

Determination of FABP4, RBP4 and the MMP-9/NGAL complex in the serum of women with breast cancer

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Abstract. Breast cancer is the most common type of cancer in females and is the leading cause of cancer-associated death among women, worldwide. The present study aimed to measure the serum levels of fatty acid-binding protein 4 (FABP4), retinol binding protein 4 (RBP4) and the MMP-9/neutrophil gelatinase-associated lipocalin (NGAL) complex in women diagnosed with breast cancer. Serum levels of the examined proteins were determined in the peripheral blood of patients via ELISA. Furthermore, whether the concentration of each protein was associated with breast cancer growth, molecular subtype, BMI, postmenopausal status, diabetes and the social background of patients was assessed. Women with invasive breast cancer demonstrated significantly higher levels of FABP4 ($P=0.008$). Additionally, considerably elevated FABP4 levels were demonstrated specifically in Luminal breast cancer cases ($P<0.01$). No significant association was recorded between RBP4 and breast cancer development. In addition, significantly lower levels of the MMP-9/NGAL complex were recorded in triple negative/HER-2 cases ($P<0.05$). BMI values appeared to influence the aforementioned associations, while significantly high serum levels of FABP4 and the MMP-9/NGAL complex were found in postmenopausal patients with breast cancer and a BMI ≥ 25 kg/m² ($P<0.05$). In addition, high levels of FABP4 were significantly associated with breast cancer patients with diabetes ($P=0.05$). However, no association was identified between RBP4, the MMP-9/NGAL complex and diabetes. In conclusion, FABP4 can be regarded as a biomarker of breast cancer growth, while both FABP4 and the MMP-9/NGAL complex may provide considerable information regarding the development of specific breast cancer subtypes. FABP4 and

the MMP-9/NGAL complex may also be able to predict the development of breast cancer in postmenopausal patients with obesity.

Introduction

Breast cancer is the most frequent developing malignancy among women with high mortality rates, worldwide (1,2). Epidemiological data revealed that in 2018 more than 2 million women were diagnosed with breast cancer, accounting for more than 600,000 cancer-associated deaths (2-4). Since 1980, the global prevalence of breast cancer provides a consistently positive trend and it has been anticipated that the tendency for the number of incidences to rise will continue in the following years (2-4). Although the 60% of deaths occur in developing countries, the breast cancer mortality rate has been reduced in most developed countries due to the establishment of novel methodologies in screening, diagnosis and cancer therapy (5,6).

Over the last years, several studies have focused on the analysis of numerous biomarkers, in order to improve the early prognosis, diagnosis and the proper breast cancer treatment (7-9). In particular, the effect of fatty acid-binding protein 4 (FABP4) on breast disease gained increased popularity, since high levels of the corresponding protein have been observed in plasma of breast cancer patients, while exogenous FABP4 significantly stimulates the proliferation of breast cancer cells (10-12). FABP4 is a member of fatty acid-binding proteins family (FABPs) which is implicated in the intracellular transportation of fatty acids in several cellular organelles (12). FABP4 is constantly expressed in macrophage and adipocyte, while it is involved in numerous cellular functions, including fatty acid uptake and storage as well as in regulation of gene expression, cell proliferation and differentiation (12,13). In macrophages FABP4 regulates inflammatory responses via activation of the NF- κ B (nuclear factor κ B) and JNK pathways (c-Jun N-terminal kinase), while in adipocytes FABP4 stimulates lipolysis and constrains lipogenesis through interaction with HSL (hormone-sensitive lipase) and PPAR γ (peroxisome proliferator-activated receptor gamma) (14-16). In regard to breast cancer development FABP4 is highly expressed in a small subgroup of tumor associated macrophages (TAMs). FABP4-positive TAM subgroup in turn assembles in the late

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stage of mammary tumor, stimulating tumor growth, (17). An alternative pathway implicates adipocytes in cases of obesity. In particular, during obesity adipocytes release FABP4 which triggers oncogenic signaling factors, including IL-6, STAT3 and ALDH1 in breast mammary tumor cells, leading to tumor progression (14,16-18).

Recent analyses have focused on the implication of retinol binding protein 4 (RBP4) in breast cancer development, as well. RBP4 is an adipokine and member of the lipocalin family of proteins (19,20). Lipocalin protein family consist of a heterogeneous group of extracellular proteins, expressed in liver and adipose tissue and they facilitate the transferring of small hydrophobic molecules, including hormones, prostaglandins, arachidonic acids and retinoids, while it is the major transporter for retinol acid (Vitamin A) (20,21). RBP4 along with retinol acid (Vitamin A) stimulate STRA6 that in turn recruit and activate Janus kinase and the transcriptional factors STAT3 or STAT5 (22). Alternatively, RBP4 alone can activate pro-inflammatory responses through JNK1, JNK2 or Toll-like receptors (23). The expression of RBP4 is associated with insulin resistance as well as with cardiovascular risk markers, including body mass index [BMI; calculated as weight (kg) divided by height (m²)], waist to hip ratio and the levels of triglyceride in serum (21). Furthermore, RBP4 is correlated with numerous types of cancer, such as prostate cancer, colon adenoma, ovarian cancer and oral cancer (24-26), but its association with breast cancer growth remains rather vague (27,28).

