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ORIGINAL ARTICLE

Interaction of Breast Cancer and Insulin Resistance on PD1 and TIM3 Expression in Peripheral Blood CD8 T Cells

Miriam Victoria Martín-Manzo[1,2] · Carlos Lara[3] · Cruz Vargas-de-Leon[4] · Julio Carrero[5] · Gloria Queipo[6,7] · Miguel Fonseca-Sanchez[6] · Nancy R. Mejia-Dominguez[8] · David Kershenobich[2,9] · Srinivas Mummidi[10] · Alejandro Zentella-Dehesa[11] · Joselin Hernandez[2,10,12]

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Abstract

Epidemiological evidence points to a link between insulin resistance (IR) and breast cancer (BrCA). Insulin plays a role in CD8+ T cells (CD8T) differentiation and function and affects adipocytokines levels. CD8T activity in BrCA is associated with favorable outcome; while PD1 and TIM3 are markers of CD8T exhaustion and play critical roles in the negative regulation of T cell responses. Patients with (BrCA) have high expression levels of PD1 on circulating. Therefore, we hypothesized that BrCA and IR could affect PD1 and/or TIM3 expression on circulating CD8T. We determine PD1 and TIM3 expression on CD8T and analyze the relationship of CD8T phenotype with serum insulin and plasma adipocytokines levels in the different groups. We enrolled four groups of treatment-naive patients: women without neoplasms (Neo-)/without IR (IR-), Neo−/with IR (IR+), BrCa/IR- and BrCa/IR+. We found interactions between BrCA and IR with respect to TIM3 on naïve and central memory (CM) CD8T subsets. Furthermore, BrCA had a greater PD1 + TIM3- CD8T frequency in CD8T subsets than Neo-. IR+ presented a significantly lower PD1 + TIM3- frequency in CD8T subsets compare to Non-IR. In addition, we found a negative correlation between insulin levels, HOMA and frequency of PD1 + TIM3- in CD8T and a positive correlation between adiponectin levels and the frequency PD1 + TIM3- in CD8T. The increased expression of PD1 on different subsets of CD8T from BrCa patients is consistent with immunological tolerance, whereas IR has a contrary effect. IR could have a deleterious role in the activation of CD8T that can be relevant to new BrCa immunotherapy.

Keywords Breast cancer · Insulin resistance · TIM3 · PD1

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✉ Joselin Hernandez
joselin.hernandezruiz@utrgv.edu

[1] Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México, Ciudad de México, México

[2] HIPAM, Unidad de Investigación en Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad de México, México

[3] Servicio de Oncología, Hospital General de México Dr. Eduardo Liceaga, Ciudad de México, México

[4] Escuela Superior de Medicina, Instituto Politécnico Nacional, Ciudad de México, México

[5] Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Ciudad de México, Mexico

[6] Departamento de Genética, Hospital General de México Dr. Eduardo Liceaga, Ciudad de México, México

[7] Facultad de Medicina Universidad Nacional Autónoma de México, Ciudad de México, México

[8] Red de Apoyo a la Investigación (RAI), CIC-Universidad Nacional Autónoma de México, Ciudad de México, Mexico

[9] Dirección General, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Ciudad de México, Mexico

[10] South Texas Diabetes and Obesity Institute, University of Texas Rio Grande Valley, Edinburg, TX, USA

[11] Programa Institucional de Cáncer de Mama, Departamento de Toxicología Ambiental y Medicina Genómica, IIB, Universidad Nacional Autónoma de México, Departamento de Bioquímica INCMNSZ; Centro de Cáncer, CM AB, Ciudad de México, Mexico

