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Profile of Plasma Galectin-3 Concentrations, Inflammatory Cytokines Levels and Lymphocytes Status in Breast Cancer under Chemotherapy

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Abstract

We investigated relationship between galectin-3 (Gal-3) levels and T lymphocytes apoptosis and the activation rates in breast cancer during chemotherapy. We used plasma samples from 112 women classified into two groups: 70 women with breast cancer (BC) and submitted to neoadjuvant chemotherapy (3 cycles) and 42 healthy women used as controls. In the group of BC, blood samples were taken before each cycle of chemotherapy and Gal-3 levels was evaluated by ELISA sandwich. Flow cytometry was used to study T cells apoptosis and activation. Before treatment, median value of Gal-3 was 6.31 ng/ml (range 1.07 - 50.74) in BC and 0.84 ng/ml (range 0.00 - 4.82) in HC. Gal-3 levels were highest in plasmas from BC ($p < 0.001$). During the same period, proportions of apoptotic T lymphocytes were highest in BC compared to HC ($p < 0.05$). Similar results were observed about T cells activation. According to clinical symptoms, we observed, in the group of patients with a low or null response to chemotherapy, a positive correlation between gal-3 levels and tumor size (rho = 0.48; $p = 0.010$). In addition, we found a dynamic relationship between gal-3 levels, tumor size and T lymphocytes apoptosis rates during treatment depending to the cure efficiency. We suggest gal-3 plasma concentrations could be used as predictive biomarker for chemotherapy efficiency in breast cancer patients.

Keywords

Galectin-3, T Lymphocyte, B Lymphocyte, Breast Neoplasms

1. Introduction

Breast cancers are the most commonly diagnosed female neoplasia worldwide and in Senegal, with epidemiological distinctiveness in developing countries including for example: the younger age of patients and the high rate of therapeutic failure [1] [2]. Hence, this highlights the necessity of new therapeutic and surveying tools.

Galectin-3 (gal-3) is encoded by the LGALS3 gene located on chromosome 14q21-q22. It is a biologically active protein that has been shown to be involved in many cellular processes like cell cycle, adhesion, activation, differentiation and apoptosis. With these activities, gal-3 is considered as a potential biomarker and therapeutic target in severe tumors such as prostate and breast cancers [3]. Similar to other galectin's family members, gal-3 is ubiquitously and able to bind specifically β-galactose-containing-glycoconjugates [4]. Previous studies showed that gal-3 could have a pro-tumor activity and participate in tumor evasion from immune-surveillance [5] [6]. Zhang *et al.* described an overexpression of gal-3 associated with chemotherapy resistance in triple negative breast cancer [7]. In addition, several hypotheses were proposed to explain tumor escape associated to gal-3 levels. For some scientists, a high increase of T cells apoptosis observed in cancers was probably involved in this phenomena [8]. On the other hand, gal-3 release might be under influence of certain pro-inflammatory cytokines such as TNF-α and IL-1 [9] [10]. In breast cancer, few data are available, particularly in sub-Saharan countries. In this study, we have assessed: 1) the predictive value of gal-3 plasmatic levels in chemotherapy response, 2) the influence gal-3 levels on activation and apoptosis of T and B lymphocytes in peripheral blood, 3) the relationship between gal-3 and pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) plasmatic levels in African women during breast cancer chemotherapy.

2. Patients and Methods

2.1. Recruitment, Samples Collection and Clinical Management

Breast Cancer (BC) patients and Healthy Controls were recruited at the Joliot Curie Institute, Cancer institute of Medicine of Cheikh Anta Diop University, of Hospital Aristide Le Dantec in Dakar (Senegal). Recruited HC are women free of known tumors or chronic inflammatory diseases. They live in Dakar.

Selected BC patients were newly diagnosed for breast cancer and had not undertaken any chemotherapy (CT) or radiotherapy. The major exclusion criteria included prior treatment with traditional plant-based remedies, blood transfusion during our screening and bacterial or viral infections such as tuberculosis,

