



Adipocyte of Obese Breast Cancer Patients Is Characterized by The Overexpression of Caveolin-1 Protein/Mediator the Main Constituent of the Plasma Membrane Vesicles Caveolae That Contain Proteins Contribute to Breast Cancer Progression

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ABSTRACT

Breast cancer (BC) is the second leading mortality cause due to poor survival rates compared to lung cancer all over the world. Recently, lifestyle increased obesity among the population globally. Since, the adipose tissues (AT) are the major contributor to the volume of the breast and adipocytes cells, which constitute AT are one of the major prominent cells play an effective role in cancer progression via releasing different mediators and adipokines. Thus, AT may display a crucial role in BC progression, especially in obese patients compared to non-obese patients, which characterized by increased AT. Interestingly, adipocytes are characterized by expressing caveolin-1 (Cav-1) protein. Cav-1 constitutes the lipid raft of caveola which contains different proteolytic enzymes inducing cancer metastasis. In this regard, the aim of the present study was to explore the level of expression of Cav-1 protein in the tissue specimen of 5 non-obese vs. 15 obese patients using immunohistochemistry (IHC) and immunoblotting techniques. Our finding demonstrates that the level of Cav-1 expression was statistically significantly low in non-obese compared to obese BC patients ($p < 0.05$). Herein, our results revealed that the highest expression of Cav-1 in obese patients compared to non-obese (control) patients can be considered as a biomarker for BC patients.

INTRODUCTION

Women with breast cancer (BC) remains the second lethal form of cancer disrupts the female's lives over the world after lung cancer (Torre, Bray, Siegel, & Ferlay, 2015; Yedjou et al., 2019). BC affects more than one woman in ten globally (Yedjou et al., 2019) resulted in nearly over 2 million cases of BC annually (MOKBEL & MOKBEL, 2019).

In Egypt, 32% of all newly diagnosed cancer among women in BC with a high mortality rate represents 15% of worldwide deaths per year (Ibrahim, Khaled, Mikhail, Baraka, & Kamel, 2014; Yehia Ibrahim, 2019).

Molecular subtypes of BC are essential for patient diagnosis, treatment decision and patient outcomes (Bouchal et al., 2019). BC are categorized into four molecular subtypes by three cell membrane markers; estrogen receptor (ER), progesterone receptor (PR), as well as the human epidermal growth factor receptor (HER2⁻) that is crucial for overall survival (OS) prediction (Nielsen et al., 2004; Shuch, Brian; Linehan, B. W. M.L.; Srivasan, 2012). The molecular intrinsic subtypes include luminal A (ER⁺ and/or PR⁺ and HER2⁻), luminal B (ER⁺ and/or PR⁺ and HER2⁺), HER2⁺ (ER⁻, PR⁻ and HER2⁺), and triple-negative (ER⁻, PR⁻ and HER2⁻).

The increase of fast food and non-healthy diets are considered threats for increasing obesity among Egyptian women and worldwide (Almuhanna, Alsaif, Alsaadi, & Almajwal, 2014) that responsible for developing various tumor forms, including breast cancer and colon cancer (Chu et al., 2019; Tiwari et al., 2019). Several studies have found that obesity can be considered as a predictor for poor prognosis in pre- and postmenopausal BC women (Chan, Sc, Norat, & Ph, 2015; Tiwari et al., 2019).

Obesity is identified as over fat accumulation in the adipose tissues (AT) which constitute mainly of cells known as adipocytes (Engin, Engin, & Gonul, 2019). Any dysfunction in AT physiological role can promote excess weight (Chu et al., 2019), which in turn affects BC by releasing certain bioactive proteins collectively termed adipokines that contribute to proliferation and migration of cancer

cells that influence the poor prognosis in patients with BC (Guero-Millo, 2004).

Caveolin-1(Cav-1) protein is the primary component of the caveola (lipid raft) membrane (Chang et al., 2017; Nouh et al., 2011). Cav-1 belongs to a group of Cav-2 and Cav-3 caveolins (Cohen, Hnasko, Schubert, & Lisanti, 2004; Williams & Lisanti, 2004; Williams et al., 2006). It is highly expressed by AT especially adipocytes of fatty mammary pads (Williams et al., 2006). Some studies considered Cav-1 protein as adipokine and exerts an effect on the role of the adjacent cell in the microenvironment (Chang et al., 2017). Cav-1, 22 kDa (Catalán et al., 2008) has been demonstrated to be elevated by numerous kinds of human cancers including kidney, liver, breast, colon (Nwosu, Ebert, Dooley, & Meyer, 2016). This elevated Cav-1 level built caveola which contains different proteases that stimulate extracellular matrix (ECM) degradation and promote cancer motility and invasion. (Victor, Anbalagan, Mohamed, Sloane, & Cavallo-medved, 2011). In addition, secreted Cav-1 proteins were increased by obesity in mice model. The objective of this study was to determine the expression levels of Cav-1 protein in AT of non-obese vs. obese BC patients by IHC and immunoblotting.

