58 ± 0.2	0.57 ± 0.9	0.688
Biochemical measurements						
Glucose (mg/L)	88.1 ± 10.8	88.8 ± 11.6	0.100	89.6 ± 10.8	90.4 ± 11.5	0.059
HbA1c (%)	5.42 ± 0.27	5.46 ± 0.12	0.091	5.55 ± 0.32	5.60 ± 0.18	0.124
Insulin (μU/mL)	9.88 ± 2.84	9.84 ± 2.80	0.709	10.0 ± 2.89	10.1 ± 2.81	0.355
HOMA-IR	21.7 ± 1.9	21.5 ± 1.7	0.003	21.9 ± 2.0	22.1 ± 1.9	0.007
Cholesterol (mg/L)	150.15 ± 28.91	152.36 ± 32.62	0.059	148.54 ± 29.89	149.75 ± 32.63	0.309
HDL (mg/L)	61.20 ± 14.61	60.17 ± 14.58	0.063	64.68 ± 14.64	63.65 ± 14.63	0.064
LDL (mg/L)	92.96 ± 25.46	91.20 ± 22.43	0.058	90.39 ± 25.48	88.64 ± 22.44	0.065
Non-HDL cholesterol (mg/L)	90.1 ± 34.7	92.2 ± 33.2	0.103	79.8 ± 34.7	81.2 ± 33.3	0.278
Triglycerides (mg/L)	133.80 ± 38.29	132.47 ± 36.94	0.352	126.50 ± 38.30	125.17 ± 36.94	0.352
Creatinine (μmol/L)	90.55 ± 15.86	88.18 ± 18.97	0.094	95.56 ± 15.87	94.76 ± 19.49	0.237
Estradiol (pg/mL)	55.26 ± 23.18	56.55 ± 10.62	0.158	52.12 ± 23.09	53.42 ± 10.71	0.056
FSH (mIU/mL)	14.20 ± 5.90	14.18 ± 6.65	0.933	16.12 ± 6.91	16.08 ± 6.79	0.878
Leptin (ng/mL)	9.52 ± 2.70	9.49 ± 2.74	0.771	10.57 ± 2.99	10.53 ± 3.03	0.726
White blood cells (10^9/L)	6.95 ± 0.42	6.93 ± 0.76	0.393	7.30 ± 0.48	7.28 ± 0.88‡	0.459
Granulocytes (10^9/L)	4.45 ± 0.67	4.51 ± 0.72	0.123	4.76 ± 0.72	4.82 ± 0.77*‡	0.034
Platelets (10^9/L)	336 ± 30	332 ± 23*	0.001	359 ± 32	355 ± 24*,‡	0.001
Fibrinogen (mg/dL)	381 ± 43	380 ± 37	0.511	402 ± 42	400 ± 36‡	0.178
hs-CRP (mg/dL)	1.51 ± 0.36	1.45 ± 0.39*	0.001	1.61 ± 0.39	1.55 ± 0.42*, ‡	0.001
Echocolor Doppler parameters						
LVEDd, mm	51.2 ± 3.68	52.1 ± 4.45	0.162	51.3 ± 3.70	51.9 ± 4.48	0.181
LVESd, mm	33.5 ± 4.45	33.3 ± 4.22	0.224	31.6 ± 4.20	31.9 ± 4.26	0.162
LAD, mm	39.6 ± 7.3	39.9 ± 5.3	0.214	40.0 ± 7.3	40.4 ± 5.3	0.298
Septum, mm	10.8 ± 2.3	10.8 ± 2.1	0.902	11.1 ± 2.1	11.2 ± 2.1	0.210
Posterior wall, mm	9.2 ± 1.8	9.1 ± 2.1	0.179	9.6 ± 1.7	9.4 ± 1.9	0.204
IMT, mm	1.0 ± 1.6	1.0 ± 1.5	0.892	1.1 ± 1.6	1.1 ± 1.5	1.000
LV mass, g	201.9 ± 53.9	203.8 ± 58.6	0.374	206.49 ± 54.6	208.27 ± 63.15	0.428
LVEF, %	58.4 ± 7.0	57.9 ± 6.1	0.145	60.9 ± 7.2	60.5 ± 6.1	0.114
FMD	6.50 ± 0.52	6.62 ± 0.61*	0.001	6.57 ± 0.58	6.76 ± 0.74*	0.001
Medications						
Oral contraceptives	304 (21.6)	297 (21.7)	0.944	312 (22.1)	303 (22.2)	0.980
Anti-hypertensive drugs	126 (8.9)	113 (8.3)	0.578	134 (9.5)	119 (8.7)	0.510
Statins (%)	311 (22.1)	323 (23.6)	0.341	321 (22.8)	325 (23.8)	0.552
Aspirin (%)	240 (17.0)	231 (16.9)	0.978	244 (17.3)	234 (17.1)	0.943
Hypoglycemic drugs (%)	151 (10.7)	141 (10.3)	0.787	161 (11.4)	149 (10.9)	0.714
Insulin	23 (1.6)	18 (1.3)	0.598	25 (1.8)	20 (1.5)	0.621

BMI: body mass index; Hb1Ac: hemoglobin A1c; FMD: flow mediated dilatation; FSH: follicular stimulating hormone; HOMA-IR: homeostasis model assessment of insulin resistance; hs-CRP: high-sensitivity C-reactive protein. HDL: high density lipoprotein; LDL: low density lipoprotein; LVEDd; left ventricle end diastolic diameter; LVESd: left ventricle end systolic diameter; LVEF: left ventricle ejection fraction; LAV: left atrium diameter; LV: left ventricle; BSA: body surface area; MPI: myocardial performance index; IMT: intima media thickness; ‡Difference in square-root breast area adjusted for BMI. * p value <0.05 vs. vs women with low-breast fat accumulation; ǂ p value <0.05 follow-up vs. baseline in any cohort.

values of FMD of the brachial artery were significantly lower in the High-BFA vs Low-BFA women from the first year to the last year of follow-up ($p < 0.05$). Regarding the mammographic measures, High-BFA vs Low-BFA women showed significant differences in percentage density, dense area, and non-dense area ($p < 0.05$). Table 1, 2 and 3.