HIV and hepatitis B (immunodeficiency). These infectious diseases were systematically investigated by the medical team responsible for the monitoring. The tumor size was assessed using tape measure and ultrasound prior to any chemotherapy. Therefore, we sorted patients in two groups regarding the median value of the tumor size: group 1 with tumor size smaller than the median value and group 2 with tumor size greater than the median value. All selected patients were managed by the same medical staff and the CT protocol was in accordance with European Sarcoma Network Working Group (ESMO) recommendations specifying one cycle every 21 days. Usually, three cycles are recommended before undertaking a thorough clinical examination to evaluate therapy response. Each patient followed a treatment protocol depending on individual clinical disease characteristics and general health status [11]. Besides the assessments of the tumor size, drugs as doxorubicin (A), epirubicin (E), cyclophosphamide (C), methothrexate (M), 5-fluorouracil (F) were administered in 5 different neoadjuvant chemotherapy protocols (drug combinations: AC, CMF, FAC, FEC, EC). In terms of doses, drugs were administrated every three weeks: 10 - 20 mg/m^2 of body surface for doxorubicin, 60 - 120 mg/m^2 of body surface for epirubicin, 500 - 4000 mg/m^2 of body surface for cyclophosphamide and 40 - 60 mg/m^2 of body surface for methotrexate. 5-FU was used with to 1000 mg/m^2 every 3 weeks for 3 cycles.

After chemotherapy, clinical responses to chemotherapy were assessed according to RECIST1.1 criteria and patients were classified into 3 groups: complete response (CR), partial response (PR) and null response (NR = stable and progressive disease) [11].

Peripheral blood from all study participants (BC and HC) was sampled using EDTA vacutainers (Marque, ville, pays). For BC, sample collection was based on the treatment protocol. Three samples were collected per BC, corresponding to one before each CT cycle and are designated: Sample 1 (S1), Sample 2 (S2) and Sample 3 (S3). However, only one sample was obtained per HC. All samples were immediately centrifuged, and sera were stored at −80˚C and PBMCs isolated.

2.2. Ethics Statement and Procedure

Informed consent was obtained from each participant and/or relatives prior to inclusion, after providing written or verbal information in their native language. The protocol 0196/2016/CER/UCAD was approved by Institutional Ethics Committee of Cheikh Anta Diop University (Dakar, Senegal).

2.3. Determination of Serum Levels of Gal-3 and Inflammatory Cytokines

Plasma samples were stored at −80˚C until performing the assay. Studied biological parameters were galectin-3, TNF-α, IL-1 and IL-6 levels in peripheral blood. They were measured by ELISA sandwich kits (Invitrogen™, Carlsbad, California, USA). According to the manufacturer's instructions, the lyophilized

standards were reconstituted to obtain for each parameter a stock of known concentration and serially diluted to generate a standard curve. Diluted capture antibodies were added to each well of the microtiter before incubation overnight at 4˚C. For each parameter, after washing with the appropriate solution with an adequate volume, diluted standards or samples were added and plates were incubated for 2 hours at room temperature. Detection antibodies were added to each well after a second wash and incubated for 1 hour at room temperature. Avidin-HRP was added to each well and incubated for 30 min at room temperature, before adding the substrate (15 - 20 min) and then stop solution. Optical densities were immediately determined using a microplate (Thermo Fisher™, Waltham, Massachusetts, USA) reader set to 450 nm. The results are expressed in pg/ml for cytokines and in ng/ml for galectin-3.

2.4. Isolation and Analysis/Purification of T and B Lymphocytes by Flow Cytometry

From BC patients and HC, Peripheral blood mononuclear cells (PBMCs) were isolated using standard Ficoll density gradient (Ficoll-Paque™ PREMIUM Marlborough, UK) centrifuged within four hours after blood collection. Cells were washed once in 10% FBS/RPMI and once in phosphate-buffered saline (PBS) (Thermo Fisher™, Waltham, Massachusetts, USA) before a staining step for 30 minutes at 4˚C in FACS buffer (0.5% BSA/2mM EDTA/PBS) containing anti-FcγR (eBioscience™, San Diego, California, USA) to inhibit non-specific bindings. To identify T and B lymphocytes populations, 50 µl of PBMC suspension (1.106 cells/µl) were stained with three different panels of monoclonal antibodies labeled with fluorochromes. Panel 1: (CD3-PE, CD4-APC, CD8-PerCP, CD69-FITC), Panel 2: (CD3-PE, CD19-APC, CD69-FITC, HLADR-PercP) and Panel 3: (CD3-PeCy5, CD69-FITC, CD19-APC, Apo2.7-PE) (Becton Dickinson®, Franklin Lakes, New Jersey, USA). Stained cells were washed and suspended in 0.5%BSA/2mM EDTA/PBS and sorted in a FACS Calibur (Becton Dickinson®, Franklin Lakes, New Jersey, USA) using Cell Quest Pro® software. Results were analyzed by Flowjo® software version 10.0.6 (Becton Dickinson®, Franklin Lakes, New Jersey, USA). Apoptotic and activated T and B lymphocytes were Apo2.7+ and CD69+ (early activation for both T and B cells) or HLADR+ (late activation only for T lymphocytes) respectively.