MATERIALS AND METHOD

Patients' Samples:

For the purpose of patients' enrolment in this study, Institutional Review Board (IRB) approval was obtained before the surgery and the protocol was approved by the ethics committee of Ain Shams University (Cairo, Egypt). Each patient signed a written consent form prior to participation, including agreements for enrolment in this study and publication of data. Patients who were pre-operatively diagnosed as BC cases by clinical examination,

mammography, ultrasound and Tru-cut biopsy in breast clinic of Ain Shams University hospitals have participated in the present study. The human AT was collected from 20 participants undergoing modified radical mastectomy (MRM) or conservative surgery. Patients enrolled in our study were classified into two subgroups; non-obese BC patients (n=5) and obese BC patients (n=15).

Sample Collection and Handling:

Within hr as soon as possible after Dr. El-Shinawi's surgery AT specimens were carried to the laboratory from the operating room. (Fig.1A) Each tissue specimen was divided into 2 parts; one fixed at 10% PBS-formalin buffered solution for IHC diagnosis and the second part was processed for further biochemical and molecular studies.

Culture of Tumor-Associated Adipose Tissue (TAAT) Isolated During Breast Surgery:

The method of maintaining the tissue specimens was performed under an aseptic condition. TAAT provided by the surgeon during breast cancer surgery was transferred directly to the lab (Fig 1A). AT specimens (Fig 1B) were washed twice with Phosphate Buffered Saline (PBS) and a total amount of about 200-300 mg AT was sliced into very small pieces. The TAAT fragments were distributed equally into a tissue culture dish (Greiner bio-one, Frickenhausen, Germany) supplemented with 2 ml DMEM-F12 media containing 10% FBS and incubated for 24 h in the incubator. The media were discarded was discarded on the next morning, 1 ml serum-free media were added and incubated for 24 h in a humidified atmosphere of 5% CO₂ at 37 °. Finally, TAAT conditioned media was collected and stored at -80°C for cytokine profiling. Alternatively, TAAT explants were kept at -80°C for future experiments.

Profiling of the Secretions of TAAT-CM Using Human Cytokine Array:

Ray-Bio cytokine antibody array-C3 (RayBiotech, Inc. GA, USA) was used to detect all different adipokines in ATCM according to the manufacturer's instructions. Briefly, the TAAT-CM was 5-fold concentrated using Amicon Ultracell10K filters (Millipore, Billerica, MA). The cytokine array membrane is incubated with 2 ml blocking buffer for 1 h at RT followed by incubation overnight at 4°C with ATCM. Finally, the membrane was probed with a biotinylated antibody cocktail overnight at 4°C followed by incubation with HRP-conjugated streptavidin overnight at 4°C. ImageJ software was used to evaluate the adipokines signal intensity. Each adipokine signal intensity value is presented as mean ± SEM (n = 5).

Immunohistochemistry (IHC):

Immunohistochemical staining was performed using 5 µm paraffin adipose tissue sections sliced with a microtome and mounted on positively charged slides as described by the author (Nouh *et al.*, 2011). In brief, AT sections were first de-waxed in xylene clearing agent and hydrated through descending serial dilutions of ethanol (100%, 90%, 70%, and 50%) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). For the heat-induced antigen retrieval process, slides were incubated in pre-heated retrieval buffer in water steamer for 30 min, followed by cooling at room temperature for 30 min. Endogenous peroxidase activity was blocked for 10 min using Dako Dual Endogenous Enzyme Block. After blocking, adipose tissue sections were incubated overnight at 4 °C with 1: 150 diluted primary antibody Cav-1 (BD Biosciences, San Diego, CA, USA). After washing, the slides were incubated with 100 µl horseradish peroxidase (HRP) linked secondary anti-mouse (EnVision + Dual Link System, Dako, Denmark) for 45 min at

room temperature. IHC signal development was carried out by adding 100 μ L of the chromogen 3-3'-diaminobenzidine (DAB+) diluted 1:50 in substrate buffer for 10-15 min depending on the appearance of a brown color. Negative control slides were run in parallel in which the primary antibody was omitted. Finally, tissue specimens were washed in phosphate-buffered saline (PBS), the nuclei were counterstained with Mayer's haematoxylin for a minute, washed in tap water following dehydration through ascending ethanol serial dilutions (70%, 90%, and 100%) and clearing steps through xylene and the specimens were mounted using Permount[®] (Fisher Scientific, Pittsburgh, PA, USA) and air-dried overnight for examination by light microscopy (Olympus, CX41, Japan). The stained area fractions were analyzed semi-quantitatively using ImageJ software (National Institutes of Health, MD, USA).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting:

