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Small Extracellular Vesicles and Survivin as Diagnostic and Prognostic Marker for Breast Cancer: A Pilot Study

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1. Abstract

The impact of breast cancer on women across the world has been extensive. Liquid biopsy has the advantages of minimal invasiveness and relies on the existence of blood unique biomarkers, which may reflect the patient's disease status. In this study, we quantified serum small extracellular vesicles and survivin protein from patients with BC to verify their potential as diagnostic and prognostic biomarkers. Blood serum samples were collected from human patients categorized as malign and benign BC, and control healthy groups. SEVs were isolated by ultracentrifugation and characterized by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and immunoblot (WB). Survivin was detected in the serum using a commercial ELISA kit. Although the concentration and size of SEVs numerically varied among experimental groups, there was no statistical significance. Otherwise, the expression of survivin protein was significantly increased in BC patients before treatment and drastically reduced in the sera after surgical excision in patients with disease remission. While SEVs concentration was not a conclusive indicator for aggressiveness and disease stage, the survivin concentrations obtained through liquid biopsy, have confirmed to be a potential diagnostic and prognostic marker in BC patients.

2. Introduction

Breast cancer (BC) is the most common cancer diagnosed in females reaching 2,3 million new cases yearly [1]. To date, the limitations of tissue biopsy have been gradually recognized in the field of precision medicine and, in this context, liquid biopsy has the advantages of minimal invasiveness, easy sample acquisition, and dynamic analysis, allowing early detection of malignant tumors while providing a longitudinal evaluation of cancer patients for the design of effective therapies [4, 30]. Liquid biopsy reveals the existence of unique biomarkers that can be detected in the circulation and reflect the patient's disease status [3, 6].

SEVs are 40 to 150 nm diameter particles secreted by cells into the extracellular space. These vesicles can modulate the tumor microenvironment, promote immune evasion, support angiogenesis, and generate metastasis. The circulating levels of tumor SEVs were

shown to be significantly higher in lung cancer patients compared to healthy controls, suggesting their use as cancer biomarkers [23].

Survivin is a member of the apoptosis inhibitor protein (AIP) family extracellularly found in circulating SEVs [13, 14]. This protein is often present in undifferentiated tissues during the embryonic and fetal periods and in almost all types of cancer. The increased expression of survivin in tumor cells is regulated by several factors, including microRNAs (miRNAs) [11] and signaling cascades of receptor tyrosine kinases (RTKs), such as phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) [10]. Survivin is also involved in tumorigenesis through several mechanisms, such as interaction with the caspase-3 and 7 proteases [9]. Therefore, this study aimed to verify whether the association of high concentrations of SEVs and survivin protein might represent a promising strategy for early BC diagnosis and prognosis.

3. Materials and Methods

3.1. Experimental Groups

Serum was collected from 31 women based on the following groups: 20 patients with malignant BC in age ranging from 23 to 74 (mean 54 years old); six patients with benign tumors with age ranging from 23 to 65 (mean 49 years old); and five healthy control women with age ranging from 23 to 68 (mean 51 years old). The inclusion criteria for the neoplastic group included women with clinical, histological and phenotypic BC diagnosis, with or without metastasis. These patients were not subjected to any type of neoadjuvant treatment before the first blood draw. For the cohort of patients with benign disease, the criteria were women with clinical, histological and phenotypic diagnosis of benign breast tumor. Finally, the control cohort was formed of healthy adult patients without comorbidities or treatments. Clinical evaluations were the responsibility of the medical staff of the Oncology Service, Gynecology and Obstetrics Department. Regarding the histopathological diagnosis, grading was carried out considering the Nottinghan's classification, which corresponds to the grading system of Scarff, Bloom and Richardson, modified by Elston and Ellis [7]. The stained tissue of histological samples was classified on the basis of the following criteria: well differentiated (Grade 1), moderately differentiated (Grade 2), and poorly differentiated (Grade 3).