Finally, neutrophil gelatinase-associated lipocalin (NGAL) is a member of the lipocalin family and it has been related with breast disease, as well (20,29). NGAL is a fundamental component of cytoplasmic granules of human neutrophils and it contributes to the chelation of bacterial siderophores, thus inhibiting iron availability and consequently it prevents bacteria to establish infection (30,31). Moreover, NGAL act as siderocalin and with sideropores and ferrous iron (Fe⁺⁺) contribute to iron uptake that is necessary in regulating the iron-dependent growth pathways (32). Cancer cells have increased need for intracellular iron, while iron uptake is pivotal in regulating cell proliferation, invasion and metastasis (32,33). NGAL expression is regarded as a considerable diagnostic and prognostic biomarker in several diseases, including inflammation and tumor growth (29,31,34). It is noteworthy that NGAL generates a complex with metalloproteinase-9 (MMP-9) that constraints MMP-9 autodegradation and subsequently leads to increased activity of MMP-9 (31,34-36). Previous studies have suggested that MMP-9/NGAL complex augments cancer growth, including breast cancer (29,31,34-36). MMP-9 is found in gelatinase granules and contribute to cancer progression, invasion and metastasis in numerous neoplastic diseases (29,37,38). Notably, a previous analysis in Greek population demonstrated that high levels of MMP-9, NGAL as well as increased levels of MMP-9/NGAL complex are associated with breast disease (26).

Nowadays, no further analyses have been conducted in order to better evaluate the influence of MMP-9/NGAL complex on the development of breast malignancy, while little is known concerning the impact of FABP4 and RBP4 on patients' susceptibility to the development of breast cancer in Greek women. Towards this end, the present study focused on the relationship of serum levels of FABP4, RBP4,

MMP-9/NGAL complex with the growth of breast cancer in the Greek population. Moreover, we examined the association of FABP4, RBP4, MMP-9/NGAL complex with the different breast cancer molecular subtypes, body mass index (BMI), diabetes, menopausal status and the social background of patients. Our goal was to elucidate whether the examined proteins are implicated in breast cancer development as well as to further investigate whether additional factors may influence this association, providing valuable information concerning the prognosis and diagnosis of breast cancer.

Materials and methods

Breast samples. In the present analysis a total of 73 women were examined. In particular, 53 women were diagnosed with breast cancer and they were further classified into different molecular types according to their immunohistochemistry (IHC) profile concerning ER (estrogen receptor), PR (progesterone receptor), HER-2 (human epidermal growth factor receptor 2) and ki67 (39,40). As a result, patients were characterized as Luminal A (n=22), Luminal B (n=8), Triple Negative-TN: ER negative, PR negative, HER-2 negative (n=15) and HER-2 positive: ER negative, PR negative, HER-2 positive (n=8). According to histopathological criteria all breast cancer cases were characterized as invasive ductal carcinoma (IDC). Moreover, the control group comprised 20 healthy women. The mean age of the examined patients and controls was 62.8±14.12 and 58.7±7.9 years, respectively, while the mean age in Luminal 64±12 years old and the mean age in TN/HER-2 group was 61±14.12 years old. In addition, 44 patients were postmenopausal and 9 were premenopausal, while 45 breast cancer patients were diagnosed with diabetes, as well. Finally, the mean BMI value of breast cancer cases was 28.1±6 kg/m², while the mean BMI value of healthy women was 27.7±4.2 kg/m². Clinical samples were collected from Anticancer Oncology Hospital of Athens 'Saint Savvas' between May 2017 and July 2018. All patients signed an informed consent form, while the study was approved from the Research Committee of the Hospital.

Sample preparation. Venous blood was collected from patients and controls between 12:00 a.m. and 14:00 p.m. Sampling was performed directly into serum vacuum tubes with clot activator, while prior to centrifugation, tubes remained at room temperature for 20-30 min to enable blood clotting. Subsequently, the samples were centrifuged at 3,000 x g for 15 min at 8°C, the serum was isolated and divided into aliquots parts, while all serums were stored at -80°C for further use.

ELISA assay for the measurement of serum levels of FABP4, RBP4 and MMP-9/NGAL complex. The evaluation of serum levels of FABP4, RBP4 and MMP-9/NGAL complex in the examined clinical samples was conducted for each individual protein in duplicates through enzyme-linked immunosorbent assay (ELISA), using the respective Human FABP4, RBP4 and MMP-9/NGAL Quantikine® ELISA kit (R&D Systems), according to the manufacturers instruction. Finally, the acquired fluorescence data were analyzed using Multiscan™ FC Microplate Photometer (Thermo Fisher Scientific, Inc.).