AT of non-obese and obese fresh BC patients obtained by doctor El-shinawi was lysed in radio-immunoprecipitation assay buffer (RIPA buffer) [25 mM Tris HCL pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate and 0.1% SDS (Sigma-Aldrich, St. Louis, MO, USA)] and then centrifuged for 10 minutes at 10,000 \times g at 4°C. The protein content of adipose tissue lysates was quantified using the Bradford assay (BioRad Laboratories, CA, USA) using Infinite[®]200 PRO NanoQuant (Tecan, Zürich, Switzerland). 25–50 μ g of AT protein lysates per lane were loaded and resolved using SDS-PAGE (12% acrylamide gel) using Mini-PROTEAN[®]II Electrophoresis Cell (BioRad Laboratories, CA, USA) apparatus and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA).

After blocking for 1 hr with 5% non-fat dry milk in TBS-0.5% Tween 20 (TBST), the membrane was probed with primary antibody against 1:5000 dilution of cav-1 (BD Biosciences, San Diego, CA, USA) overnight at 4 °C on shaker and then washed and incubated with secondary antibody conjugated with 1:10,000 diluted horseradish peroxidase (Santa Cruz Biotech, CA, USA) for 1 hr at room temperature. After washing, bound antibodies were visualized by using Pierce Enhanced Chemiluminescence immuno-blotting substrate (ECL) (Thermo Scientific, ON, Canada) according to the manufacturer's protocol, and analyzed with ImageJ software (National Institutes of Health, Bethesda, MA, USA), which quantifies the density of each band using β -actin (Santa Cruz Biotech, CA, USA) as a loading control.

Statistical Analysis:

The data are analyzed using IBM SPSS version 15.0 (SPSS, Chicago, IL, USA) and presented as mean \pm SEM. Differences among patients were evaluated using Chi-square and Student's t-test. Pearson's Rank coefficient test was used to assess the associations with patient clinical data. Two-tailed $p < 0.05$ was considered significant.

RESULTS

Clinical and Pathological Characterization of BC Patients:

Clinical and pathological characteristics of obese patients ($n = 15$) and non-obese ($n = 5$) patients who participated in the study are presented in Table 1. The mean age of obese patients was 52.5 ± 3.9 which ranged from 34 to 71 years while the mean age of non-obese patients was 52.6 ± 3.28 which ranged from 43 to 62 years. Tumor size measurements revealed that 61.54% of obese patients exhibited tumor masses less than or equal 4 cm and 38.46% of them exhibited tumor masses greater than 4 cm, whereas, in the non-obese group, 50% of the patients had a tumor size

less than or equal 4 and 50% of them having a tumor greater than 4. The tumor grade analysis among obese patients revealed that 78.57% were classified as grade II (G2) and 21.43% were grade III (G3), while 100% classified as grade 2 (G2) in non-obese. Among obese patients, 78.57% were negative for lymph vascular invasion and 21.43% were positive for lymph vascular invasion. While 100% of non-obese (control) patients were negative for lymph vascular invasion. All obese patients who underwent surgery had positive metastatic lymph

nodes: 71.43% had had lymph nodes less than or equal 4 positive lymph nodes and 28.75% had greater than 4 positive metastatic lymph nodes while in non-obese patients, 100% had ≤ 4 lymph nodes. ER, PR and HER-2 status were assessed as negative and positive for all obese and non-obese patients. Positive staining for ER, PR, and HER-2 was detected in 53.33%, 60% and 33.33% obese patients, respectively. Whereas, the positive staining for ER, PR in non-obese patients were 33.3% and 33.3%, respectively.