3.2. Liquid Biopsy

Blood was collected in tubes without anticoagulant. For SEVs quantification, we used a total of 43 tested samples, in the following moments: the first collection at diagnosis, the second collection after neoadjuvant therapy and third collection after surgical therapy. For survivin quantification, we used 11 samples, including 2 control patients and 9 BC patients (before and after surgical treatment). For the control group, a single blood sample was collected. After collection, the blood was centrifuged at 1,465 x g for 10 min at room temperature for serum separation. Next, serum was centrifuged at 300 x g for 10 min to remove remaining cells, 2,000 x g for 10 min to remove cell debris, and finally at 16,500 x g for 30 min to remove larger SEVs. All serial centrifugation were carried out at 4 ºC. Serum was aliquoted into 1.5 mL tubes and stored at -80 ºC.

3.3. Extracellular Vesicles (EVs) Isolation

EVs isolation was carried out by ultracentrifugation (UC) (Optima XE-90 Ultracentrifuge; rotor 70 Ti; Beckman Coulter). To obtain small EVs enriched content, the pellets were filtered through a 0.22 µm pore filter and ultracentrifuged at 119,700 x g for 70 min at 4 ºC. After the first UC, the pellet was diluted in phosphate-saline (PBS) and stored at -80 ºC.

3.4. Transmission Electron Microscopy (TEM)

Following UC, SEVs-containing pellets were diluted in a fixative solution (0.1 M Cacodylate; 2.5% Glutaraldehyde; 4% paraformaldehyde; pH between 7.2 and 7.4) for 2 h at room temperature. 2 mL of ultrapure milli-Q water was added for a third centrifugation at 119,700 x g for 70 min at 4 ºC, to remove the fixative solution. The pellet obtained was diluted in 20 mL of ultrapure milli-Q water and kept in a refrigeration until analysis. The contents were placed on a copper grid coated with pioloform for 5 min, and the excess was removed with moist filter paper. The grid was then inserted into a drop of 2% aqueous uranyl acetate for 3 min, removing the excess again with moist filter paper. After these processes, reading was carried out using a transmission electron microscope (FEI 200kV, model Tecnai 20, emitter LAB6).

3.5. Nanoparticle Tracking Analysis (NTA)

EVs pellets were characterized for size, morphology, and quantity. Initially, the isolated serum EVs were diluted in 50 µL of magnesium-calcium-free PBS. The dilution factor used was 1:500 in PBS. Through this liquid suspension, a laser beam passed through the sampling chamber. The suspended particles scattered the light, allowing visualization under a microscope at 20x magnification. A video camera (sCMOS in Camera Level 15 at a temperature of 37 ºC) captured five images of 30 seconds of these particles in Brownian motion. Through the Nano-Sight software (NS300; NTA 3.1 Build 3.1.45; Malvern), the particles were individually tracked, and the hydrodynamic diameter of the particles was calculated using the Stokes-Einstein equation.

3.6. Immunoblot Analysis

The EVs obtained during UC were diluted in a 50 µL lysis buffer (RIPA) and homogenized with a vortex (Phoenix AP56). Proteins were placed in 10% polyacrylamide SDS-PAGE (Bio-Rad, California, USA), running at 100 V in a chamber with Tris/Glycine/SDS 1X running buffer, for approximately 90 min. Then, a semi-dry transfer to the PVDF membrane (Thermo Scientific, Massachusetts, USA) was performed. Proteins were detected using the following antibodies: ALIX (95 kDa) (SC-49267), CD63 (34-55 kDa) (SC-15363), Calnexin (90 kDa) (SC-23954) and GRP78 (78 kDa) (SC-376768) (Santa Cruz Biotechnology, Texas, USA), di-

luted according to the manufacturer's instructions to 3% BSA in TBS-T (20 mM Tris Hcl, pH 7.5, 120 mM Nacl, 0.1% Tween20). The blots were developed using peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies as appropriate (Thermo Scientific, Massachusetts, USA). Finally, the membrane was exposed to the Clarity Western ECL detection solution (Bio-Rad, California, USA) and subsequent analysis performed by the photo documentation Fusion Fx. All analyses were conducted qualitatively.