[12] Research Deparment, Universidad Mexico Americana del Norte, Reynosa, Mexico

Introduction

Breast cancer (BrCA) is the most frequent malignancy in women which impacts over 1.5 million of women each year and is the leading cause of cancer-related deaths among women in the world accounting for 570,000 deaths in 2015 [1]. Recent epidemiological evidence points to a link between insulin resistance (IR) and BrCA, some of these studies have shown that IR is a risk factor in BrCA patients and that it can be used as a prognostic factor for these patients [2–4]. However, the potential pathophysiological mechanisms of this association are not fully understood, but hyperinsulinemia, which is often a driver and consequence of IR [5], has been identified as a potential factor that participates in BrCA initiation and progression by multiple mechanisms [6]. Insulin alters adipocytokines concentration; it decreases adiponectin levels [7] and stimulates leptin synthesis and secretion [8]. It is known that these adipocytokines influence the progress of the tumor and have effects in T lymphocytes [9]. Nevertheless, little is known about the effects of insulin or IR on CD8 T cells, which have a crucial role in the prevention and control of BrCA [10, 11]. Recently a study in a murine model has proven that the silencing of the insulin receptor impairs selective T cell functions causing a decrease of CD8 T cells cytotoxicity in response to alloantigens [12]. Furthermore, Tregs from visceral adipose tissue (VAT) of diet-induced obese mice with IR have a significant reduction in IL10 expression at mRNA and protein level. This effect could be mediate by the high level in insulin in this model [13]. This panorama indicates that hyperinsulinemia and IR can influence activation and differentiation of immune cell populations. On the other hand, it was observed that in children with diabetes type one, the percentage of PD1+ (Programmed cell death protein 1) CD8 T cells is altered before and after 4–6 months of insulin therapy [14]. PD1 is a member of the B7 / CD28 family that inhibits T cell proliferation and production of interferon-γ (IFN-γ), tumor necrosis factor-α (TNFα) and IL-2 and reduces T cell survival through binding to ligands, PD-L1 or PD-L2 [15, 16], regulating the balance between T cell activation, tolerance, autoimmune diseases and cancer immune escape [17]. During responses to chronic pathogens and tumors, PD1 expression can limit antigen specific protective immunity [18]. For example, PD-1 expression in the tumor infiltrating lymphocytes (TIL) of BrCA patients is associated with lower survival and correlates with poor prognostic factors such as larger tumor size, high histological grade and triple negative types [19, 20]. In addition, expression of PD1 on CD8 T cells of peripheral blood of patients with BrCA in the early stages is higher than in patients with benign lesions [21]. In fact, PD-1 expression is a hallmark of "exhausted" T cells in conjunction with other molecules like TIM3 (T cell immunoglobulin and mucin domain 3). This phenotype is common in other tolerant cells of immune system [15, 22, 23]. In melanoma, it has been reported that PD1 + TIM3+ CD8 T cells are more dysfunctional than cells expressing only PD1+ [24]. In contrast to PD1 that has always an inhibitory effect, TIM3 has been reported to be bifunctional, acting as either inhibitor or as activator, depending on the cell context [25]. However, it is not known if this phenotype is overexpressed in BrCa patients and if RI can increase or counteract it. Therefore, in this study we evaluated in peripheral blood CD8 T cells from patients with BrCA and/or IR, the expression of PD1 and/or TIM3, individually and in conglomerate.

Materials and Methods

Study Population

We analyzed 31 treatment-naïve BrCA patients (16 without IR (Non-IR) and 15 with IR (IR+)) and 34 women without neoplasms (Neo-) (17 Non-IR and 17 IR+). The clinical data of the individuals that participated in this study are summarized in Table 1. Women with diabetes or known comorbidities (autoimmune disease, viral infections, hyperthyroidism, hypothyroidism, acute infectious process) were excluded. We obtained 20 mL of fasting venous blood to analyze glucose, insulin, leptin, adiponectin, hematic biometry and expression of PD1 and TIM3 on CD8 T cells.

Glucose, Insulin, IR and Adipocytokines Determination

Fasting glucose and insulin levels were determined in the Central Laboratory of General Hospital of Mexico "Dr. Eduardo Liceaga", Mexico City. Serum glucose and insulin were determined in fresh by measurement of rate oxygen consumption with LX-20 and LX-20 PRO equipment and by using the paramagnetic particle chemiluminescent immunoassay Beckman Coulter Access Ultrasensitive Insulin Reagent, respectively. The cut-off score to define IR was homeostasis model assessment (HOMA-IR) >2.5. The plasma for adiponectin, and leptin was stored at −80 °C until its determination with BioVendor's human ELISA and PEPROTECH's human ELISA commercial kits, respectively.

Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation with Histopaque®-1077 (Sigma) and immediately subjected to flow cytometry analysis. All experiments were performed using fresh cells. PBMCs were stained with the following fluorochrome-conjugated monoclonal antibodies: CD3-FITC (clone OKT3), CD8-Alexa 700 (clone Hit88a), CD45RA-PeCy5 (clone HI100), CCR7-PECy7, (clone G043H7), TIM3-PE (clone F38-2E2), PD1-APC (clone

Table 1 General women and tumor characteristics

Characteristics	Neo- Non- IR $n = 17$	Neo- IR $n = 17$	BrCA Non-IR $n = 16$	BrCA IR $n = 15$
Age ± SD	52 ± 9	51 ± 7	49 ± 9	49 ± 7
TNM classification				
Stage II	NA	NA	3	4
Stage III	NA	NA	9	8
Stage IV	NA	NA	4	3
Tumor diameter cm ± SD				
Stage II	NA	NA	2.3 ± 1	2.9 ± 0.2
Stage III	NA	NA	8.3 ± 3.8	7.6 ± 3.8
Stage IV	NA	NA	9.0 ± 3.1	8.9 ± 3.8
Histological grade				
G1	NA	NA	1	1
G2	NA	NA	11	6
G3	NA	NA	3	4
No Data			1	4
Histological type				
Ductal	NA	NA	14	14
Lobular	NA	NA	1	1
Mixed	NA	NA	1	0
Molecular Classification				
No data	NA	NA	1	1
Luminal A	NA	NA	4	2
Luminal B	NA	NA	8	9
Her2-neu	NA	NA	2	2
Triple Negative	NA	NA	1	1
IMC	27.2 ± 3.7^{a}	32.6 ± 5.3^{b}	28.1 ± 4.8^{ab}	32.5 ± 6.5^{b}
Postmenopausal	11	9	7	6
With Metabolic Syndrome	1	10	0	10
Hypertension	2	3	3	5
Triglycerides ≥ 150 mg/dl	6	9	6	10
HDL Cholesterol < 39 mg/dl	8	7	1	4
Obesity	4	13	8	10

Quantitative variables are described as means and ± standard deviations (SD) and qualitative variables are defined as frequencies

Neo-: women without neoplasms, BrCA: Breast cancer patients, Non-IR: non-insulin resistant, IR: insulin resistant, NA: does not apply. Different letters indicate significant differences between groups ($p < 0.05$)

EH12.2H7); all antibodies from Biolegend. Data acquisition was performed with cytometer BD FACScantoII, the data were processed with FACS Diva Version 6.1.3. Gating strategy can be seen in Online Resource 1.

Statistical Analysis

The sample size calculation was carried out in the R program using the balanced one-way analysis of variance power calculation, using a 0.05 type I error probability, a 0.8 type II error probability and an effect size of 0.57. The effect size was obtained from PD1 mean fluorescence intensity (MFI) graphic between control and BrCA groups from Poschke's et al. article (2012) [21]. The result of the sample size calculation was 9 subjects per group. The normal distribution and homoscedasticity were evaluated by the Kolmogorov-Smirnov and Levene tests, respectively. Comparisons of means and frequencies from Table 1 were performed with the Student's t tests or one-way ANOVA and $\chi 2$ test, respectively. Differences between groups were analyzed using two-way ANOVA (factor 1: BrCA, present or absent; factor 2: IR, present or absent) or the generalized linear model gamma function. One-way ANOVA and Tuckey analysis were used to compare different groups in case an interaction between the two factors

was observed. Correlations were calculated with the Spearman or Pearson correlation coefficient. Subsequently, Principal Component Analysis (PCA) was done using immunological variables that were found to be significant in the one-factor ANOVA to determine possible clusters. Next, the scores of the first principal components were clustered using a Fuzzy C-Means (FCM) clustering. Additionally, simple correspondence analysis (CA) was performed in order to better visualize the association between the computed immunological clusters and the groups of Neo- and BrCA patients, IR+ or Non-IR. Finally, we analyzed immunological clusters to determine any possible relationships between the immunological variables of the subjects. SPSS v.21.0 software and R Statistical Software v.3.4. were used and statistical significance was set at $p < 0.05$ for all tests.