Table 1. Clinical and pathological data of obese and non-obese breast cancer patients

Characteristic	Obese BC (N = 15)	Non-obese BC (N = 5)	P value
Age (years)			
Range	34-71	43-62	0.98 ^a
Mean \pm SEM	52.5 \pm 3.9	52.6 \pm 3.28	
Tumor size (cm)			
≤ 4	8 (61.54%)	2 (50%)	1 ^b
> 4	5 (38.46%)	2 (50%)	
NA	2	1	
Lymph node status, n (%)			
≤ 4	10 (71.43%)	4 (100%)	0.52 ^b
> 4	4 (28.57%)	0	
NA	1	1	
Tumor grade, n (%)			
G1	0	0	0.55 ^b
G2	11 (78.57%)	4 (100%)	
G3	3 (21.43%)	0	
NA	1	1	
Lymphovascular invasion, n (%)			
Negative	11 (78.57%)	3 (100%)	0.59 ^b
Positive	3 (21.43%)	0	
NA	1	2	
ER, n (%)			
Negative	7 (46.67%)	2 (66.67%)	1 ^b
Positive	8 (53.33%)	1 (33.33%)	
NA	0	2	
PR, n (%)			
Negative	6 (40%)	2 (66.67%)	0.56 ^b
Positive	9 (60%)	1 (33.3%)	
NA	0	2	
HER-2, n (%)			
Negative	10 (66.67%)	3 (100%)	0.52 ^b
Positive	5 (33.33%)	0	
NA	0	2	

Data are expressed as mean \pm SEM

NA = not available

*significant P value calculated by ^aStudent's t-test or ^bPearson Chi-Square

Profiling of Adipokines Secreted From AT Explants BC Patients:

ATCM secreted of AT explants from BC patients after 24 h was subjected to RayBio™ cytokine antibody array 3 cytokines (Fig. 2). The secreted ATCM from BC patients showed that the monocyte chemoattractant protein (MCP-1), interleukin (IL-6), and interleukin (IL-8) cytokines are the highest adipokines secreted by AT. The cytokines array image was quantified using ImageJ software.

Overexpression of Caveolin-1 Protein in AT of Non-Obese Versus Obese BC Patients:

Assessment of the expression of Cav-1 in paraffin-embedded tissue sections of non-obese versus obese AT patients with BC using IHC to stain

Cav-1 (Figs. 3A, B& C). A statistically significant difference between the expressions of Cav-1 protein was detected in obese compared to non-obese tissues ($P < 0.05$).

Caveolin-1 Protein Level Is Higher in the AT of Obese Than Non-Obese Breast Cancer Patients:

Immunoblotting analysis was used for assessment of Cav-1 expression in tissue lysates of obese vs. non-obese BC adipose tissue patients to confirm IHC results. Western Blot results (Figs. 4A, B& C) showed the expression of Cav-1 (22 KDa) was significantly ($p < 0.05$) overexpressed in obese ($n = 10$) compared to non-obese patients ($n = 5$) by ImageJ software and normalized against the loading control B-actin.