3.7. Elisa Essay

Serum samples were centrifuged at 2,000 x g speed for 10 min to remove debris and diluted 1:2 in 75 BP diluent. To detect the protein survivin (ab183361, Abcam, UK), the protocol was followed according to the manufacturer's instructions. Briefly, survivin and reaction blank standards for the calibration curve were prepared and added to the appropriate wells. Afterwards, 50 µL of the previously diluted serum samples were added to the wells. Next, 50 µL of antibody cocktail was added to the entire plate. The wells were sealed with adhesive plastic strips, incubated at room temperature for 1 h in a shaker at 400 rpm. Then, three washes with 1x PT buffer were performed and 100 µL of TMB was added to each well. For 10 min, the plate was incubated in the dark on a plate shaker at room temperature and 100 µL of the stop solution was added to the wells. After 1 min shaking, the absorption of each well was determined at 450 nm by an ELISA plate reader (Multiskan, THERMO FISHER, USA). Survivin protein levels were reported in pg/mL. All experiments were performed in duplicate.

3.8. Statistical Analysis

Statistical calculations were performed using Microsoft Excel and GraphPad PRISM 9 software (GraphPad Software, La Jolla, CA, USA). Statistical significance between two or more paired groups was determined using Student's t-test and ANOVA test, representing two-tailed tests, unless otherwise stated. All quantitative data were expressed either as the median with interquartile ranges or using means with standard errors. The statistical significance was set at $P < 0.05$.

4. Results

4.1. Invasive ductal carcinoma is the most common BC histological type

Invasive ductal carcinoma comprised most of the malign histopathological diagnoses in women (14 patients/70%). Considering the molecular diagnosis, HER2+ and luminal B comprised 40% (8 patients) and 35% (7 patients), respectively, triple negative comprised 20% (4 patients) and luminal A comprised 5% (1 patient) of the molecular diagnosis. For the benign cohort, fibroadenoma comprised most of the histopathological diagnoses (4 patients/66%).

4.2. EVs isolated from the serum of BC patients shared characteristics of SEVs

EVs were isolated from serum by UC according to a standard procedure described in previous studies, being the isolated vesicles characterized in accordance with the International Society for Extracellular Vesicles (ISEV) guidelines [26]. The isolated SEVs were validated by NTA, TEM and Immunoblot. The NTA and TEM showed the general features of SEVs derived from BC patients. SEVs isolated from the serum of patients had a typical "donut-like" appearance by transmission electron microscopy. Additionally, the immunoblot analysis confirmed the presence of ALIX and CD63 proteins in SEVs enriched pellets. Additionally, the absence of Calnexin and GRP78 protein expression was used to ensure that the SEVs enriched pellets were not contaminated with cellular debris. The presence of ALIX and CD63 (positive controls) and the absence of Calnexin and GRP78 (negative controls) in the samples allowed to validate the presence of SEVs and was used for qualitative analysis. For comparison criteria and bias control, a CF41 cell lysate was used in which the expression of ALIX, CD63, Calnexin, and GRP78 proteins was identified. Collectively, our data demonstrated that the SEVs isolated from serum had all the characteristics of exosomes.

4.3. The concentration of SEVs did not vary in pre- and post-treatment blood collections

SEVs average concentrations were not statistically different in the following groups: BC patients before surgical resection (1.93 e+09 particles/mL¬), BC patients after neoadjuvant therapy (3,42 e+09), BC patients after surgical resection (3.58 e+09 particles/mL), benign tumor group (3.01 e+09 particles/mL), and control group (2.36 e+09 particles/mL). The size of the SEVs was quite similar among the experimental groups and the SEVs average size was 135 nm. The concentrations and sizes of SEVs obtained from the cohorts are provided in Figures 1 and 2.

4.4. The concentration of survivin was significantly reduced after mastectomy

In addition to the isolation and characterization of serum SEVs, we analyzed the concentration of survivin protein present in the BC patient serum using an ELISA assay (Figure 3). Mean values of survivin concentration were significantly higher in BC patients before mastectomy (160 pg/mL) compared to its concentrations after mastectomy (61 pg/mL) and in the control group (48 pg/mL).