Statistical analysis. Data are presented with mean, standard deviation and median, while statistical analysis was conducted considering whether they followed a normal distribution or not, respectively. Normality was examined through D'Agostino - Pearson, Shapiro-Wilk or Kolmogorov-Smirnov tests. In particular, FABP4 data follow a non normal distribution, whereas RBP4 and MMP-9/NGAL complex data follow a normal distribution. Student's t-test or Mann-Whitney non-parametric test were used to examine the association of the respective proteins with breast cancer development, BMI, menopausal status, diabetes and social background of patients. One way Anova was used for statistical analysis of the expression of RBP4, MMP-9/NGAL in Luminal, TN/HER-2 cancers and controls, followed by the Tukey post hoc test for pair-wise comparison, while Kruskal-Wallis non parametric test was performed for statistical analysis of the expression of FABP4 in the respective breast cancer molecular subtypes, followed by the Dunn multiple comparison post hoc test. P values were regarded as statistically significant at the 0.05 cut off level. All analyses were carried out with the GraphPad Prism 6 (GraphPad Software, Inc.).

Results

FABP4, RBP4, MMP9/NGAL complex and breast cancer. In the present analysis the serum levels of FABP4, RBP4 and MMP9/NGAL complex were measured in a total of 73 women, 53 of which have been diagnosed with breast cancer, while 20 cases were used as control group. The differences that occur at protein levels between patients and controls were evaluated, in order to investigate whether the concentrations of FABP4, RBP4 and MMP9/NGAL complex are related with breast cancer in the studied population. The mean and median serum concentrations of the respective proteins are presented in Table I. According to our results it was demonstrated that the median concentration of FABP4 in breast cancer cases was significantly higher than that of healthy women (median concentration; 27,740 pg/ml vs. 22,115 pg/ml, $P=0.0078$) (Tables I and II, Fig. 1). However, no considerable association was recorded between cancer cases and controls, regarding the serum levels of RBP4 and MMP9/NGAL complex, respectively (Tables I and II; Fig. 1).

FABP4, RBP4, MMP9/NGAL complex and molecular subtypes. The relationship of the corresponding proteins with the development of breast disease was further examined considering the molecular classification of breast cancer into different molecular subtypes (34,35). Hence, the examined cases were classified into two major groups, containing the Luminal cohort (Luminal A and Luminal B) and TN/HER-2 group (HER-2 positive and Triple Negative). In regard to FABP4 serum levels, it was demonstrated that the median concentration of the respective protein in Luminal cases was considerably higher than that of healthy women (median concentration; 37,105 pg/ml vs. 22,115 pg/ml, $P=0.0002$), while high levels of FABP4 were recorded in Luminal group when compared with that of TN/HER-2 group (median concentration; 37,105 pg/ml vs. 25,040 pg/ml, $P=0.0073$) (Tables I and II; Fig. 2). Although the median concentration of FABP4 in TN/HER-2 cluster was found to be increased compared to healthy women, the difference was not considered as statistically significant ($P=ns$). In

addition, no significant association was recorded between the groups of breast cancer cases and the serum levels of RBP4 protein (Tables I and II, Fig. 2).

Interestingly, differences in serum levels of MMP9/NGAL complex were found between breast cancer subtypes. In particular, slightly elevated levels of MMP9/NGAL complex were recorded in Luminal cohort compared to control group, but this association did not reach the limits of statistical significance (mean concentration-ng/ml; 50.97 ± 4.191 vs. 47.96 ± 7.566 , $P=ns$) (Tables I and II). In contrast, the serum levels of MMP-9/NGAL complex were found to be substantially decreased in TN/HER-2 group. In particular, the mean concentration of MMP-9/NGAL complex in TN/HER-2 group was considerably lower than that of control group (mean concentration-ng/ml; 28.59 ± 3.78 vs. 47.96 ± 7.566 , $P=0.03$) and Luminal cohort, as well (mean concentration-ng/ml; 28.59 ± 3.78 vs. 50.97 ± 4.191 , $P=0.0002$) (Tables I and II; Fig. 2).

FABP4, RBP4, MMP9/NGAL complex and BMI. A stratified analysis according to body mass index (BMI) values was performed in order to evaluate whether the BMI in combination with serum levels of FABP4, RBP4 and MMP9/NGAL complex, may be used as valuable biomarkers for the development of breast disease. In particular, breast cancer cases and controls were classified into two groups, comprising specimens with BMI values ≥ 25 kg/m² and specimens with BMI values < 25 kg/m² (Tables III and IV). Our results indicate that in clinical samples with BMI ≥ 25 kg/m² the median concentration of FABP4 was significantly higher when compared with that of healthy women (median concentration 37,180 pg/ml vs. 24,445 pg/ml, $P=0.0023$) (Tables III and IV). In contrast, in samples with BMI < 25 kg/m² no significant alterations were detected in FABP4 levels between cancer and control cases. Moreover, no significant differences were recorded in RBP4 and MMP9/NGAL serum levels between cancer cases and controls, neither when BMI ≥ 25 kg/m² nor when BMI < 25 kg/m². Merging outcomes derived from BMI clusters and the different cancer groups it was revealed that when BMI ≥ 25 kg/m² the median concentration of FABP4 was significantly augmented in Luminal cases compared either to TN/HER-2 (median concentration 45,595 pg/ml vs. 22,960 pg/ml, $P=0.018$) or healthy women (median concentration 45,595 pg/ml vs. 24,445 pg/ml, $P=0.0009$) (Tables III and IV). However, no considerable changes in FABP4 levels were detected in cases with BMI lower than 25 kg/m². In regard to RBP4 serum levels, no significant associations were identified between the concentration of the respective protein and BMI groups within the different molecular subtypes of breast disease (Tables III and IV). Finally, considering results derived from MMP9/NGAL complex, it was observed that in TN/HER-2 cases with BMI ≥ 25 kg/m² the mean concentration of the respective complex was significantly lower than that of Luminal samples (mean concentration-ng/ml; 30.59 ± 6.45 vs. 50.83 ± 3.7 , $P=0.014$) (Tables III and IV). Nevertheless, no further associations were found between the concentration of MMP9/NGAL complex and BMI cohorts within the divergent subtypes of breast malignancy (Tables III and IV).