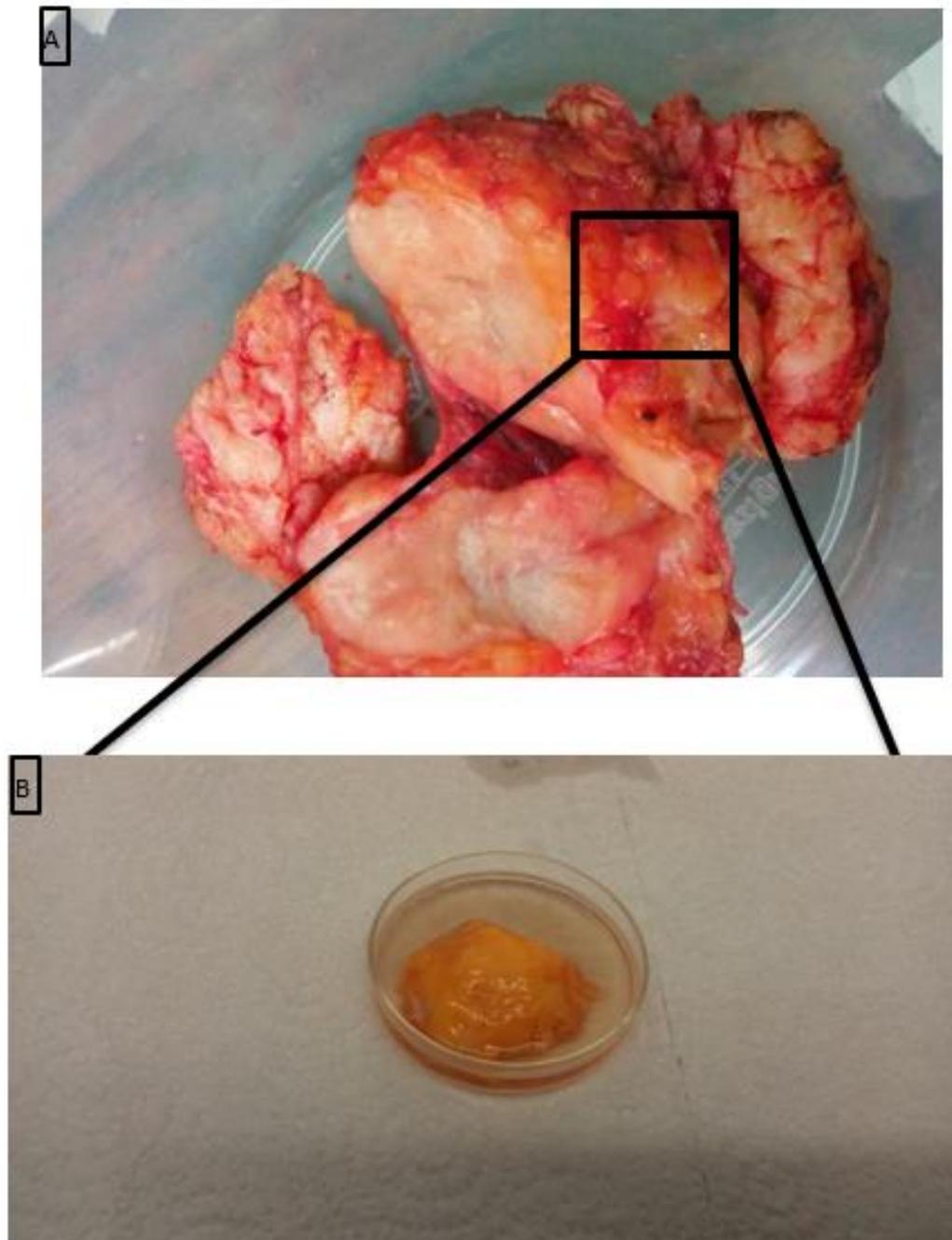


Fig. 1. A) Representative photograph of excision breast carcinoma tissue during modified radical mastectomy (MRM) showing how fat cells enveloping cancer tissue. B) Under sterile conditions, AT was sliced into very small pieces. The fragments were distributed equally into a tissue culture dish (supplemented with media containing FBS).