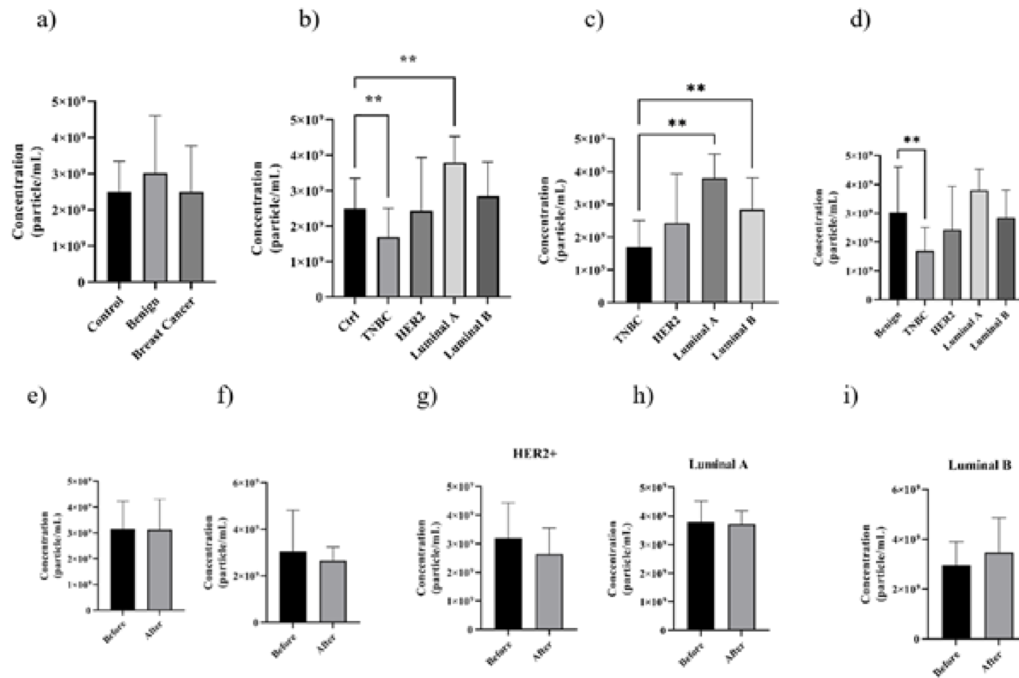

Figure 1: Graphics representing concentration of SEVs. a) Comparison between the control group x benign group x BC group showed no statistical significance; b) Comparison between the control group with each subtype (Ctrl x TNBC, Ctrl x HER2, Ctrl x Luminal A, Ctrl x Luminal B) showed statistical significance in t test for Ctrl x TNBC e Ctrl x Luminal A; c) Comparison of all types of BC groups (TNBC x HER2 x LUM A x LUM B) showed statistical significance in t test for TNBC x LUM A and LUM B; d) Comparison between all BC group types and benign group showed statistical significance in t test only in benign x TNBC; e) Comparison between SEVs concentration upon diagnosis (first collection) and after neoadjuvant therapy (second collection) in general BC groups showed no statistical significance; f) In the same way, comparison between SEVs concentration upon diagnosis (first collection) and after surgical therapy (third collection) in general BC groups showed no statistical significance; g) Comparison between first and second collection in HER2 group showed no statistical significance; h) Comparison between first and second collection in LUM A group showed no statistical significance; i) Comparison between first and second collection in LUM B group showed no statistical significance. Data analysis used GraphPad Prism 9 for ANOVA and t-test.

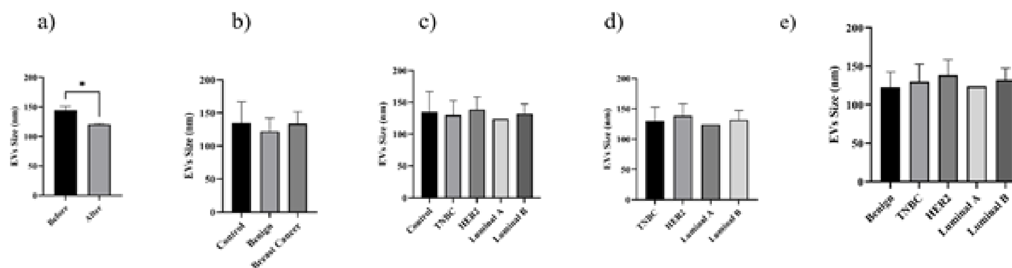

Figure 2: Graphics representing size of SEVs. a) Comparison between the size of SEVs in the first and third collection of the BC group showing statistical significance; b) Comparison between the control group x benign group x BC group showed no statistical significance; c) Comparison between the control group with each subtype (Ctrl x TNBC, Ctrl x HER2, Ctrl x Luminal A, Ctrl x Luminal B) showed no statistical significance; d) Comparison of all types of BC groups (TNBC x HER2 x LUM A x LUM B) showed no statistical significance; e) Comparison between all BC group types and benign group also showed no statistical significance. Data analysis used GraphPad Prism 9 for ANOVA and t-test.