FABP4, RBP4, MMP9/NGAL complex, menopausal status, diabetes and social background. Finally, we further evaluate

Table I. Mean and median concentrations of FABP4, RBP4 and MMP-9/NGAL complex in the serum of the examined patients.

Group	FABP4 (pg/ml), mean \pm SD	FABP4 (ng/ml), median	RBP4 (pg/ml), mean \pm SD	RBP4 (ng/ml), median	MMP-9/NGAL (pg/ml), mean \pm SD	MMP-9/NGAL (ng/ml), median
Breast cancer (n=53)	38,324 \pm 3,250	27,740	27,368 \pm 1,274	26,650	41.26 \pm 3.24	37.89
Control (n=20)	23,193 \pm 1,462	22,115	30,138 \pm 1,765	27,705	47.96 \pm 7.56	40.23
Luminal (n=30)	45,298 \pm 4,659	37,105	26,755 \pm 1,278	26,605	50.97 \pm 4.19	53.21
TN/HER-2 (n=23)	29,227 \pm 3,684	25,040	28,167 \pm 2,445	27,200	28.59 \pm 3.78	24.48
Control (n=20)	23,193 \pm 1,462	22,115	30,138 \pm 1,765	27,705	47.96 \pm 7.56	40.23

FABP4, fatty acid-binding protein 4; RBP4, retinol binding protein 4; NGAL, neutrophil gelatinase-associated lipocalin; TN, triple negative.

Table II. P-values were calculated to estimate the differences in protein levels between patients with breast cancer and controls, and among different breast cancer subtypes.

Groups	P-values		
	FABP4	RBP4	MMP-9/ NGAL
Breast cancer vs. control	0.008	0.260	0.420
Luminal vs. control	<0.001	0.130	0.700
TN/HER-2 vs. control	0.420	0.320	0.030
Luminal vs. TN/HER-2	0.007	0.600	<0.001

FABP4, fatty acid-binding protein 4; RBP4, retinol binding protein 4; NGAL, neutrophil gelatinase-associated lipocalin; TN, triple negative.

the relationship of the tested serum proteins with menopausal status, diabetes, and social background of the examined patients including smoking and exercise. Considering our results, it was observed that serum FABP4 levels are significantly increased in postmenopausal breast cancer patients when compared with that of premenopausal patients (median concentration 32,720 pg/ml vs. 14,550 pg/ml, $P=0.0005$) (Table V). In addition, in the cohort of postmenopausal patients the concentration of FABP4 protein is significantly higher in individuals with BMI ≥ 25 kg/m² than patients with BMI < 25 kg/m² (median concentration 40,050 pg/ml vs. 24,610 pg/ml, $P=0.03$) (Table VI). In addition, the link of FABP4 with diabetes reached the limits of statistical significance, as the serum FABP4 concentration was found to be higher in breast cancer patients with diabetes when compared with that of patients with no diabetes (median concentration 54,310 pg/ml vs. 27,460 pg/ml, $P=0.05$) (Table VII).

Although the serum levels of MMP9/NGAL complex are not modified considering the menopausal status of patients, it was observed that MMP9/NGAL complex in postmenopausal individuals is significantly higher in patients with BMI ≥ 25 kg/m² than patients with BMI < 25 kg/m² (mean concentration ng/ml; 46.21 \pm 3.51 vs. 30.34 \pm 5.15, $P=0.016$) (Table VI). Moreover, no significant association was recorded between MMP9/NGAL complex and diabetes in breast cancer patients as well as no significant association was detected regarding RBP4 serum levels either with menopausal status or diabetes (Table VII). Furthermore, it was revealed that smoking and exercise do not influence the serum levels of FABP4, RBP4 and MMP9/NGAL in breast cancer patients.

Discussion

The stratification of breast cancer severity lies on numerous factors, including TNM stage, tumor grade, lymphatic invasion as well as the expression of the hormonal receptors ER, PR and HER-2 (39,40). Although these factors offer crucial information concerning the risk profile of patients and they significantly contribute to select the most suitable therapeutic approach (41,42), today, there is a growing interest in

Table III. Mean and median concentrations of FABP4, RBP4 and MMP-9/NGAL complex in the serum of patients separated according to BMI values.