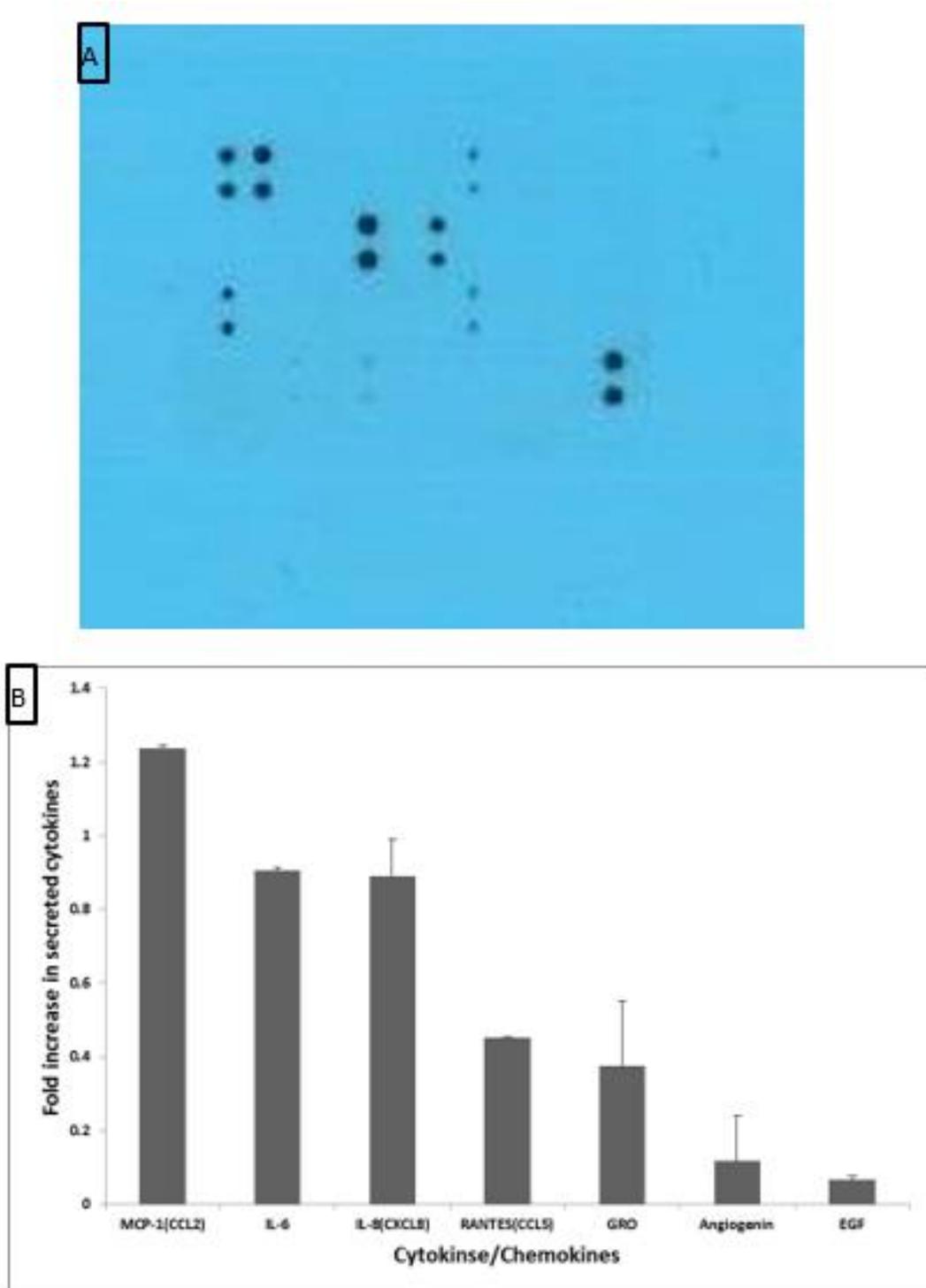


Fig. 2. Chart illustrates the secreted cytokines/chemokines from AT BC patients after 24 hr. A) Representative RayBio™ cytokine antibody array 3 of secreted ATCM from BC patients after 24 hr. B) Bars represent mean of signal intensity value of each cytokine secreted by AT explants from BC patients (n = 5). The cytokine array image was quantified using ImageJ software

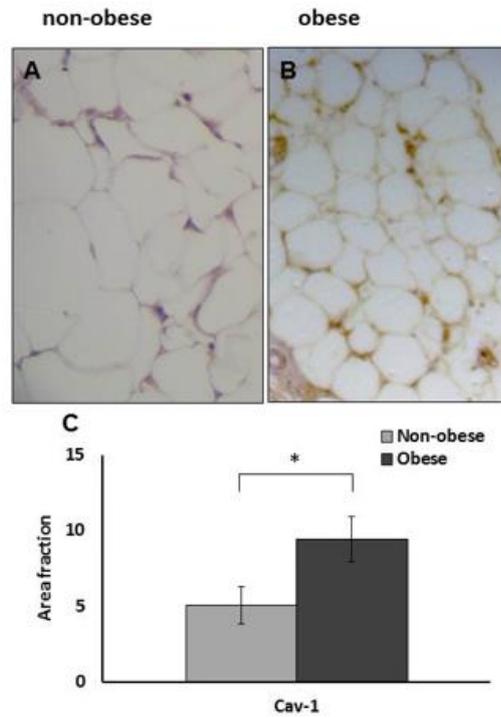


Fig. 3. Representative fields of immunostaining of Cav-1 (brown color) in paraffin embedded adipose tissue sections showing high density of adipocyte cells positive for Cav-1 in (B) obese (n = 10) compared to (A) non-obese patients (n = 5). (F) Bars represent the area fraction of obese relative to non-obese patients calculated by using ImageJ program. The data represent the mean \pm SEM. * P < 0.05 as determined by Student's *t* test.