3.4. Serum expression of inflammatory cytokines, ET-1 and TSP-1 at follow-up

-**Serum ET-1 expression**: as seen in Fig. 1, as compared to the baseline, the High-BFA vs Low-BFA women over-expressed ET-1 from the 1st year of follow-up until the study end ($p < 0.05$). Fig. 2, **panel A**. Compared with baseline, the Low-BFA women showed overexpression of

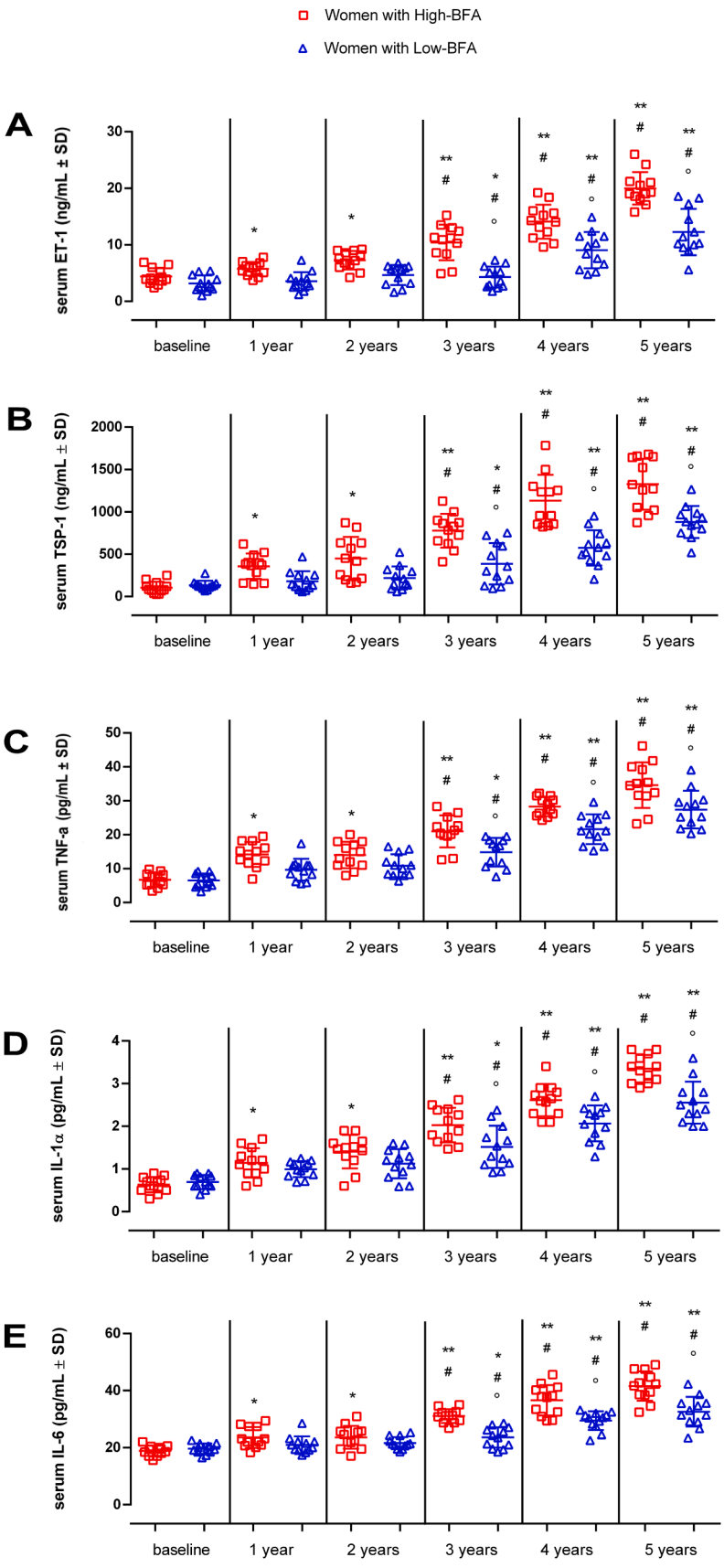
Table 3
Clinical characteristics of study population at 4th and 5th year of follow-up.