2.5. Statistical Analysis

The statistical analyses were performed using Statview® software version 5.1 (SAS institute inc., Cary, North Carolina, USA). Differences between groups were evaluated with nonparametric Kruskal-Wallis H and Mann-Whitney U tests for continuous variables. Correlation analyses were performed using the nonparametric Spearman Rho test. Relationships between continuous dependent variables and age or tumor size as dichotomy nominal variables (high and low) have been performed using the Multiple Linear Regression (MLR) where dummy variable coding has been used for the tumor size status variable which has

two categories. Graphs were performed in Statview® and GraphPad Prism® V5.03 for Windows (GraphPad, Playa La Jolla, California, USA). A p value < 0.05 was considered significant.

3. Results

3.1. Study Subjects, Clinical Data and Response to Chemotherapy

This study included 112 individuals aged from 20 to 78 years and distributed in two groups: 70 Senegalese Breast cancer (BC) patients (46 years range 24 - 78) and 42 healthy controls (HC) with no history of malignancy (26 years range 20 - 49). Peripheral blood from patients and controls was collected as described previously. The clinical and therapeutic characteristics of the patients are illustrated in Table 1.

Table 1. Clinical and therapeutic characteristics of the 70 breast cancer patients.

Characteristics	N (70 patients)
Histological type	
– Invasive tubular carcinoma	43
– Invasive lobular carcinoma	3
– Medullary carcinoma	1
– Mucinous carcinoma	1
– Mixed carcinoma	1
– nd[a]	21
SRB (Scarff Bloom Richardson) grade	
– I	4
– II	33
– III	6
– nd	27
Chemotherapy protocol	
– AC	25
– FAC	9
– CMF	7
– EC	2
– FEC	2
– nd	25
Chemotherapy response	
– CR	8
– PR	39
– NR	20
– nd	3

a. nd*: data not available; A: doxorubicin; E: epirubicin; C: cyclophosphamide; M: methotrexate; F: 5-fluorouracil; CR: complete response; PR: partial response; NR: no response.

In terms of clinical aspects, most of the patients had a high Scarff Bloom Richardson (SBR) grade tumor. A rate of 50.8% of patients had grade II tumor and 9.2% had grade III versus 6.2% with grade I. The median value of tumor size was 60 mm (range 3 - 200).

After 3 neoadjuvant chemotherapy cycles, rate of patients with complete (CR), partial (PR) and no or low response (NR) to the cure were respectively 11% (n = 8), 59% (n = 39) and 29% (n = 20). For 3 patients, the response to the chemotherapy wasn't assessed.

3.2. Galectin-3 Plasma Concentration

Median value of the gal-3 plasma concentration was 6.31 ng/ml (range 1.07 - 50.74) in patients group compared to versus 0.84 ng/ml in the controls. The gal-3 concentration was significantly higher in the patients group (Figure 1). After chemotherapy, this concentration seemed to be decreasing in patients with total and half therapy responses albeit without any statistical significance (Figure 1). We also found a positive correlation (Figure 2) between gal-3 concentration and tumor size ($p = 0.010$; rho = 0.48), before the first chemotherapy cure. For BC patients depending on therapy responses, a positive correlation was observed only in patients with no responses to chemotherapy ($p = 0.041$; rho = 0.68). Therefore, we sorted patients in two groups regarding their tumor size: group 1 (n = 11) with tumor size smaller than 60 mm (median value of tumor size) and group 2 (n = 19) with tumor size greater than 60 mm. We found a positive correlation only in group 2 ($p = 0.019$; rho = 0.55).

3.3. TNF-α, IL-1 and IL-6 Plasma Concentrations and Relationship with Gal-3

Before chemotherapy, median values in the BC group of TNF-α, IL-1 and IL-6 plasma concentrations were respectively 8.66 pg/ml (range 2.67 - 79.40), 4.530 pg/ml (range 3.01 - 16.77), 2.39 pg/ml (range 0.60 - 18.20) in BC group compare

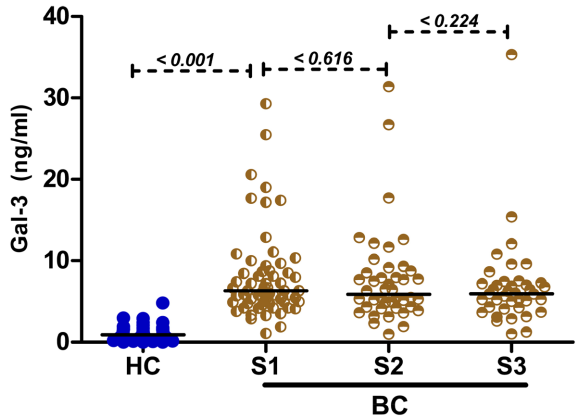

Figure 1. Galectin-3 plasmatic concentration's comparison between patients during the survey and controls groups. HC: healthy controls; S1: breast cancer patients before the first chemotherapy cycle; S2: breast cancer patients before the second chemotherapy cycle; S3: breast cancer patients before the third chemotherapy cycle.