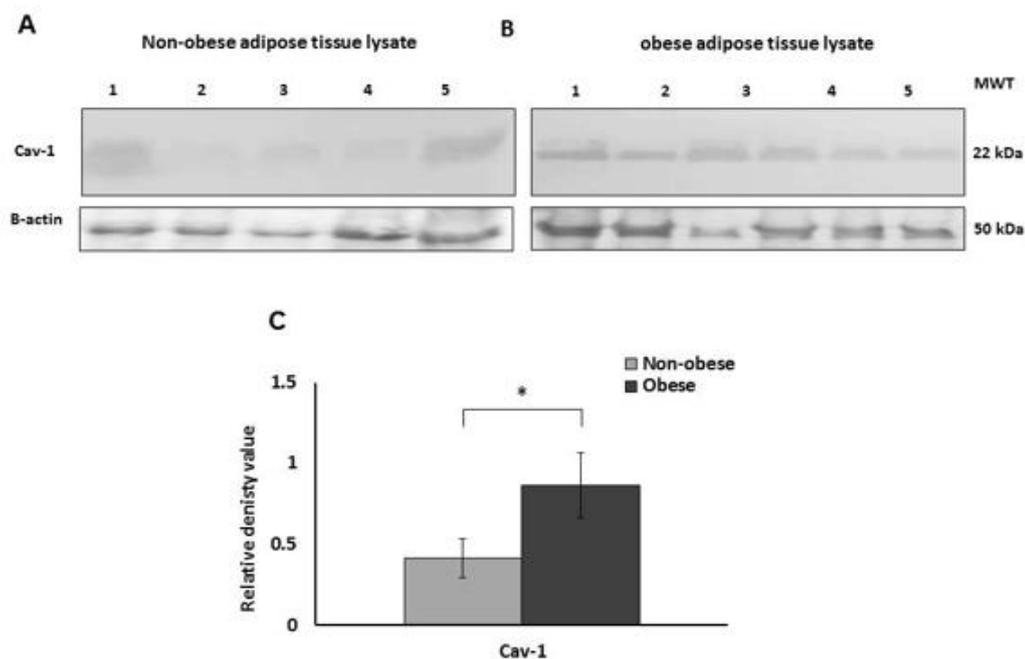


Fig. 4. Representative immunoblots membranes showing Cav-1 protein expression in (B) adipose tissues of obese relative to (A) non-obese breast cancer patients. (A)1-5 Lanes represent adipose tissue lysates of different non-obese patients showing weak expression of Cav-1 (22 kDa). (B)1-5 Lanes represent adipose tissue lysates of different obese patients showing increased expression of Cav-1 (22 kDa). (C) Bars represent the relative density values of detected protein bands assessed by ImageJ software and normalized against the loading control B-actin, showing statistically significant higher expression of Cav-1 in obese (n = 10) than in non-obese (n = 5) adipose tissues. The data represent mean \pm SEM. * P < 0.05 as determined by Student's *t* test.

DISCUSSION

In the present study, our data further demonstrated that the highest secreted adipokines are IL-6, IL-8, and MCP-1 and there is a high expression of Cav-1 *protein* level in adipose breast carcinoma tissues by adipocytes of obese vs. non-obese.

As mentioned by the author Catalan that there is a positive correlation between the secretion of MCP-1 and Cav-1 (Catalán *et al.*, 2008). Our study also confirmed this where we found more expression of MCP-1 by obese breast cancer patients. In other words, we found that there is an increase also in secreted Cav-1 in addition to MCP-1 but the secretion increased significantly in obese patients compared to non-obese patients.

Furthermore, the expression of IL-6 found to be correlated with the expression of caveolin-1 and poor prognosis in breast cancer patients (Podar *et al.*, 2003).

In the caveolae, the membrane regions which is rich in cholesterol and glycosphingolipids characterized by obtaining the main protein Cav-1 (Chang *et al.*, 2017). Besides previous studies that Cav-1 implicated in endocytosis and signaling, there is a wide variety of studies showed that Cav-1 is altered in several cancer types such as liver, colon, breast, kidney, prostate, and plays diverse functions in cancer development by secretion of MMPs which degrade ECM (Nwosu *et al.*, 2016). In addition, Cav1 promote breast cancer development and metastasis by affecting various aspects of epithelial–mesenchymal transition (EMT) (Bailey & Liu, 2008; Gai, Lu, Tu, Liang, & Zheng, 2014; Goetz, Lajoie, Wiseman, & Nabi, 2008; Qian *et al.*, 2019). As mentioned in the study conducted by Taher and colleagues that human and mouse prostate cancer cell lines promotes cancer cell survival and growth *in vitro* via secreting Cav-1 and

thus considered as a biomarker and therapeutic target for cancer treatment (Tahir *et al.*, 2001).

It should be mentioned that in obesity, adipocytes are hypertrophied with a change in the adipokine secretive profile of obese adipose tissue. Our findings agree with a study conducted by Chang and co-workers that obesity changed Cav-1 secretion as reported that adipose tissues from normal chow diet-fed mice far less than that secreted high-fat diet-fed mice and that lack of Cav-1 has been linked to loss of fat tissue (Chang *et al.*, 2017). In addition, they found that ERK1/2 might play a role in obese subjects in comparison to non-obese subjects. Interestingly, inhibition of ERK1/2 activation in obese patients with PD98059, a MEK1/2 inhibitor, prevented the Cav-1 secretion (Chang *et al.*, 2017).