A, BMI ≥ 25 kg/m ²						
Cancer subtype	FABP4 (pg/ml), mean \pm SD	FABP4 (pg/ml), median	RBP4 (pg/ml), mean \pm SD	RBP4 (pg/ml), median	MMP-9/NGAL (pg/ml), mean \pm SD	MMP-9/NGAL (pg/ml), median
Breast cancer (n=33)	42,509 \pm 4,378	37,180	25,846 \pm 1,203	25,900	44.09 \pm 3.65	45.11
Luminal (n=22)	51,005 \pm 5,442	45,595	26,571 \pm 1,391	26,090	50.83 \pm 3.75	53.21
TN/HER-2 (n=11)	25,516 \pm 4,038	22,960	24,396 \pm 2,332	23,050	30.59 \pm 6.44	31.69
Control (n=14)	25,345 \pm 1,728	24,445	30,368 \pm 2,081	27,885	52.92 \pm 9.88	40.23
B, BMI < 25 kg/m ²						
Cancer subtype	FABP4 (pg/ml), mean \pm SD	FABP4 (pg/ml), median	RBP4 (pg/ml), mean \pm SD	RBP4 (pg/ml), median	MMP-9/NGAL (pg/ml), mean \pm SD	MMP-9/NGAL (pg/ml), median
Breast cancer (n=33)	31,419 \pm 4,396	26,050	29,878 \pm 2,686	28,875	36.59 \pm 6.13	25.5
Luminal (n=30)	29,603 \pm 6,701	28,555	27,262 \pm 3,058	31,560	51.36 \pm 12.53	42.9
TN/HER-2 (n=23)	32,629 \pm 6,021	25,595	31,623 \pm 4,014	27,600	26.75 \pm 4.42	24.05
Control (n=20)	18,172 \pm 1,331	18,185	29,602 \pm 3,618	26,015	36.37 \pm 9.72	40.13

Patients were divided into two groups according to BMI: ≥ 25 and < 25 kg/m². FABP4, fatty acid-binding protein 4; RBP4, retinol binding protein 4; NGAL, neutrophil gelatinase-associated lipocalin; TN, triple negative; BMI, body mass index.

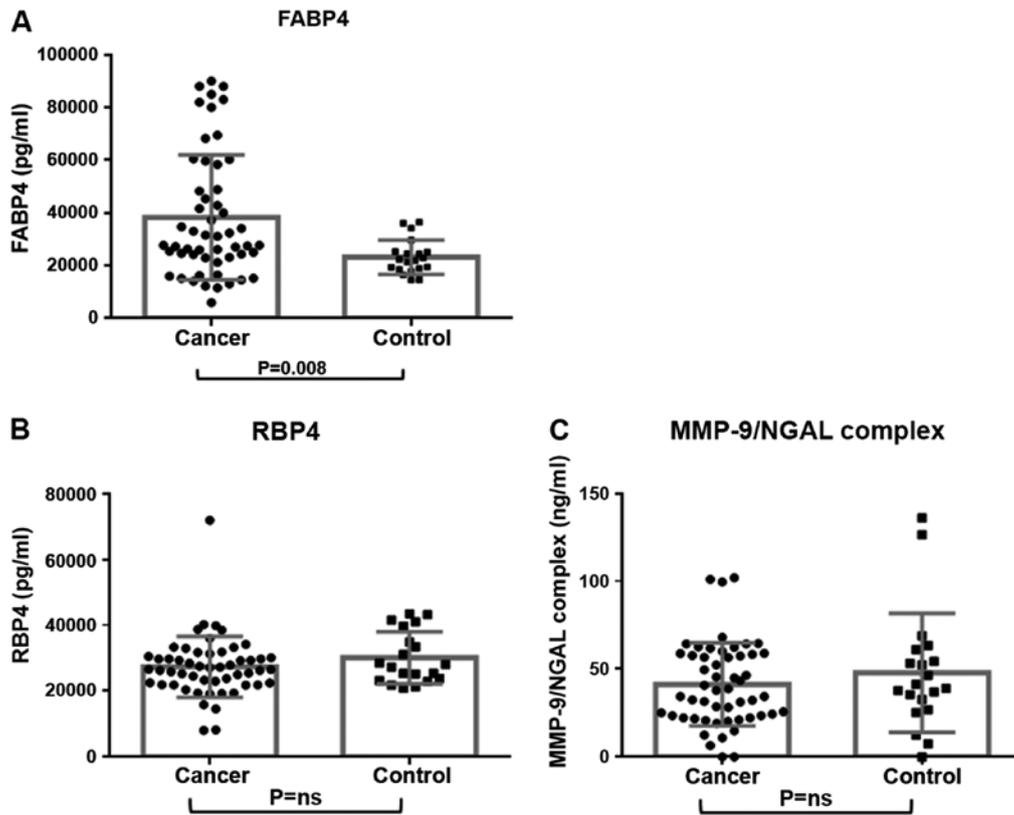


Figure 1. Association of breast cancer cases or control group patients and the expression of FABP4, RBP4 and MMP-9/NGAL. The concentration of the respective proteins are demonstrated, with bars indicating the mean with 95% confidence interval. Serum concentrations of (A) FABP4, (B) RBP4 (C) MMP-9/NGAL were detected. FABP4, fatty acid-binding protein 4; RBP4, retinol binding protein 4; NGAL, neutrophil gelatinase-associated lipocalin.