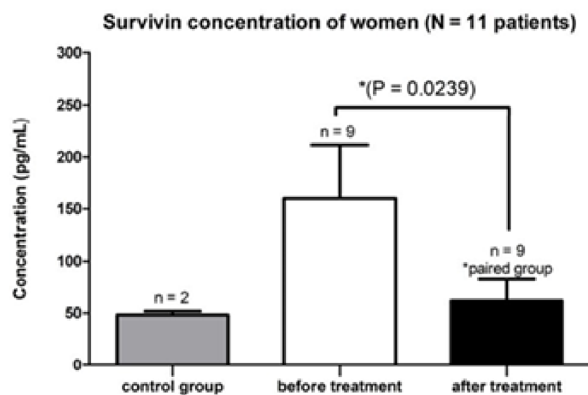

Figure 3: Survivin concentration in BC patients (n=11) was enhanced at the time of diagnosis, but dramatically reduced after treatment, like the values of the control group. Data analysis was performed using GraphPad Prism.

5. Discussion

Extracellular vesicles (EVs) have emerged as excellent mediators of intercellular communication and are increasingly recognized as a potential source of biomarkers for "liquid biopsy" of many cancers [5]. They are selective in tumor cell signaling and are continuously secreted in body fluids from the initial stages of the disease [22].

Small extracellular vesicles (SEVs), also called exosomes, are very abundant within the liquid biopsy, reaching 1.95 × 1012 particles per milliliter of blood in the original human plasma, and up to 10% of all the circulating exosomes may be tumor-derived exosomes in BC patient, depending on tumor stage [29]. Many studies claim that SEVs signal the presence of neoplastic cells in the patient's body and the interference of this signaling on the host's immunological defenses, facilitates the spread of the neoplasm and the appearance of metastasis [20]. In this context, SEVs role in cell-to-cell communication in cancer patients could prepare distant environments to generate metastasis [8, 24].

Overall, other studies reported higher amounts of exosome-derived protein in cancer patients than in healthy controls [2, 19, 27]. Our results demonstrated that SEVs concentration did not show a statistically significant difference, before or after neoadjuvant therapy and surgical excision of the primary tumor. However, if tumor-derived exosomes comprise up to 10% of circulating SEVs, it is reasonable not to find a significant difference in the serum concentration because this increase may be numerically imperceptible. Alternatively, when considering the EV protein's content, it can vary according to the state of the organism, and hence signal the occurrence of a neoplasm. Numerical variation really did not prove to be a reliable parameter under our experimental conditions.

Due to the constant influx of exosomes, the exosomal release–uptake dynamics of different cells, and the lack of fine characterization of exosome origin, it is difficult to ascertain whether the number of tumor SEVs is different compared to that of normal cells [27]. In the study by Chiu and colleagues (2016), three breast cell lines (MCF10A, MCF7, and MDA-MB-231) were used to measure the exosome secretion rate from each single cell under normal culture conditions. MCF7 and MDA-MB-231 cells had a similar exosome secretion rate of 60–65 exosomes per hour. However, the MCF10A cells secreted about 2.8x more exosomes than MCF7 or MDA-MB-231 cells [2]. This difference in the BC cell lines may be related to our results regarding the high variation in SEVs concentration. The efficiency of SEVs isolation may depend on multiple factors linked to the quality of the blood sample, including donor age, medical history, diet, time of sample collection, choice of anticoagulant, and venipuncture. In fact, the physical forces during blood drawing can activate platelets and trigger the release of platelet-derived SEVs, altering the quality of the blood samples [18], and all those factors should be considered to explain the obtained results.

Generally, besides SEVs concentration, the cargoes sorted into them can not only supply additional characteristics for their identification; but also provide promising biomarkers for diagnosis, treatment monitoring, and prognosis prediction in patients with cancer, which offers a new tool for liquid biopsy [30].