Clinical variables	4 YEARS OF FOLLOW-UP			5 YEARS OF FOLLOW-UP		
	High-BFA women (n 1410)	Low-BFA women (n 1366)	P value	High-BFA women (n 1410)	Low-BFA women (n 1366)	P value
General characteristics						
Age (years)	49.1 ± 3.3	49.3 ± 3.4	0.116	50.1 ± 3.3	50.3 ± 3.4	0.116
BMI (kg/h2)	27.65 ± 2.48	27.49 ± 2.19	0.071	27.80 ± 2.50	27.62 ± 2.20	0.044
Waist/hip	1.105 ± 0.006	1.106 ± 0.001	0.184	1.129 ± 0.006	1.128 ± 0.009	0.181
Systolic blood pressure (mmHg)	124.4 ± 10.8	125.1 ± 10.2	0.179	123.5 ± 10.9	124.2 ± 10.1	0.079
Diastolic blood pressure (mmHg)	73.4 ± 6.8	73.9 ± 7.8	0.272	72.2 ± 6.6	72.6 ± 7.9	0.148
Heart rate	68.8 ± 10.3	68.9 ± 6.9	0.763	67.8 ± 9.3	67.7 ± 6.8	0.746
Risk factors						
Family history of ischemic heart disease, (%)	/	/	/	/	/	/
Hypertension (%)	198 [14]	167 (12.2)	0.174	211 [15]	181 (13.3)	0.214
Dyslipidemia (%)	361 (25.6)	337 (24.7)	0.601	377 (26.7)	352 (25.8)	0.591
Prediabetes (%)	317 (22.5)	255 (18.7)*	0.015	347 (24.6)	277 (20.3)*	0.007
Type 2 diabetes(%)	73 (5.2)	53 (3.9)	0.121	90 (6.4)	63 (4.6)*	0.046
Current smoking (%)	120 (8.5)	122 (8.9)	0.745	118 (8.4)	120 (8.8)	0.746
Mammography measures						
Density, %	18.2 ± 0.2	55.1 ± 0.3*	0.001	17.9 ± 0.4	55.1 ± 0.6*	0.001
Dense Area, cm2	24.2 ± 0.6	95.8 ± 15.9*	0.001	26.3 ± 0.7	99.2 ± 17.3*	0.001
Breast area, cm2	163.4 ± 10.2	164.3 ± 11.6	0.130	158.2 ± 9.0	160.9 ± 9.4	0.154
Non dense Area, cm2	132.2 ± 7.0	64.8 ± 6.2*	0.001	130.6 ± 6.0	63.1 ± 5.3*	0.001
Breast Area for BMIx10^{-1}, cm	0.57 ± 0.7	0.57 ± 0.1	0.822	0.57 ± 0.2	0.56 ± 0.6	0.558
Biochemical measurements						
Glucose (mg/L)	97.6 ± 19.2	95.1 ± 15.4*	0.001	108.9 ± 18.4	105.6 ± 16.1*	0.001
HbA1c (%)	5.58 ± 0.27	5.62 ± 0.12	0.152	5.66 ± 0.32	5.68 ± 0.18	0.042
Insulin (μU/mL)	10.3 ± 2.92	10.2 ± 2.95	0.370	11.6 ± 3.01‡	10.5 ± 3.03*	0.001
HOMA-IR	24.8 ± 2.4	24.0 ± 2.2*	0.001	28.8 ± 2.7	27.5 ± 2.5*‡	0.001
Cholesterol (mg/L)	145.8 ± 28.81	147.6 ± 32.60	0.124	144.84 ± 28.82	146.65 ± 32.62	0.122
HDL (mg/L)	68.61 ± 14.32	67.58 ± 14.58	0.061	70.12 ± 14.61	69.15 ± 14.60	0.080
LDL (mg/L)	85.47 ± 23.42	83.85 ± 20.62	0.059	81.50 ± 22.32	79.96 ± 19.67	0.054
Non-HDL cholesterol (mg/L)	77.1 ± 34.8	78.9 ± 33.2	0.163	75.7 ± 34.8	77.5 ± 33.2	0.163
Triglycerides (mg/L)	112.30 ± 32.14	111.18 ± 31.01	0.350	107.28 ± 30.71	106.21 ± 29.61	0.350
Creatinine (μmol/L)	95.83 ± 18.01	94.53 ± 19.83	0.071	98.13 ± 18.45	96.80 ± 20.31	0.071
Estradiol (pg/mL)	50.02 ± 20.89	51.19 ± 9.60	0.087	47.85 ± 19.99	48.98 ± 9.20	0.055
FSH (mIU/mL)	18.19 ± 8.78	18.14 ± 10.10	0.889	18.55 ± 8.98	18.50 ± 10.30	0.892
Leptin (ng/mL)	11.1 ± 3.32	11.09 ± 3.20	0.936	11.59 ± 3.29	11.54 ± 3.33	0.691
White blood cells (10^9/L)	7.36 ± 0.45	7.29 ± 0.79*	0.004	7.48 ± 0.47	7.41 ± 0.80*‡	0.005
Granulocytes (10^9/L)	4.87 ± 0.79	4.78 ± 0.77*	0.002	4.93 ± 0.80	4.84 ± 0.78*‡	0.003
Platelets (10^9/L)	360 ± 32	356 ± 24*	0.001	367 ± 33	363 ± 25*,‡	0.001
Fibrinogen (mg/dL)	417 ± 46	380 ± 37*	0.001	420 ± 428	416 ± 40*‡	0.020
hs-CRP (mg/dL)	1.66 ± 0.39	1.59 ± 0.43*	0.001	1.70 ± 0.41	1.63 ± 0.44*, ‡	0.001
Echocolor Doppler parameters						
LVEDd, mm	50.9 ± 3.51	51.9 ± 4.30	0.162	51.1 ± 3.48	51.3 ± 4.28	0.178
LVESd, mm	33.5 ± 4.26	33.4 ± 4.42	0.544	31.6 ± 5.10	32.0 ± 4.89	0.135
LAD, mm	40.6 ± 7.8	40.9 ± 8.3	0.327	41.8 ± 6.2	42.3 ± 3.8	0.290
Septum, mm	12.0 ± 2.1	12.1 ± 2.0	0.199	12.9 ± 2.2	12.8 ± 2.1	0.221
Posterior wall, mm	9.6 ± 2.0	9.5 ± 2.4	0.234	9.6 ± 2.5	9.5 ± 3.0	0.341
IMT, mm	1.1 ± 2.0	1.1 ± 1.9	0.946	1.1 ± 2.6	1.1 ± 2.4	0.971
LV mass, g	215.5 ± 55.9	216.1 ± 65.2	0.795	219.89 ± 57.9	217.09 ± 66.2	0.236
LVEF, %	58.9 ± 6.8	58.6 ± 5.6	0.204	59.7 ± 6.7	59.3 ± 5.6	0.088
FMD	6.60 ± 0.59	6.80 ± 0.74*	0.001	6.78 ± 0.76	7.06 ± 0.86*	0.001
Medications						
Oral contraceptives	321 (22.8)	308 (22.5)	0.927	328 (23.3)	314 (23.0)	0.899
Anti-hypertensive drugs	155 (11.0)	138 (10.1)	0.483	178 (12.6)	158 (11.6)	0.426
Statins (%)	328 (23.3)	331 (24.2)	0.579	337 (23.9)	336 (24.6)	0.701
Aspirin (%)	247 (17.5)	238 (17.4)	0.988	256 (18.2)	240 (17.6)	0.724
Hypoglycemic drugs (%)	170 (12.1)	158 (11.6)	0.733	180 (12.8)	165 (12.1)	0.623
Insulin	27 (1.9)	24 (1.8)	0.866	31 (2.2)	27 (2.0)	0.782

BMI: body mass index; Hb1Ac: hemoglobin A1c; FSH: follicular stimulating hormone; HOMA-IR: homeostasis model assessment of insulin resistance; hs-CRP: high-sensitivity C-reactive protein. HDL: high density lipoprotein; LDL: low density lipoprotein; LVEDd; left ventricle end diastolic diameter; LVESd: left ventricle end systolic diameter; LVEF: left ventricle ejection fraction; LAV: left atrium diameter; LV: left ventricle; BSA: body surface area; MPI: myocardial performance index; IMT: intima media thickness; ‡Difference in square-root breast area adjusted for BMI. * p value <0.05 vs women with low-breast fat accumulation (group 2); ǂ p value <0.05 follow-up vs. baseline in any cohort.