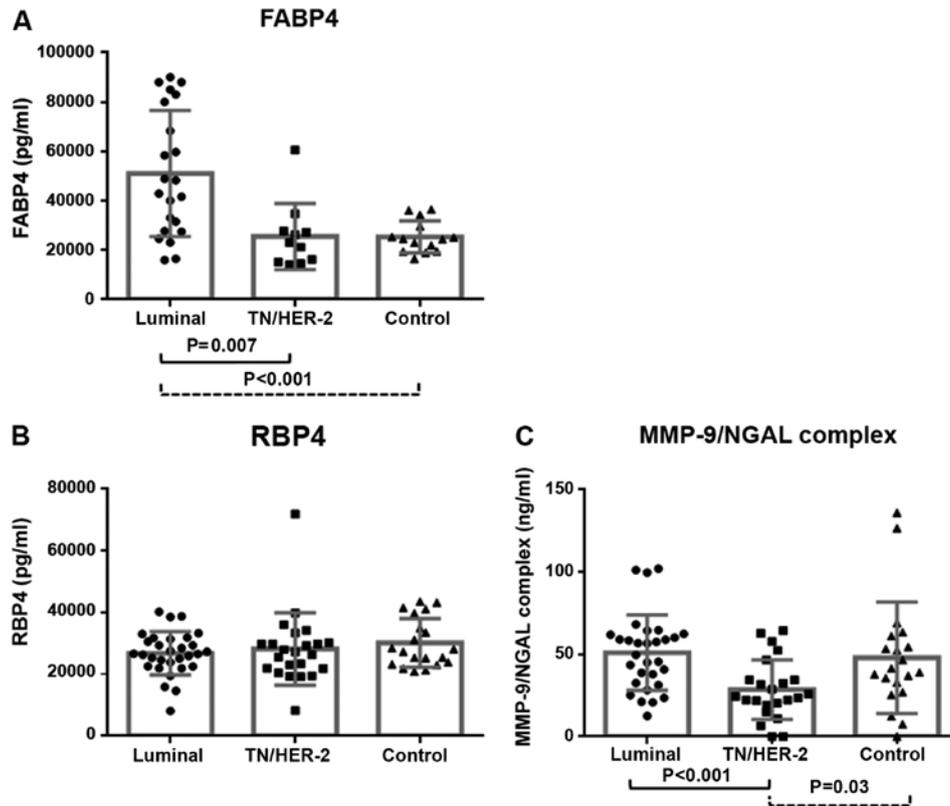


Figure 2. Association between breast cancer subtypes or control group patients and the expression of FABP4, RBP4 and MMP-9/NGAL. The concentration of the respective proteins are demonstrated, with bars indicating the mean with 95% confidence interval. Serum concentrations of (A) FABP4, (B) RBP4 and (C) the MMP-9/NGAL complex were detected. FABP4, fatty acid-binding protein 4; RBP4, retinol binding protein 4; NGAL, neutrophil gelatinase-associated lipocalin.

Table IV. P-values were calculated to assess the differences in protein levels between patients and controls, and among the different breast cancer subtypes.

A, BMI ≥25 kg/m ²			
Groups	P-values		
	FABP4	RBP4	MMP-9/ NGAL
Breast cancer vs. control	0.002	0.070	0.300
Luminal vs. control	<0.001	0.100	0.800
TN/HER-2 vs. control	0.540	0.069	0.070
Luminal vs. TN/HER-2	0.018	0.430	0.014

B, BMI <25 kg/m ²			
Groups	P-values		
	FABP4	RBP4	MMP-9/ NGAL
Breast cancer vs. control	0.055	0.900	0.900
Luminal vs. control	0.120	0.600	0.362
TN/HER-2 vs. control	0.061	0.700	0.400
Luminal vs. TN/HER-2	0.900	0.250	0.090

FABP4, fatty acid-binding protein 4; RBP4, retinol binding protein 4; NGAL, neutrophil gelatinase-associated lipocalin; TN, triple negative; BMI, body mass index.

the establishment of novel biomarkers that will enable the accurate prognosis and diagnosis of breast disease (8,9,41,42). Hence, the present analysis focused on the study of serum FABP4, RBP4 and MMP9/NGAL complex levels in women diagnosed with breast cancer along with healthy women, while results were further associated with the different breast cancer molecular subtypes, BMI, menopause status diabetes and social background of patients.

In the present study differential serum levels of FABP4, RBP4 and MMP9/NGAL complex were detected in breast cancer cases, while different serum levels of the corresponding proteins were observed between the different molecular subtypes of breast disease, as well. In particular, increased serum concentration of FABP4 was detected in the examined breast cancer cases compared to control group, supporting previous findings (10-12,17,43,44). Although substantially increased serum FABP4 levels were recorded in Luminal breast cancer samples, the concentration of the respective protein was found to be decreased in TN/HER-2 cases. These results imply that FABP4 may be a key element in the development of specific breast cancer molecular subtype and that each subtype uses different metabolic pathways. Indeed, receptor positive breast cancers are associated with gene signature involved in de novo lipogenesis, fat acid mobilization and oxidation while TNBC are associated with genes expressed in exogenous lipid uptake (45). This may support the hypothesis

Table V. Mean and median concentrations of FABP4, RBP4 and MMP-9/NGAL complex in the serum of patients with breast cancer separated according to menopausal status.