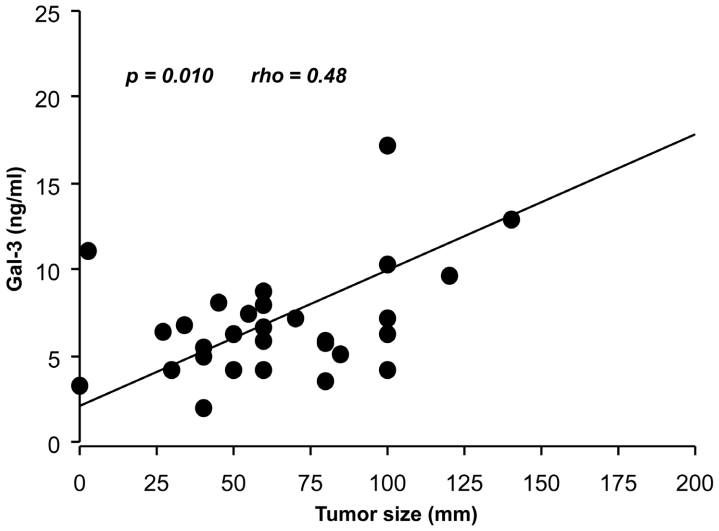

Figure 2. Relationship between gal-3 and tumor size before the start of chemotherapy.

to 5.89 pg/ml (range 1.84 - 16.95), 4.57 pg/ml (range 3.30 - 11.63), 1.65 (range 0.86 - 7.05) in the HC group.

However, a comparison of plasma inflammatory cytokines concentrations between patients and healthy controls has shown higher TNF-α rates in the BC patients compare to HC group (p = 0,024) (Figure 3). There weren't any differences in the levels of IL-1 and IL-6 between the two groups.

Further, no correlation was found between gal-3 plasma concentration and the inflammatory cytokines in both HC and BC groups ($p > 0.05$).

3.4. Lymphocyte's Apoptosis and Activation Rates

Prior to the first chemotherapy cure, FACS results have shown statistically higher T and B lymphocytes apoptosis and activation rates in the patients' group (Figure 4 and Figure 5).

Median values of T and B lymphocytes apoptosis, were respectively, 0.73% (range 0.00 - 2.63) and 0.99% (range 0.00 - 3.470) in the patients group in comparison to 0.13% (range 0.00 - 1.53) and 0.285 (0.00 - 1.00) found in the HC. After the first chemotherapy cure, we found an elevation in the lymphocytes apoptosis rates (Figure 3). Both the T and B lymphocytes apoptosis rates were significantly increased ($p < 0.05$).

The median values of CD69+ T and B lymphocytes, were respectively 2.39% (range 0.00 - 10.94) and 1.360% (range 0.00 - 61.50) in the patients' group compared to 1.28% (range 0.52 - 5.78) and 0.83 (range 0.00 - 5.630) detected in the HC. Similar to the lymphocytes apoptosis rates, there was an increase in CD69+ T and B lymphocytes after the first chemotherapy cure. We have observed no effect of the first chemotherapy cure on HLA - DR+ T lymphocytes in patients (2.93%) and healthy HC (0.73%) as shown in (Figure 5). However, before the first chemotherapy cycle, CD69+ CD8+ T lymphocytes rate was higher in the PR group than in the other groups (p = 0.008) (Figure 6).