ET-1 from the 3rd year of follow-up until the study end ($p < 0.05$). Fig. 2, **panel A**. Compared with the previous study time point, at the third, fourth, and fifth years of follow-up, High-BFA women, as well as Low-BFA women, overexpressed ET-1 ($p < 0.05$). Fig. 2, **panel A**. Finally, the High-BFA vs Low-BFA women overexpressed serum ET-1 at the third, fourth, and fifth years of follow-up ($p < 0.05$). Fig. 2, **panel A**.

-**Serum TSP-1 expression**: As compared to the baseline, the High-BFA women over-expressed TSP-1 from the 1st year of follow-up until the study end ($p < 0.05$). Fig. 2, **panel B**. Compared with baseline, the Low-BFA women showed overexpression of TSP-1 from the 3rd year of follow-up through the study end ($p < 0.05$). Fig. 2, **panel B**. Compared with the previous study time point, at the third, fourth, and fifth years of follow-up, the High-BFA women, as well as the Low-BFA women, overexpressed TSP-1 ($p < 0.05$). Fig. 2, **panel B**. Finally, the High-BFA vs



(caption on next page)

Fig. 2. In the figure, we reported the scatter plots of serum expression levels of endothelin-1 (panel A), thrombospodin-1 (panel B), and inflammatory cytokines (panel C-E) in women with high breast fat accumulation (High-BFA, red color) vs women with low-breast fat accumulation (Low-BFA, blue color) at baseline and for each year of follow-up. In **panel A**, the bar graphs showed the expression of ET-1, at different follow-up phases, in High-BFA vs Low-BFA women. In **panel B**, the serum TSP-1 expression, at different follow-up phases, in High-BFA vs Low-BFA women. In **panel C, D** and **E** (**panel C-E**) the serum expression of inflammatory cytokines (TNF-α, IL-1α and IL-6), at different follow-up phases, in High-BFA vs Low-BFA women. TNF-α: tumor necrosis factor alpha; IL-1α: interleukin 1 alpha; IL-6: interleukin 6.
* is for statistical significant vs. baseline, same group ($p < 0.05$); ** is for statistical significant vs. baseline, same group ($p < 0.01$); # is for statistical significant vs previous time point, same group ($p < 0.05$); ° is for statistical significant vs women with high breast fat accumulation (High-BFA), ($p < 0.05$), at the same time point.

Low-BFA women overexpressed serum TSP-1 at the third, fourth, and fifth years of follow-up ($p < 0.05$). Fig. 2, **panel B**.

-**Serum expression of inflammatory cytokines:** As compared to baseline, the High-BFA women over-expressed the inflammatory cytokines (TNF-α, IL-1α, and IL-6) from the 1st year of follow-up until the study end ($p < 0.05$). Fig. 2, **panel C-E**. As compared to baseline, the Low-BFA women showed an over-expression of the inflammatory cytokines (TNF-α, IL-1α, and IL-6), from the 3rd year of the follow-up until the study end ($p < 0.05$). Fig. 2, **panel C-E**. As compared to the previous study time point, at the third, fourth, and fifth year of follow-up, the High-BFA women, as the Low-BFA women, over-expressed inflammatory cytokines (TNF-α, IL-1α, and IL-6) ($p < 0.05$). Fig. 2, **panel C-E**. Finally, the High-BFA vs Low-BFA women overexpressed serum inflammatory cytokines (TNF-α, IL-1α, and IL-6) at the third, fourth, and fifth years of follow-up ($p < 0.05$). Fig. 2, **panel C-E**.

We evaluated the effects of **hormonal status and menstrual phase** on endothelial function and circulating biomarkers. No significant differences were detected for FMD (mean difference = 0.12 %, $p = 0.42$), ET-1 (mean difference = 0.09 pg/mL, $p = 0.47$), or TSP-1 (mean difference = 0.13 ng/mL, $p = 0.39$), suggesting limited phase-related variability within this subset of patients.

3.5. Primary study endpoints: rate of ED and MACE in the study cohorts

At 1 year of follow-up, we had a total of 1007 (36.2 %) patients with the ED: 620 (44 %) High-BFA vs 387 (28.3 %) Low-BFA women ($p < 0.05$). In the supplementary files, we reported the ED rate for each year of follow-up in High-BFA vs Low-BFA women. The Kaplan-Meier curves show the cumulative risk of ED (Fig. 3) in High-BFA vs Low-BFA women at 5 years of follow-up. Regarding MACE, at follow-up end, we had a total of 192 (6.9 %) MACE: 158 (11.2 %) High-BFA vs 34 (2.5 %) Low-BFA women ($p < 0.05$). In the supplementary files, we reported the number of MACEs per year of follow-up for High-BFA vs Low-BFA women. The Kaplan-Meier curves show the cumulative risk of MACE in High-BFA vs Low-BFA women at 5 years of follow-up. Figs. 3 and 4.

The ED was predicted by pre-T2DM (1.140, CI 95 % (1.031–1.676); $p < 0.05$) and TSP-1 (1.081, CI 95 % 1.007–1.121; $p < 0.05$). Table 4. The MACE was predicted by the FMD (1.189, CI 95 % 1.055–1.461; $p < 0.05$), LVEF (1.030, CI 95 % 1.005–1.056; $p < 0.05$), WBC (1.605, CI 95 % 1.395–1.825; $p < 0.05$), ET-1 (1.459, CI 95 % 1.206–1.765; $p < 0.05$), TSP-1 (1.170, CI 95 % 1.014–1.321; $p < 0.05$), and High-BFA (1.968, CI 95 % 1.962–2.013; $p < 0.05$). Table 4.

In the supplementary figure, we reported the serum levels of ET-1 and TSP-1 in the overall study population, divided into those with vs. those without ED (panel A and B), and in those with vs. those without MACE (panels C and D) at 1 year of follow-up. In Fig. 5, we reported the graphical abstract of the study.

4. Discussion

The important and novel finding of the study is that excess breast fat is associated with a higher rate of ED and MACE at 5 years of follow-up in pre-menopausal High-BFA vs Low-BFA women. From the first year of follow-up until the study end, High-BFA vs Low-BFA women showed lower FMD values and over-inflammation, and higher serum levels of ET-1 and TSP-1 ($p < 0.05$). Intriguingly, the ED was predicted by pre-T2DM and TSP-1 serum values. The MACE was predicted by FMD, LVEF, WBC, ET-1, TSP-1, and diagnosis of High-BFA. Furthermore, ET-1 was an independent predictor of MACE, while TSP independently predicted both ED and MACE at the end of follow-up.