Menopause status	FABP4			RBP4			MMP-9/NGAL		
	Mean ± SD (pg/ml)	Median (pg/ml)	P-value	Mean ± SD (pg/ml)	Median (pg/ml)	P-value	Mean ± SD (pg/ml)	Median (pg/ml)	P-value
Postmenopause (n=44)	42,446±3,558	32,720	<0.001	28,069±1,474	27,435	0.211	41,52±3.08	39.76	0.900
Premenopause (n=9)	18,172±3,259	14,550		23,941±1,802	24,550		39,97±12.39	22.29	

FABP4, fatty acid-binding protein 4; RBP4, retinol binding protein 4; NGAL, neutrophil gelatinase-associated lipocalin.

Table VI. Mean and median concentrations of FABP4, RBP4 and MMP-9/NGAL complex in the serum of postmenopausal women with breast cancer, divided according to BMI values.

Postmenopause	FABP4			RBP4			MMP-9/NGAL		
	Mean \pm SD (pg/ml)	Median (pg/ml)	P-value	Mean \pm SD (pg/ml)	Median (pg/ml)	P-value	Mean \pm SD (pg/ml)	Median (pg/ml)	P-value
BMI \geq 25 kg/m ² (n=31)	46,802 \pm 4471	40,050	0.030	27,334 \pm 1,320	27,590	0.440	46.21 \pm 3.52	45.56	0.016
BMI $<$ 25 kg/m ² (n=13)	32,058 \pm 7556	24,610		29,821 \pm 3,949	25,900		30.34 \pm 5.15	25.17	

FABP4, fatty acid-binding protein 4; RBP4, retinol binding protein 4; NGAL, neutrophil gelatinase-associated lipocalin; BMI, body mass index.

Table VII. Mean and median concentrations of FABP4, RBP4 and MMP-9/NGAL complex in the serum of patients with breast cancer, separated according to diabetes.

Diabetes	FABP4			RBP4			MMP-9/NGAL		
	Mean \pm SD (pg/ml)	Median (pg/ml)	P-value	Mean \pm SD (pg/ml)	Median (pg/ml)	P-value	Mean \pm SD (pg/ml)	Median (pg/ml)	P-value
yes (n=45)	51,184 \pm 7,827	54,310	0.050	32,323 \pm 6,011	26,605	0.100	31.71 \pm 5.64	27.90	0.200
no (n=8)	36,083 \pm 3,491	27,460		26,487 \pm 1,058	27,500		42.96 \pm 3.65	40.77	

FABP4, fatty acid-binding protein 4; RBP4, retinol binding protein 4; NGAL, neutrophil gelatinase-associated lipocalin.

that specific metabolic lipid pathways are probably accelerated or moderated depending on tumor ER and HER-2 status. Interestingly, a previous study concerning the tissue analysis of breast tumors showed that the concentration of FABP4 was higher in HER-2 tumors and lower in Luminal A tumors, suggesting that this discrepancy lies mainly on differences in lipid metabolic pathways that have been observed in the different breast cancer subtypes (43,46). Taking all these data into account it was anticipated that the different serum FABP4 levels that were detected in Luminal and TN/HER-2 breast cancers might be observed due to a possible stronger association between lipid metabolic status and breast cancer subtypes and therefore different levels of FABP4 might be circulating in the serum of patients. However, further analyses are required in order to confirm this hypothesis.

In regard to RBP4, previous research studies have recommended that RBP4 is not associated with breast cancer development, while the expression of RBP4 gene seems to be down regulated in breast cancer cases (27,47). In contrast, a more recent study revealed for the first time that increased serum levels of RBP4 are related with breast cancer in menopausal women (28). In the present analysis no significant association between the serum levels of RBP4 and the development of breast cancer was observed in the tested patients (27,47). Interestingly, a previous analysis by Formelli *et al* (27) suggested that low plasma RBP4 levels are found in postmenopausal women (≥ 55 years old) and these patients exhibited poor prognosis of breast disease. In the present analysis, no significant association was recorded between serum levels of RBP4 and the development of breast cancer, while no significant relationship was described between RBP4 and menopausal status of the examined breast cancer patients.