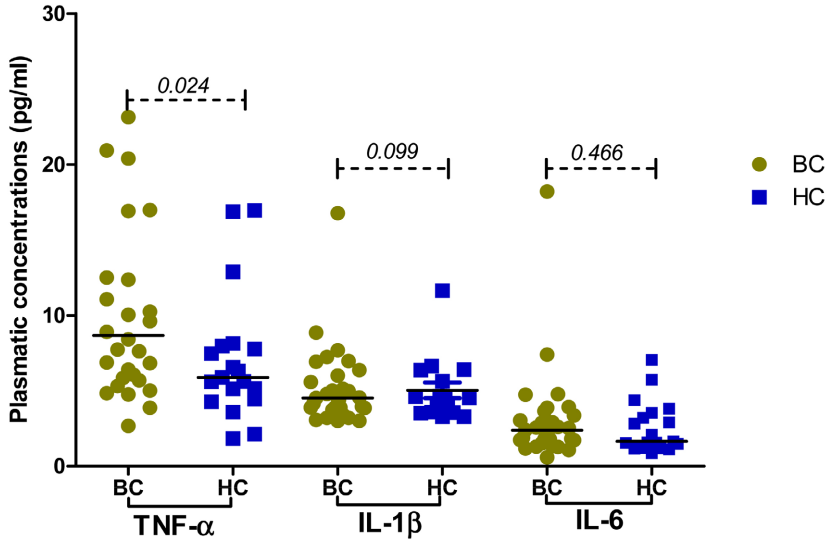

Figure 3. TNF-α (a), IL-1 (b), IL6 (c) plasma concentrations comparison between patient and control groups. HC: healthy controls; BC: breast cancer patients.

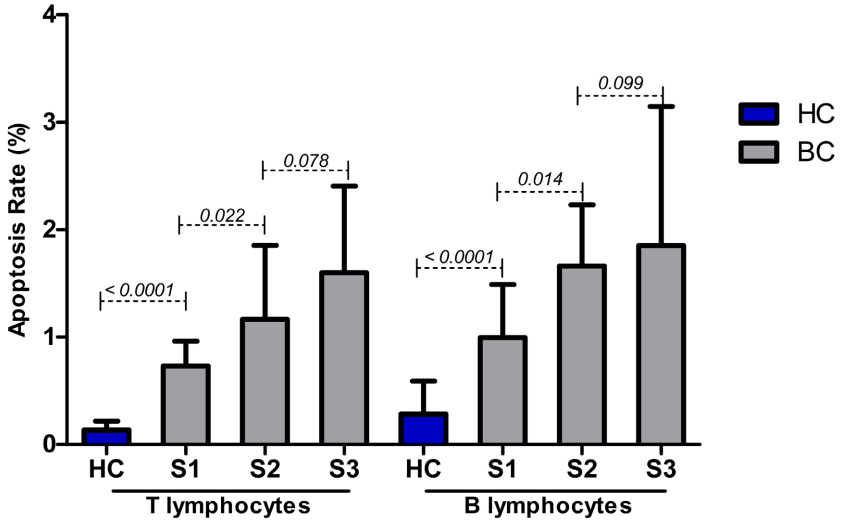

Figure 4. T and B lymphocytes apoptosis rate variations between patient and control groups. HC: healthy controls; S1: breast cancer patients before the first chemotherapy cycle; S2: breast cancer patients before the second chemotherapy cycle; S3: breast cancer patients before the third chemotherapy cycle.

3.5. Relationship between Gal-3 Plasma Concentration, Lymphocyte Apoptosis and Activation

There wasn't any correlation between gal-3 concentration and B lymphocytes apoptosis. But our results had shown a dynamic relationship between gal-3 concentration and T lymphocytes apoptosis (Table 2). Before therapy began, there was a negative correlation ($p = 0.048$; rho = −0.39) which became positive after the 2nd chemotherapy cure ($p = 0.017$; rho = 0.75). Also, there wasn't any statistically significant correlation between the gal-3 plasma concentration and lymphocytes activation.

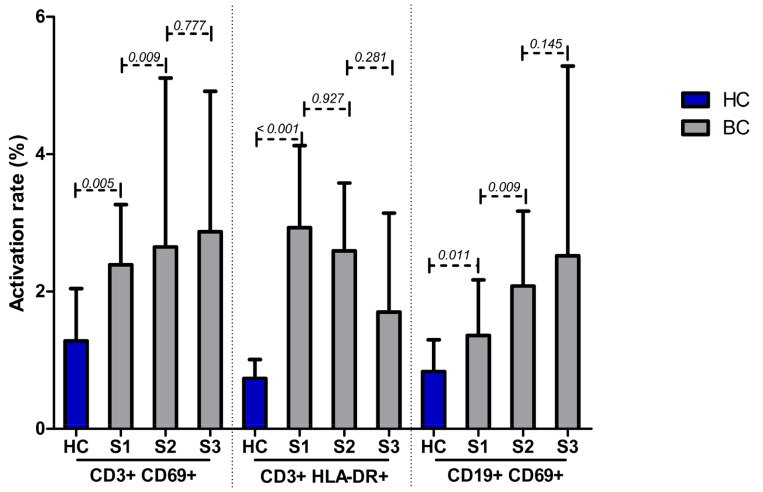

Figure 5. T and B lymphocytes activation rate variation between patient and control groups. HC: healthy controls; S1: breast cancer patients before the first chemotherapy cycle; S2: breast cancer patients before the second chemotherapy cycle; S3: breast cancer patients before the third chemotherapy cycle.