We further analyzed the survivin protein concentration in serum samples of BC women to verify its behavior in BC patients. As a member of the apoptosis inhibitory protein (PIA) family that is secreted extracellularly primarily via exosomes, increased survivin concentration is consistent with unfavorable clinical-pathological parameters [13, 14]. Its extracellular traffic throughout the tumor microenvironment may be responsible for increasing the aggressive status of the tumor, while minimizing therapeutic outcomes [23, 9]. Gonda and colleagues (2018) reported that exosomal survivin is not just a passenger but plays an active role in the uptake of its carrier vesicle. The high expression of survivin was identified in SEVs (exosomes) obtained from breast cancer cells [12, 16, 17, 25]. In this study, we have observed higher concentrations of survivin in the serum of BC women before mastectomy and a marked drop in its concentration following surgical treatment of patients, corroborating previous data. Kocoglu and colleagues (2018) reported that serum survivin levels were significantly higher in human cancer patients compared to healthy subjects (196 pg/mL vs. 117 pg/mL, respectively). The optimal cut-off value of serum survivin was determined at >120.8 pg/mL, and its serum levels above this cut-off value were associated with 4.2 times increased risk of cancer. Compared to our results, in the control group and BC group, we obtained lower minimum and maximum values and lower median values following the same pattern. All the control patients and post mastectomy patients showed survivin concentration values below the cut-off value, whereas the median values in BC groups, before mastectomy, were above the cut-off value.

Li and colleagues (2020) demonstrated that the survivin expression induced resistance to chemotherapy and radiation and targeting

survivin in experimental models improved overall patient's survival. That seems to be very useful information during the patient's treatment, but our experimental design involved the collection of punctual samples and not various longitudinal samples from the same patient, so that we could not corroborate these findings. In the same way, a meta-analysis focused on the relationship between survivin expression and overall survival suggested that high survivin expression played an unfavorable prognostic role for patients with BC [25]. Another study reported that the treatment with the antineoplastic drug paclitaxel caused the release of SEVs uniquely enriched with survivin, and its expression was closely correlated with poor patient's prognosis, chemotherapy resistance, and tumor recurrence [16]. Also, Veiga and colleagues (2019) evaluated the importance of survivin associated with BC samples and concluded that survivin represents a biomarker and a prognostic factor for BC, bringing new light and possibilities for treating mammary carcinoma.

Overall, these findings highlight the potential of using the serum survivin levels as a reliable biomarker for diagnosing BC and consider survivin as a potential candidate for targeted therapy. Therefore, our results corroborate previous scientific data and confirm the importance of survivin as a diagnostic and prognostic biomarker for mammary neoplasia

6. Conclusion

Based on our experimental conditions, SEVs concentration did not vary considerably in BC patient groups, before and after surgical treatment, and in benign tumor and control groups, showing not to function as a reliable biomarker for BC diagnosis and prognosis. Conversely, serum survivin levels, obtained through liquid biopsy, was lower after tumor excision close to the control patients, thereby proving its potential use as a diagnostic and prognostic parameter for BC.

7. Declaration of Interest Statement

7.1. Ethics approval and consent to participate

The study was submitted to the Research Ethics Committee (CEP) of FAMERP and was duly approved, being registered under the protocol number 001-004391/ 2019. Patients were able to participate in the study after signing the Free and Informed Consent Form, according to the Institution's Code of Ethics.

7.2. Authors Contribution

AAN and DAPCZ have made substantial contributions to conception and design. DSC, AAN, GMLN, LLZ, BOL have been involved in the acquisition of biological material. BLVG, GAF and PMSR have been involved in isolation and characterization of extracellular vesicles. DSC has been involved in surviving essay, statistical analysis and bioinformatics. AAN, JCS, NABJ, SB and DAPCZ were involved in the interpretation of data. AAN, FAF, PMS, JCS, LGAC, NABJ, SB and DAPCZ have been involved in drafting the manuscript or revising it critically for important intellectual content. DAPCZ has given final approval of the version to be published. Each author has participated sufficiently in the work to take public responsibility for appropriate portions of the content and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

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