Nowadays, little is known concerning the impact of MMP-9/NGAL complex on breast disease. A previous analysis derived by our group observed for the first time significantly increased serum levels of MMP-9/NGAL complex in breast cancer cases (29). In contrast to previous findings, we observed low levels of MMP-9/NGAL complex in breast cancer cases compared to healthy women but this association did not reach the limits of statistical significance. It is important to underline that significantly lower levels of MMP-9/NGAL complex were found in TN/HER-2 group compared to either healthy women or Luminal breast cancers ($P < 0.05$). One possible explanation of the contradictory results could be the age group of the examined population, since in the previous study the mean age of the tested women was 52.8 years old, while in the present analysis the mean age of the examined patients was 62.8 years old (29). As a result, the age of the tested population may influence the MMP-9/NGAL complex levels (48,49). An additional explanation could be the molecular subtype of breast cancer cases. Notably, in the previous analysis by our group the breast lesions were classified into four groups including sclerosis adenosis, atypical ductal hyperplasia, ductal carcinoma *in situ* and invasive carcinoma, while there was no available data concerning the molecular subtype of breast cancer (29). As a consequence the composition of breast cancer cases in TN/HER-2 subtype may influence the serum levels of MMP-9/NGAL complex and consequently might affect the prognostic value of MMP-9/NGAL complex in developing

breast cancer. As a consequence, more analyses are needed in order to examine whether low serum MMP-9/NGAL complex levels might indicate the development of TN/HER-2 breast cancer in the examined women.

Finally, in order to assess the impact of obesity on the described associations, a stratified analysis based on BMI values and the serum levels of the corresponding proteins was carried out. According to our results it was revealed that FABP4 in patients with high BMI values ($\text{BMI} \geq 25 \text{ kg/m}^2$) is considerably increased, supporting previous findings (11). Moreover, patients diagnosed with Luminal breast cancer and $\text{BMI} \geq 25 \text{ kg/m}^2$ exhibited significantly higher levels of FABP4 ($P < 0.02$). In addition, the serum levels of RBP4, MMP-9/NGAL complex are not influenced by BMI value, supporting previous studies (28,29). However the concentration of MMP-9/NGAL complex was found to be significantly decreased in patients with TN/HER-2 breast cancer and $\text{BMI} \geq 25 \text{ kg/m}^2$ when compared to patients with Luminal subtype. It is significant to highlight that in patients with $\text{BMI} < 25 \text{ kg/m}^2$ the previously described associations did not reach the limits of statistical significance. Taking all these data into consideration it was suggested that BMI values regulate the described associations and thus BMI should be taken into consideration in order to draw more accurate conclusions concerning the prognosis of breast disease.

In addition, when protein serum levels were considered together with menopausal status, BMI and diabetes, it was observed that FABP4 concentration is significantly increased in postmenopausal patients with high BMI values as well as in breast cancer patients with diabetes, supporting previous findings (11,17,50). Although FABP4 and estrogens have been associated with obesity and obesity-associated breast cancer development, a recent analysis proved that FABP4 and estrogens have an independent impact on obesity related breast cancer growth (51). Interestingly, MMP9/NGAL complex was significantly associated in breast cancer development in postmenopausal women. As it was described above no considerable association was found between MMP9/NGAL complex and breast cancer development. However, this association reaches the limits of statistical significance in postmenopausal women with $\text{BMI} \geq 25 \text{ kg/m}^2$. As a consequence, further analyses are required in order to confirm these outcomes as well as to examine whether MMP9/NGAL complex in association with estrogens contribute to the growth of breast cancer. To the best of our knowledge this is the first study that implicates MMP9/NGAL complex to the development of breast cancer in postmenopausal women.

Our results imply that there is a bidirectional interaction between circulating FABP4 and ER/PR receptors in tumor micro-environment. The relationship between lipid metabolism and breast cancer molecular subtypes varies considerably regarding menopausal status, BMI, presence of diabetes. Adipocytes and secreted fatty acid proteins induce resistance against to hormonal therapy, HER-2 targeting agents and radiotherapy (52). From a clinical perspective, the therapeutic interventions targeting multiple molecular pathways that are implicated in tumor establishment and progression could be more effective. That is to say the specific blockade of FABP4 may improve the efficacy of endocrine therapies and moderate resistance. Further studies should be conducted in order to better evaluate the complexity

of lipid metabolism and the impact of obesity-related proteins as a diagnostic, prognostic and therapeutic tool in specific group of patients. This study has some limitations. The number of participants was small, while all specimens were collected from a single institute. As a consequence, further analyses are required in a larger population scale derived from different tertiary care Greek hospitals in order to reinforce our findings.

In conclusion, our results support the initial recommendation that FABP4 can be regarded as a powerful biomarker of breast cancer development, while its strong association with obesity indicates that FABP4 is a key therapeutic target for treatment of obesity-related breast cancer (17). To the best of our knowledge this is the first study that describes the association of serum FABP4 and MMP-9/NGAL levels in Luminal and TN/HER-2 breast cancer subtypes, respectively, suggesting that the measurement of these two factors may provide significant information concerning not only breast cancer development but also the growth of specific breast cancer subtype, in order to better evaluate the interaction of the respective circulating proteins in specific group of patients (postmenopausal, obese, diabetes). The present analysis anticipated that the metabolic profile of breast cancer subtype, the menopausal status and BMI of the examined women should be taken into consideration in order to select the most suitable biomarker, offering crucial information regarding the personalized prognosis, diagnosis and the proper therapy of breast disease.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on request.

Author's contribution

GB, EZ and FZ designed the present study. DB collected and analyzed samples, and interpreted results. GB, DT and EK analyzed and interpreted the data. DT and GB wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients provided their written informed consent prior to enrolment. The current study was approved by the 'Saint Savvas' General Anticancer Hospital Ethics Committee (approval no. 1617017278).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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