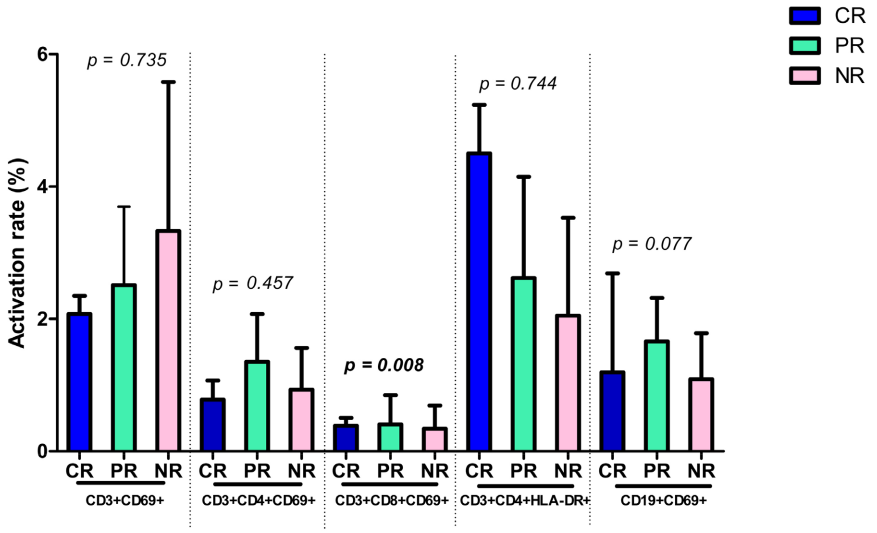

Figure 6. T and B lymphocytes activation rate regarding the response to chemotherapy. CR: complete response patients; PR: partial response patients; NR: no or low response patients.

Table 2. Evolution of the T and B lymphocytes apoptosis relationship with gal-3 plasmatic concentrations; the rho coefficients and p-values have been obtained by a Spearman's rank correlation test.

	Gal-3 concentrations		
	S1	**S2**	**S3**
% Apoptotic T lymphocytes	*rho* = −0.39 *p* = 0.048	*rho* = −0.13 *p* = 0.608	*rho* = 0.75 *p* = 0.017
% Apoptotic B lymphocytes	*rho* = −0.22 *p* = 0.252	*rho* = −0.03 *p* = 0.895	*rho* = 0.62 *p* = 0.051

4. Discussions

In this study, we have investigated the relationship between gal-3 plasma level and the pro-inflammatory cytokines, the lymphocytes apoptosis and activation in Senegalese women suffering from breast cancer.

The main aim was to determine if gal-3 could be implicated in mammary carcinoma immune evasion and also to assess the potential of the gal-3 protein as a predictive biomarker to neoadjuvant chemotherapy. In the current study, most of the women diagnosed had a high grade tumor. Furthermore, few of them had shown a complete response to chemotherapy. A previous investigation in Senegal, led to the same conclusion [1]. Thus, there is evidence that new predictive tools and therapeutic targets are needed. It has been widely reported that gal-3 could fulfill this role, both as biomarker and therapeutic target [7] [12].

Our results showed that the gal-3 plasma levels increased in breast cancer patients compared to healthy people. Further, the positive correlation we found between this protein and tumor size could be explained by the gal-3 pro-tumor activity, described in numerous investigations [13].

Among the galectin protein family members, gal-1 and gal-3 are known to be implicated in apoptosis [14] [15]. Using an *in vitro* experiment, Fukumori and colleagues had demonstrated that gal-3 triggers T cell apoptosis through stimulation of CD7 and CD29 molecules [8]. That protein could also enhance apoptotic processes by directly binding to CD45 and CD71 [16]. With an *In situ* Nick Translation assay, Zubieta and colleagues had reported positive a correlation between gal-3 expression by melanoma cells and tumor associated T lymphocytes apoptosis rates [15]. However, our results don't highlight the same relationship in peripheral blood. In the patients' group, we observed a highly elevated T lymphocytes apoptosis rate as compared to the HC. Despite this, there was a negative correlation between this lymphocyte apoptosis rate and gal-3 plasma levels. That linkage was changing through the survey and became a positive correlation after the second chemotherapy cycle. Thus, we hypothesize that chemotherapy was responsible for the modification by inducing tumor cell lysis, liberation of antigens specially gal-3, and then T cell apoptosis by hyperactivation [17].