Results

CD8 T Cell Subsets Proportions Are Modified in Patients with Breast Cancer

CD8 T cells were divided into four subsets based on CCR7 and CD45RA markers: Naïve (Naïve, CCR7 + CD45RA+), Central memory (CM, CCR7 + CD45RA-), Effector Memory (EM, CCR7-CD45RA-) and terminally differentiated memory cells (TEMRA, CCR7-CD45RA+) were identified using the method described in materials and methods. When analyzing the frequencies of these subsets by two-way ANOVA, no interaction was detected between BrCA and IR. However, as reported before, BrCA patients had significantly lower percentage of Naïve CD8 T cells ($p = 0.005$) and higher frequency of EM ($p = 0.002$) and TEMRA CD8 T cells ($p = 0.001$) than Neo- [21] (Fig. 1). In contrast, no difference was detected when comparing IR+ and Non-IR. It is known that CD8 T cell subsets are inherently different in the expression of inhibitory receptors, which are tightly linked to the differentiation status, and this link is present in healthy immune homeostasis; for instance, PD1 is particularly expressed in EM and TEMRA cells, while TIM3 is mainly present on naïve T cells [26]. We found PD1 expression on CM, EM and TEMRA populations, TIM3 expression on Naïve and on the CM groups, and co-expression of PD1 and TIM3 in the CM subsets. Because patients with cancer have a lower frequency of Naïve and a higher frequency of EM and TEMRA cells, correlations were made between the percentage of this subsets, the percentage of the different subpopulations within the subsets (PD1 + TIM3-, PD1-TIM3, PD1 + TIM3+, PD1-TIM3-) and the expression of PD1 and TIM3 within the subsets. We took into consideration the correlation found between Naïve cells percentage and Naïve TIM3 expression ($r = 0.4$, $p = 0.002$) for the subsequent comparative analysis.

Interactions between Breast Cancer and Insulin Resistance Were Observed in TIM3 Expression on Naïve and CM CD8 T Cells Subsets

A generalized linear model for TIM3 expression on Naïve CD8 T cells revealed an interaction between BrCA and IR ($p = 0.04$). In Neo- group, Post-hoc Tuckey analysis showed that Non-IR have less TIM3 expression on Naïve CD8 T cells than IR+ ($p = 0.01$). BrCA patients Non-IR or IR+ have less expression than Neo-, ($p = 0.001$, $p < 0.001$ respectively) but there was no difference between IR+ and Non-IR in BrCA patients (Fig. 2). We also found that there is no overall effect of either BrCA or IR factors for TIM3 expression in CM PD1-TIM3 + T CD8 cells, but there is a significant crossover interaction ($p = 0.02$), suggesting that the effect of IR on TIM3 expression is inverse, depending on BrCA presence or absence. We didn't find any other interaction between BrCA and IR in the rest of the variables analyzed, percentage of the distinct subpopulations (PD1 + TIM3-, PD1-TIM3, PD1 + TIM3+, PD1-TIM3-) or expression of PD1 and TIM3 in CD8 T cells subsets.

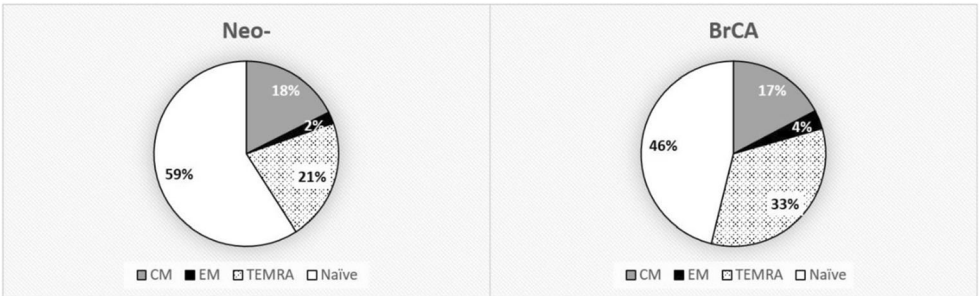

Fig. 1 BrCA patients had significantly lower percentage of Naïve CD8 T cells subsets and a higher frequency of EM CD8 T cells subsets. Frequency of Naïve, CM, EM and TEMRA CD8 T cell subsets in breast cancer patients (BrCA) and women without neoplasms (Neo-)