In this propensity-matched cohort of pre-menopausal High-BFA vs. Low-BFA women, the higher rate of ED was associated with impaired FMD, higher hs-CRP and fibrinogen levels, and elevated serum ET-1 and TSP-1 levels. ED is an early marker of atherosclerosis and increases the risk of CVDs and MACE [13]. ED could be diagnosed by the FMD of the brachial artery [13]. FMD is a marker of coronary endothelial function

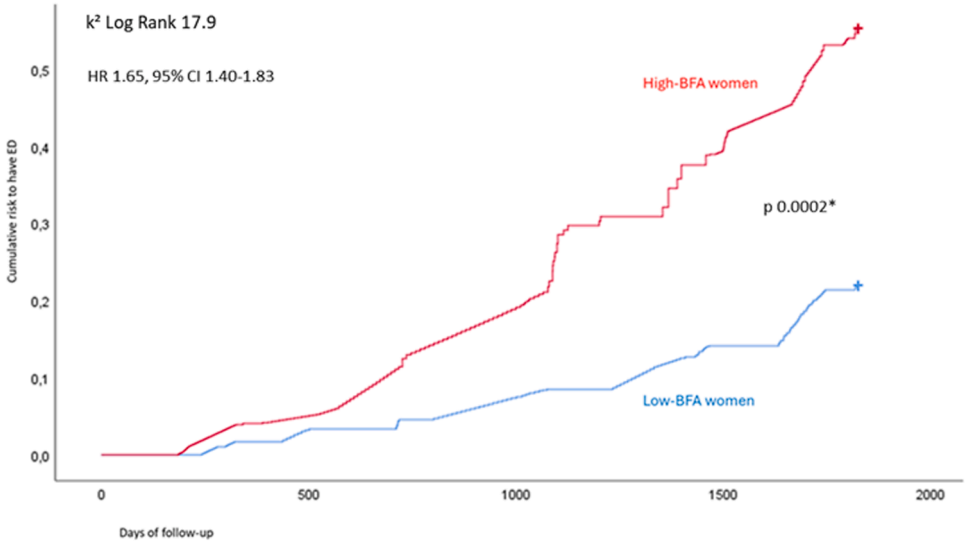


Fig. 3. In this figure, the representation of Kaplan curves for the cumulative risk of having Endothelial Dysfunction (ED) at 5 years of follow-up in women with high (High-BFA, red color) vs women with low-breast fat accumulation (Low-BFA, blue color). ED: endothelial dysfunction; * is for statistical significant: $p < 0.05$.

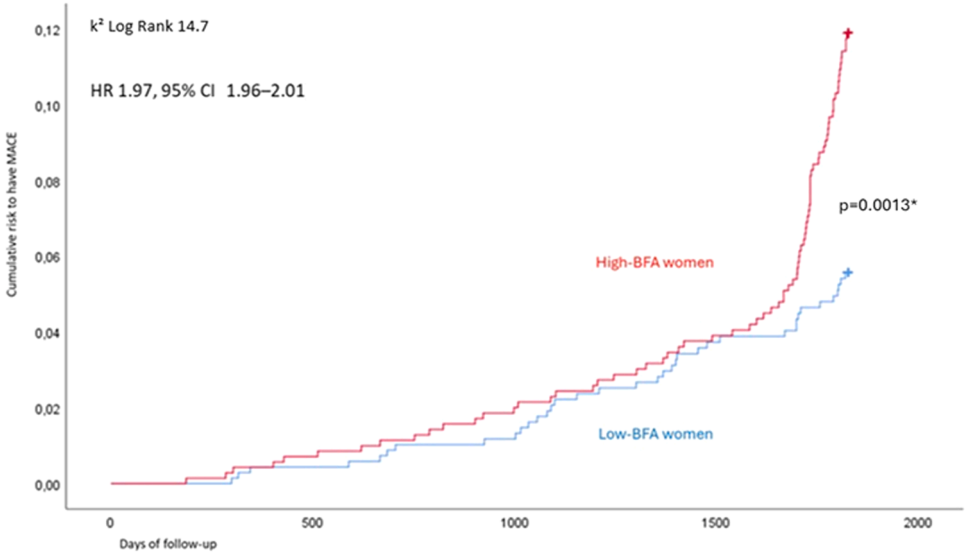


Fig. 4. In this figure, the representation of Kaplan curves for the cumulative risk of having Major Adverse Cardiac Events (MACE) at 5 years of follow-up in women with high (High-BFA, red color) vs women with low-breast fat accumulation (Low-BFA, blue color). MACE: major adverse cardiac events; * is for statistical significant: $p < 0.05$.