Although, gal-3 might be related to pro-inflammatory cytokines such as TNF-α and IL-1β [9] [10]. We didn't found any relationship, in peripheral blood, between gal-3 levels and TNF-α, IL-1 and IL6.

Our study has shown that the gal-3 concentration in peripheral blood is increased in breast cancer. The lack of relationship between gal-3 and lymphocytes apoptosis that found, doesn't mean that the inducement of T lymphocytes death through gal-3 activity, isn't implicated in the tumor escape to immune response. Further, a high gal-3 plasmatic concentration is associated with high tumor size, particularly in patients without response to chemotherapy. Hence, gal-3 might be a predictive biomarker of chemotherapy response in Senegalese women, suffering from breast cancer. Moreover, gal-3 is also an interesting potential target

in anticancer therapy. So far, there is no published data from clinical trials of galectin-3 blockade therapy in breast neoplasms. However, the use of the combination of gal-3 inhibitor the GR-MD-02 with pembrolizumab (anti-PD-1 monoclonal antibody) has shown encouraging results in patients with melanoma and HNSCC (Head and Neck Squamous Cell Carcinoma). The immune-mediated adverse events were reduced in comparison with pembrolizumab used alone [18]. The same strategies could be adopted in breast neoplasms: combining galectin-3 inhibitors with existing immunotherapy. Several *in vitro* and mice model studies have highlighted the potential benefits of targeting galectin-3 in breast neoplasms. As an example, Pectasol was able to inhibit growth and induce apoptosis of the MDA-MB-231 breast cancer cells [19]. The gal-3 inhibitors: GM-CT-01, GCS-100, B210 antigalectin-3 antibody were able *in vitro* to remove galectin-3 from T lymphocyte membranes, thus reestablishing anti-tumor functions of CD8+ and CD4+ cells . Hence, anti-galectin-3 therapy could be used in combination with adoptive T cell immunotherapies.

Conflicts of Interest

The authors report no conflict of interest.

Consent for Publication

Written informed consent was obtained from all the participants.

Ethics Approval and Consent to Participate

This research was approved by Institutional Ethics Committee of Cheikh Anta Diop University (Dakar, Senegal), Reference number: 0196/2016/CER/UCAD.