Our findings are in accordance with a study that showed that Cav-1 expression may contribute to the formation of more caveolae the membrane lipid raft that encloses different non-active proteases, cytokines, inflammatory mediators involved in cancer motility, invasion and metastasis. In addition, we found that Cav-1 secretion in CAAT is markedly associated with obesity, so the high expression of cav-1 may emerge as a biomarker for obese breast cancer patients and its targeting may represent a therapeutic strategy. However, our findings should be verified in a large group of breast cancer patients.

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ARABIC SUMMARY

الخلايا الدهنية المكونة للنسيج الدهني لمرضى سرطان الثدي ذوات الوزن الزائد تتميز بزيادة إنتاج بروتين الكافيولين-1 والذي يعتبر احدى المكونات الاساسية للانبعاج الكافيولى فى غشاء الخلية ويساهم فى تفاقم سرطان الثدي

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أكثر أشكال السرطان انتشارا وثاني أشكال مميتة وقاتلة بعد سرطان الرئة يؤثر لا يزال سرطان الثدي على أكثر من امرأة واحدة في عشرة أشخاص على حياة المرأة والأسرة في جميع أنحاء العالم، حيث انه يؤثر مما أدى إلى ما يقرب من مليوني حالة من سرطان الثدي سنويًا. وفي مصر، ٣٢٪ من كل على مستوى العالم حالات السرطان التي تم تشخيصها حديثاً بين النساء هي سرطان الثدي، ويمثل ١٥٪ من الوفيات على مستوى العالم سنويًا. أن التكوين البيولوجى والجزئى لسرطان الثدي يمثل أنواع مختلفة لتشخيص المريض وتحسين نتائج المرضى. وقد تم تصنيف المرضى إلى أربعة أنواع فرعية جزيئية اعتماداً على تعبير مستقبلات الهرمونات مثل هرمون الإستروجين وهرمون البروجسترون بالإضافة إلى مستقبل عامل نمو البشرة وهذه الأنواع الجزيئية تنقسم إلى أربع أنواع هي النوع الأول هو ليومينال إيه الذي يمتاز بوجود مستقبل هرمون الاستروجين و/أو مستقبل هرمون البروجسترون، والنوع الثاني هو ليومينال بي الذي يمتاز بوجود مستقبل هرمون الاستروجين و/أو مستقبل هرمون البروجسترون بالإضافة إلى مستقبل عامل نمو البشرة أما النوع الثالث فهو مستقبل عامل نمو البشرة الموجب الذي يمتاز بوجود مستقبل عامل نمو البشرة فقط وأخيراً النوع الرابع وهو السالب الثلاثي الذي يمتاز بعدم وجود أي من مستقبل هرمون الاستروجين أو مستقبل هرمون البروجسترون أو مستقبل عامل نمو البشرة. وقد وجد ان التغير النمطى لحياة المرأة فى العشرين سنة الماضية ومن اهمها زيادة الوزن بسبب الاعتماد على الوجبات السريعة غير الصحية من العوامل المسؤولة عن ظهور وقد اثبتت عدة دراسات أن السمنة هي دالة التنبؤ .اورم حميدة وسرطانية مختلفة، بما في ذلك سرطان الثدي يتم تحديد السمنة على أنها زائدة عن الحد من تراكم .السيئ في حالات سرطان الثدي قبل وبعد انقطاع الطمث بحيث يمكن لأي اختلال وظيفي في الأدوار تعزيز الوزن الزائد والسمنة، الذي الدهون في الأنسجة الدهنية بدوره يؤثر على سرطان الثدي عن طريق افراز بعض البروتينات الحيوية النشطة بشكل جماعي والتي يطلق عليها مسمى ادبيوكاينز وهي تسهم في انتشار وهجرة الخلايا السرطانية التي تحفز نشاط المرضى الذين يعانون إلى عائلة من الكافيولين احد المكونات الرئيسية لغشاء الكافيولا المنبعج . وينتمي ان بروتين من سرطان الثدي. ثلاثة بروتينات اخرى كافيولا ٢ و كافيولا ٣. ويتم التعبير عنه على مستوى عالٍ بواسطة الأنسجة الدهنية، وهو التي ثبت أنها مرتفعة بأنواع عديدة من متوفر بكثرة في الأنسجة الدهنية، لذا فهو يعمل كادبيوكاين ، السرطانات بما في ذلك الكلى والكبد والثدي والقولون. لذلك فان الهدف من هذه الدراسة أن يحدّد او يتم قياس مستوى الكافيولين فى المرضى ذوات الوزن الزائد والمعتدل باستخدام كيمياء الخلايا المناعية والامينوبلوتنتج.