and an independent predictor of cardiovascular events in patients with and without established atherosclerosis [13]. In pre-menopausal non--obese women, breast fat accumulation was associated with impaired FMD. The impaired FMD could increase the risk of MACE by about 1.2-fold. This effect was independent of leptin levels, which are adipocyte-derived markers of ED [14]. Fat accumulation increases leptin levels [14] and promotes sympathetic nervous system activation [15]. Women with High-BFA vs. Low-BFA exhibited greater impairment of endothelial function and elevated serum levels of the inflammatory markers, ET-1 and TSP-1 at follow-up. Chronic inflammation and oxidative stress promote LDL oxidation, vascular inflammation, endothelial apoptosis, and reduced NO bioavailability [16]. This leads to vasoconstriction, platelet activation, leukocyte infiltration, and foam cell formation, then contributing to ED, atherosclerotic plaque progression, and increased risk of MACE [16,17]. Non-HDL cholesterol is an emerging biomarker of CVDs, as it reflects the cholesterol content of atherogenic lipoproteins [12]. Non-HDL cholesterol could contribute to atherosclerotic progression, ED, and increase the risk of MACE [18,19]. Regarding **cardiovascular outcomes**, we identified High-BFA, reduced LVEF, elevated WBC count, and increased ET-1 and TSP-1 as independent predictors of MACE. High-BFA was associated with a 1.968-fold higher risk of MACE despite similar BMI. However, regional adiposity, particularly mammary fat, could exert distinct adverse effects on vascular and metabolic functions. Conversely, elevated WBC values (systemic inflammation), further increased MACE risk by 1.6-fold, underscoring the detrimental role of chronic inflammation in premenopausal women with High-BFA [1–3]. These adverse effects occurred independently of changes in heart rate and blood pressure. Indeed, breast fat accumulation could promote insulin resistance, increasing the risk of prediabetes and diabetes, both of which are detrimental to endothelial function [1–3,13]. In our cohort, prediabetes increased the risk of ED and worsened endothelial function through multiple pathways [1–3]. Conversely, reduced LVEF was associated with higher rates of MACE [19]. The LVEF reflects impaired cardiac function, reduced NO bioavailability, and worsens endothelial dysfunction [19]. Notably, ED may worsen cardiac function in a vicious cycle, ultimately leading to MACE [19]. In this setting, breast fat accumulation could further impair endothelial function by increasing ET-1 and TSP-1. This could promote inflammation and reduce vasodilation [6–8]. ET-1 drives ROS production, decreases NO bioavailability, and induces vasoconstriction [14]. Conversely, ET receptor antagonists could improve endothelial function in both experimental models and patients with CVDs [20,21]. Indeed, ET receptor antagonism reduces neutrophil infiltration and myeloperoxidase activity in ischemic myocardium [22]. In our study, elevated ET-1 increased MACE risk by 1.4-fold, reinforcing its link with inflammation, oxidative stress, endothelial dysfunction, and adverse outcomes [20–22]. TSP-1, an extracellular matrix component, also contributes to vascular dysfunction by binding CD47, inhibiting cyclic guanosine monophosphate (cGMP) production, and blocking NO-mediated vasorelaxation [20–25]. TSP-1 expression is regulated by redox signaling and NO and further modulates vascular tone and angiogenesis through VEGF pathways [25]. Notably, TSP-1 potently inhibits this chemotactic response and downstream pro-angiogenic signals [25] through two subdomains that independently suppress neovascularization by blocking FGF-2 and VEGF activity in ECs [26]. From experimental models, TSP-1 inhibition improves insulin sensitivity and energy balance [26]. In humans, TSP-1 is overexpressed in endothelial cells from obese and diabetic subjects [27]. In our study, higher TSP-1 levels increased the risk of ED and MACE, suggesting its role as an early marker of vascular dysfunction and CVDs. These findings support the evaluation of TSP-1 as a diagnostic and prognostic biomarker, as well as a potential therapeutic target in pre-menopausal women with breast fat accumulation. In this context, breast adiposity may represent a novel, non-invasive marker for early detection of cardiovascular risk in women. Integrating mammographic data into risk assessment could enhance preventive strategies, particularly in pre-menopausal women, and support the development of sex-specific approaches in preventive cardiology. These findings open the door to further studies to explore CVDs mechanisms beyond traditional atherosclerosis risk factors promoted by over-inflammation/oxidative stress.

4.1. Study limitations

The study evidences few limitations. The data are representative of a cohort of pre-menopausal High-BFA vs Low-BFA women. Thus, the study results must be supported by larger randomized clinical trials to be translated into clinical practice. From our analysis, we identified the High-BFA as an early predictor of ED and MACE in premenopausal women. On the other hand, social, economic, and political barriers might hinder clinical adoption of breast fat as a cardiovascular risk factor and thus reduce screening uptake. Indeed, adding the evaluation of ET-1 and TSP-1 into routine assessments might raise costs and require

Table 4
Cox regression analysis for Endothelial Dysfunction (A) and Major Adverse Cardiac Events (B) at 5 years of follow-up.

A) ED	UNIVARIATE ANALYSIS			MULTIVARIATE ANALYSIS		
Risk factors	**HR**	**CI 95 %**	**P value**	**HR**	**CI 95 %**	**P value**
Age	0.953	0.934–0.973	0.001*	1.049	0.991–1.101	0.051
BMI	1.019	0.989–1.049	0.228			
HOMA	0.922	0.823–1.034	0.165			
Smoking	0.950	0.757–1.193	0.659			
Hypertension	1.007	0.786–1.291	0.954			
Dyslipidemia	0.914	0.772–1.081	0.294			
preT2DM	1.297	1.085–1.850	0.004*	1.140	1.031–1.676	0.012*
High breast fat accumulation	1.014	1.012–1.020	0.001*	0.972	0.926–1.017	0.082
FMD	1.014	0.874–1.182	0.832			
Leptin	0.992	0.963–1.022	0.595			
LVEF	1.027	1.016–1.037	0.001*	0.996	0.972–1.021	0.757
WBC	0.985	0.867–1.118	0.813			
hCRP	1.215	0.974–1.516	0.085	0.374	0.227–1.616	0.479
IL-1	0.999	0.989–1.001	0.376			
Fibrinogen	1.001	0.999–1.030	0.465			
Oral contraceptives	2.270	1.957–2.633	0.001*	0.694	0.474–1.017	0.061
Beta blockers	0.172	0.049–0.261	0.031*	0.322	0.182–1.572	0.101
ET-1	0.936	0.802–1.092	0.401			
TSP-1	1.125	1.008–1.250	0.001*	1.081	1.007–1.121	0.001*
non-HDL-C	0.996	0.948–1.002	0.401			
B) MACE	**UNIVARIATE ANALYSIS**			**MULTIVARIATE ANALYSIS**		
Risk factors	**HR**	**CI 95 %**	**P value**	**HR**	**CI 95 %**	**P value**
Age	0.970	0.933–1.008	0.125			
BMI	1.074	1.013–1.138	0.017*	1.197	0.904–1.298	0.081
HOMA-IR	0.572	0.450–0.728	0.001*	0.737	0.357–1.520	0.409
Smoking	1.279	0.872–1.877	0.208			
Hypertension	1.092	0.690–1.726	0.708			
Dyslipidemia	0.990	0.721–1.360	0.951			
preT2DM	1.295	0.921–1.820	0.137			
High breast fat accumulation	2.712	1.008–3.060	0.001*	1.968	1.962–2.013	0.012*
FMD	1.338	1.002–1.788	0.028*	1.189	1.055–1.461	0.001*
Leptin	0.896	0.845–0.950	0.001*	0.882	0.735–1.059	0.177
LVEF	1.054	1.004–1.144	0.021*	1.030	1.005–1.056	0.020*
WBC	1.706	1.541–1.921	0.010*	1.605	1.395–1.825	0.020*
hs-CRP	1.021	0.666–1.564	0.924			
IL-1	0.998	0.995–1.001	0.135			
Fibrinogen	0.995	0.991–0.999	0.012*	0.994	0.989–1.089	0.090
Oral contraceptives	1.468	1.083–1.991	0.013*	0.926	0.627–1.368	0.698
Beta blockers	1.066	0.659–1.725	0.793			
ET-1	0.931	0.796–1.089	0.371	1.459	1.206–1.765	0.001*
TSP-1	1.231	1.010–1.417	0.001*	1.170	1.014–1.321	0.001*
non-HDL-C	0.999	0.995–1.002	0.488			