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Supplemental

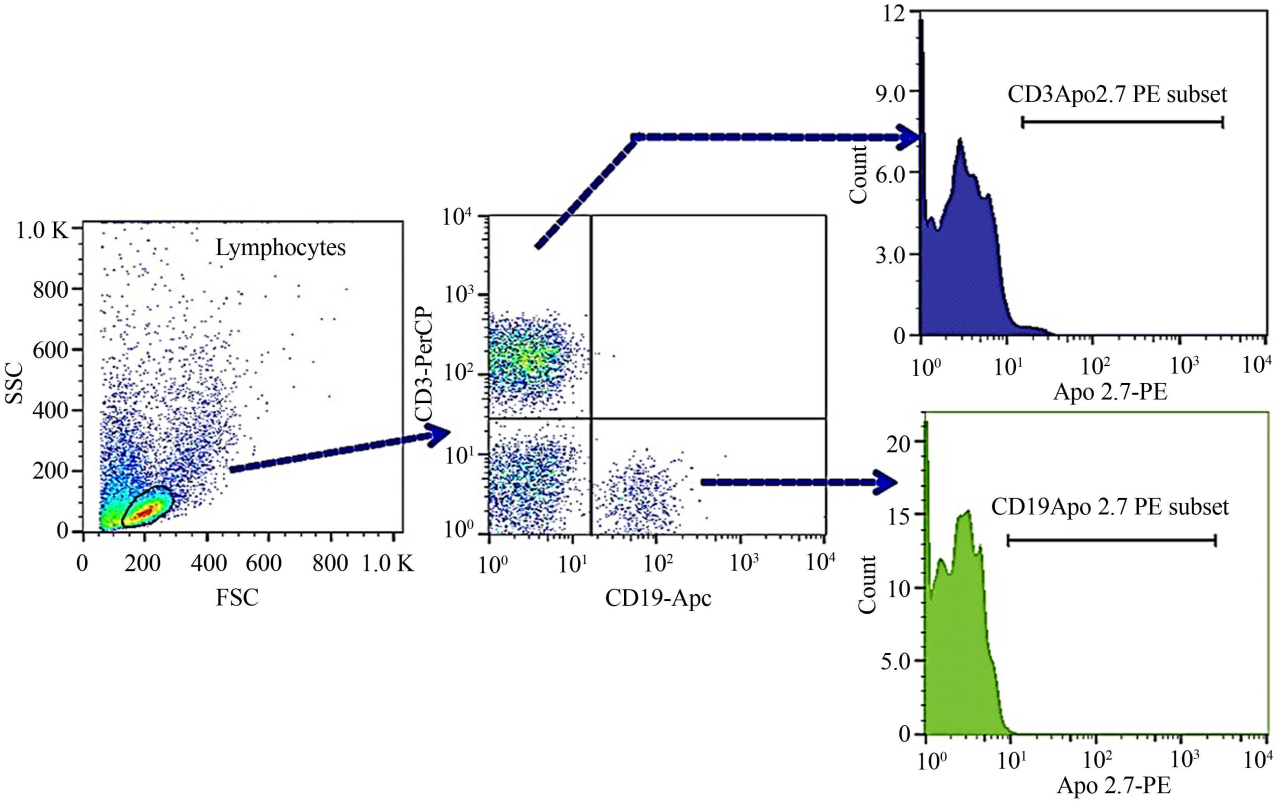

Figure S1. Gating strategy for T and B lymphocytes apoptosis assessment; SSC = Side Scatter; FSC = Forward Scatter.

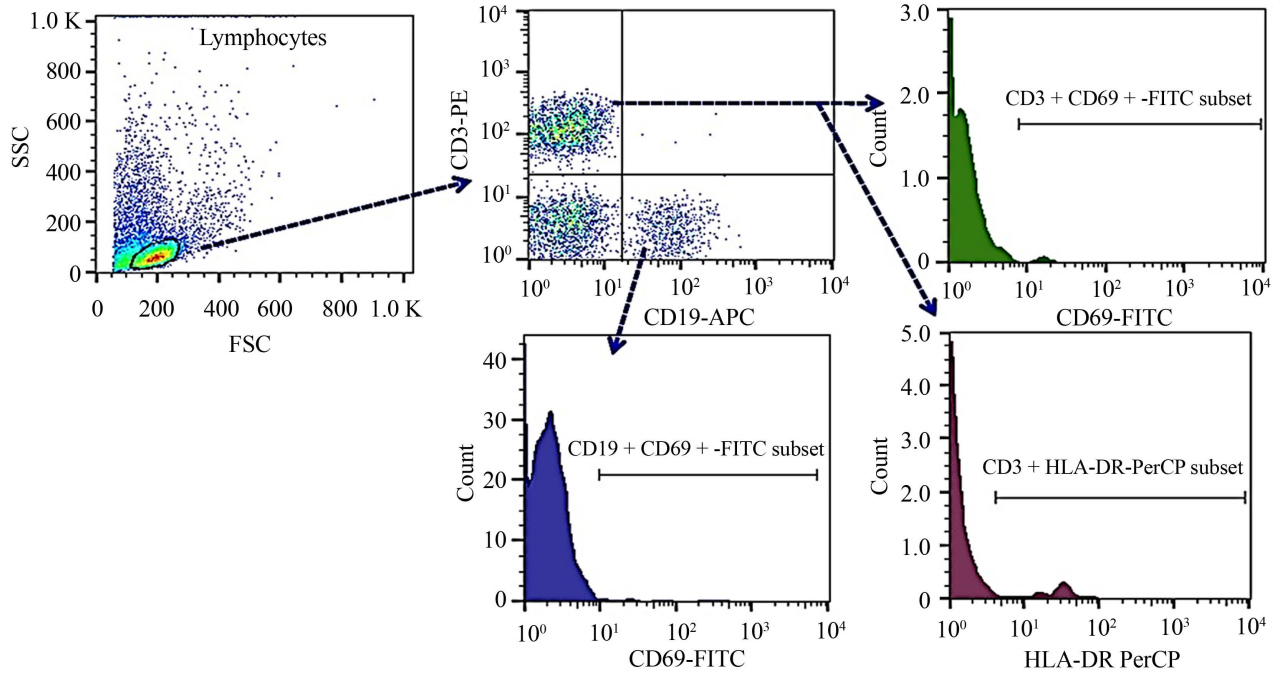

Figure S2. Gating strategy for T and B lymphocytes activation assessment; SSC = Side Scatter; FSC = Forward Scatter.