BMI: body mass index; ET-1: endothelin 1; FMD: flow mediated dilatation; High-BFA: High breast fat accumulation; HOMA-IR: homeostasis model assessment of insulin resistance; hs-CRP: hs-CRP: high-sensitivity C-reactive protein; IL-1: interleukin 1; LDL: low density lipoprotein; LVEF: left ventricle ejection fraction; preT2DM: pre type 2 diabetes mellitus; TSP-1: thrombospondin 1; WBC: white blood cells; *$p < 0.05$ (statistical significant); HR: Hazard ratio; CI: confidence of interval.

additional resources. This, in the absence of sex-specific cardiovascular guidelines, might delay implementation, particularly in under-resourced settings. Conversely, we used a single time-point measurements of ET-1 and TSP-1, which may not capture relevant longitudinal changes during the follow-up period. Again, the reduced number of serum samples analyzed with ELISA compared to the initial screened population may introduce representativeness bias despite the careful matching strategy. Biomarker analyses (ET-1, TSP-1, and cytokines) were performed in a small sub-cohort of 240 women derived from the institutional biobank. Although baseline characteristics were comparable with the rest of the study population, this limited sample size and biobank-based selection may introduce selection bias and constrain the generalizability of the findings. Therefore, these results should be considered exploratory and hypothesis-generating. On the other hand, to compare the representativeness of the biomarker sub-cohort ($n = 240$; 120 High-BFA and 120 Low-BFA) with the entire propensity score–matched population ($n = 2776$), we performed a comparative analysis of baseline demographic, clinical, and metabolic variables. Notably, we did not show significant differences in general characteristics (Age, BMI, blood pressure, heart rate etc.), risk factors, mammography measures, biochemical measurements, inflammatory markers, echocolor Doppler parameters (cardiac-derived and FMD values) and medications (all $p > 0.05$), (supplementary files). However, this could confirm that the biomarker subgroup was representative of the overall matched cohort.

Notably, while some per-unit hazard ratios were close to 1, these values largely reflect the scale of measurement rather than a lack of association. When effects were expressed by SD and quartiles, risk gradients were clearer and directionally consistent. Moreover, even modest relative risks can yield non-trivial absolute differences at the population level for common exposures. In this setting, because some of the hazard ratios observed in this study were close to 1.0 (ranging from 1.03 to 1.08), this could indicate relatively small effect sizes at the individual level. Although these associations reached statistical significance, their clinical impact should be interpreted with caution. In large prospective cohorts, even modest HRs can achieve significance due to high statistical power, but the magnitude of effect may be limited for individual risk prediction. Nevertheless, such small relative increases may still hold population-level relevance, particularly when exposure is common or when multiple low-grade risk factors act cumulatively over time

Thus, we acknowledge that the individual-level clinical impact of small per-unit changes is limited; thus, these biomarkers should be considered components of a broader multivariable risk profile, not standalone decision thresholds. Indeed, some associations were small on a per-unit scale; although standardized and categorical presentations

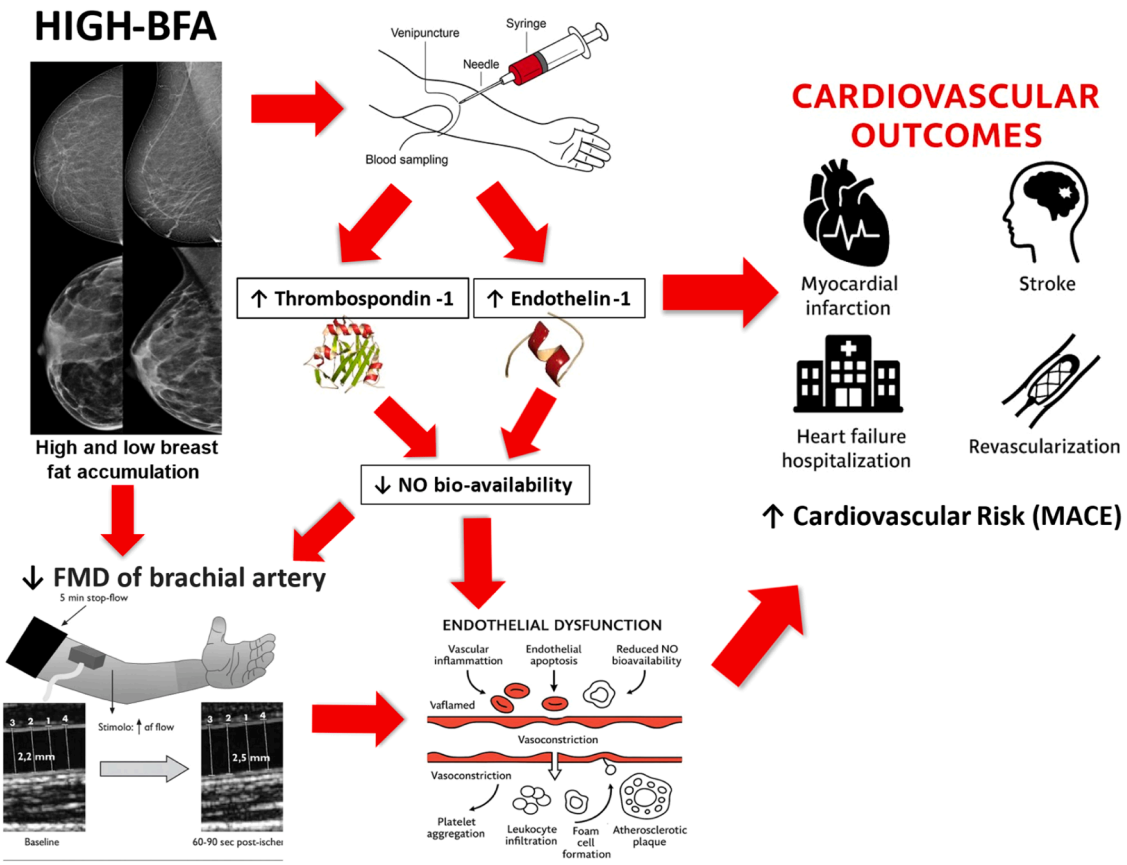


Fig. 5. In this figure we reported the Graphical Abstract of the study. In the upper left part of the image, the mammography of breast with high (High-BFA) vs low fat accumulation (Low-BFA). The High-BFA is reported in the superior part; the Low-BFA is reported in inferior part. The mammographic images are in cranio-caudal and oblique projections. The other parts of the figure represent the central pathogenic mechanisms implied in the worsening of the cardiovascular outcomes: flow-mediated vasodilation of brachial artery (FMD), the endothelial dysfunction and the serum expression of thrombospondin-1 and endothelin-1. The mechanistic link between serum and echographic biomarkers of endothelial dysfunction, breast fat density and MACE is reported by red arrows and black arrows. The black arrow indicates the increase (↑) or reduction (↓) in thrombospondin and endothelin-1 levels and in NO availability, respectively, associated with increased MACE and reduced FMD. NO: nitric oxide; MACE: major adverse cardiac events.

improved interpretability, the clinical relevance at the individual level is modest and may be influenced by residual confounding and measurement variability. Despite these challenges, our findings open new opportunities to reduce sex-based disparities in cardiovascular care, promote targeted preventive strategies, and guide future health policies. Conversely, we acknowledge that hormonal status and menstrual phase can modulate endothelial function and circulating biomarkers, such as the menstrual cycle timing which was not standardized for all premenopausal women. Thus, some degree of intra-group variability may persist. On other hand, our exploratory analysis in the subset of women with available cycle data showed no significant phase-related differences in FMD, ET-1, or TSP-1. These findings are consistent with prior evidence indicating that, although sex hormones influence vascular tone, their short-term variability may have limited impact in large-scale observational settings. Thus, future studies are recommended to incorporate cycle phase control or hormonal profiling to further refine the interpretation of vascular biomarker results in premenopausal cohorts.

In the current study we did not perform experiments as ex vivo study in animal and cellular models to study and reproduce the effects of breast fat accumulation on the endothelial function, and the activation of ED biomarkers via modulation of NO bioavailability and inflammatory distress. Conversely, we did not use specific treatments to study the expression of these molecular and cellular pathways of ED, and their effects on the rate of ED and MACE at follow-up. Furthermore, future research is needed to assess the function of ET-1 and TSP-1 and integrated by imaging-based endothelial function studies. In this context, our study shows the single-center design without site clustering. However, standardized protocols and operator training minimized potential technician-related bias. Conversely, although FMD is a well-established marker of endothelial function, additional measures such as pulse wave velocity (PWV) and coronary flow reserve (CFR) could provide a more comprehensive assessment of ED. In this context, the measurement of carotid–radial PWV, which reflects the vasodilatory response to locally induced ischemia, is a promising non-invasive method for evaluating endothelial function [28]. Indeed, aortic PWV is an indicator of arterial stiffness and is considered a reliable predictor of cardiovascular risk [29]. Notably, although FMD assessment was performed by experienced operators using standardized protocols, the technique remains operator-dependent, and despite good intra- and inter-observer reproducibility (CV 10.5 % and 12.3 %, respectively), residual measurement variability cannot be completely excluded.

Finally, limiting the study population to an Italian cohort could raise important concerns about generalizability, particularly regarding ethnic variations in breast density and cardiovascular risk. Indeed, authors found clinically significant differences in breast density prevalence across racial/ethnic groups, and they showed that Asian women tend to have higher mammographic density than White or African American women, even after adjusting for age and BMI [30,31]. Conversely, while our findings demonstrate a strong association between BFA and adverse cardiovascular outcomes, these results should be interpreted as hypothesis-generating. Indeed, this is limited by the observational nature of our study, where the causal relationships cannot be definitively

established. Again, the study focused on women with age range of 40–55 years, and this restricts generalizability to other populations. These limitations call for prospective, mechanistic, and interventional studies to further explore the causal pathways linking BFA to cardiometabolic risk. However, the study results cannot drive us toward definitive conclusions and must be assessed in future studies.

5. Conclusions

Pre-menopausal women with high BFA showed systemic inflammation, higher ET-1 and TSP-1 levels, and increased rates of ED and MACE over 5 years. High BFA and elevated TSP-1 independently predicted long-term cardiovascular risk, supporting their role as biomarkers for risk stratification in this population. These findings suggest a link between adiposity-related endothelial dysfunction and cardiovascular risk, though the associations remain non-causal and hypothesis-generating.

The central illustration summarizes the main purpose and findings of our investigation, which aimed to evaluate the link between breast fat accumulation and ED in premenopausal women. As shown, women with high breast fat accumulation exhibited higher levels of ET-1 and TSP-1, leading to impaired FMD and increased MACE at 5-year follow-up. Prospective studies are needed to confirm whether ET-1 and TSP-1 can serve as reliable biomarkers of vascular dysfunction and long-term cardiovascular risk in premenopausal women with high BFA.

Submission declaration

This submitted and revised article has not been published previously, is not under consideration for publication elsewhere. All the authors approved the current submission, and if accepted, the article will not be published elsewhere in the same form, in English or in any other language, including electronically, without the written consent of the copyright-holder.

Data availability

the data underlying this article will be shared on reasonable request to the corresponding author.

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Declaration of generative AI in scientific writing

None to declare.

CRediT authorship contribution statement

Celestino Sardu: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Giovanni Francesco Nicoletti:** Conceptualization. **Maria Consiglia Trotta:** Data curation. **Gorizio Pieretti:** Funding acquisition. **Gianluca Gatta:** Methodology. **Roberto Grella:** Methodology. **Nunzia D' Onofrio:** Methodology, Conceptualization. **Maria Luisa Balestrieri:** Project administration, Methodology. **Daniele La Forgia:** Methodology. **Ludovica Marfella:** Methodology. **Salvatore Cappabianca:** Project administration, Methodology. **Domenico Cioffi:** Methodology. **Francesco Iovino:** Methodology. **Giuseppe Signoriello:** Software, Methodology. **Carmine Pizzi:** Methodology. **Michelangela Barbieri:** Methodology. **Giuseppe Paolisso:** Supervision, Methodology, Investigation. **Raffaele Marfella:** Writing – review & editing, Validation, Methodology, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ajpc.2025.101349.

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