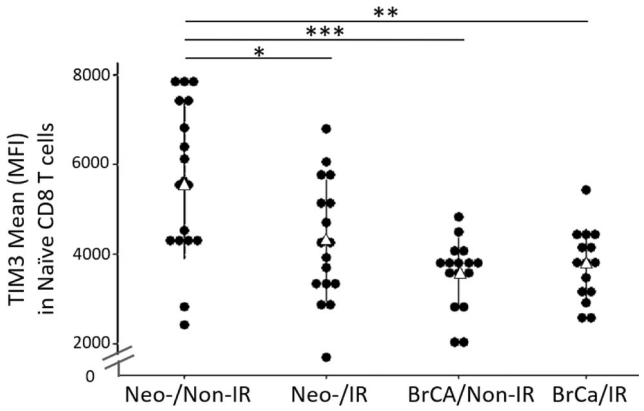

Fig. 2 Interaction between BrCa and IR for TIM3 expression on Naïve T cells. Mean fluorescence intensity (MFI) of TIM3 on Naïve CD8 T cells in Neo-/Non-IR, Neo-/IR, BrCa /Non-IR, BrCA/IR. White triangle represents the mean MFI of TIM3 expression on CD8 T cells. Vertical lines within the groups represents the range of Standar deviation (SD). *$p < 0.05$**$p < 0.01$, ***$p < 0.001$

Breast Cancer and Insulin Resistance Induce Changes in the Frequency of CD8 T Cells Expressing TIM3 and PD1

As we mention above, we found TIM3 expression on Naïve (Fig. 3a) and CM (Fig. 3b) subsets. Unexpectedly, BrCA patients had a lower percentage of PD1-TIM3 + CD8 T cells in these subsets compared with Neo- ($p = 0.003$). BrCA patients had greater PD1 + TIM3-CD8 T cell frequency in CM ($p = 0.004$, Fig. 4a), EM($p < 0.001$, Fig. 4b) and TEMRA($p = 0.006$, Fig. 4c) CD8 T cells subsets than Neo-. By contrast, IR+ women present a lower frequency than Non-IR (CM $p = 0.036$, EM $p < 0.001$, TEMRA $p = 0.005$, Fig. 4d-f). Furthermore, PD1-TIM3- frequency was lower in BrCA than in Neo- in EM ($p < 0.001$) and TEMRA ($p < 0.001$) subsets, whereas it was higher in IR+ than Non-IR women ($p = 0.001$, $p = 0.05$ respectively). In Naïve and CM subsets, the percentage of PD1-TIM3- is higher in IR+ than in Non-IR ($p = 0.03$, $p = 0.02$ respectively).

Insulin Concentration and HOMA Are Associated with the Frequency of PD1 + TIM3- CD8 T Lymphocytes, Adiponectin and Leptin Concentration

As we observed that CD8 T cells percentage expressing PD1 in IR+ is lower than Non-IR regardless of the presence or absence of BrCA, and hyperinsulinemia is often both a result and a driver of IR, we hypothesized that insulin levels and HOMA could be related with the percentage of CD8 T cells expressing PD1. We found a negative correlation between insulin levels, HOMA and the frequency PD1+ TIM3- in the EM cells ($r = -0.3$ $p = 0.03$ for both) (Fig. 5a, c) and in the TEMRA cells ($r = -0.3$ $p = 0.007$; $r = -0.3$ $p = 0.01$ respectively) (Fig. 5b, d). We also found a negative correlation between insulin levels, HOMA and adiponectin concentration ($r = -0.4$ $p = 0.002$; $r = -0.4$ $p = 0.003$ respectively) (Fig. 6a, b) and a positive correlation between insulin levels, HOMA and leptin concentration ($r = 0.4$ $p = 0.001$ for both) (Fig. 6c, d).

Adiponectin Concentration Is Associated with the Frequency of PD1 + TIM3- CD8 T Lymphocytes

As we mention above insulin alters adipocytokines concentration, which have effects in T lymphocytes, so we analyze the relation between adiponectin, leptin and the frequency of the variables that were significantly different. We found a positive correlation between adiponectin levels and the frequency PD1 + TIM3- in the CM cells ($r = 0.3$ $p = 0.007$) (Fig. 7a), in the EM cells ($r = 0.3$ $p = 0.008$) (Fig. 7c) and in the TEMRA cells ($r = 0.2$, $p = 0.048$) (Fig. 7e). Also, we detected a negative correlation between adiponectin levels and the frequency of PD1-TIM3- in the CM lymphocytes ($r = -0.3$ $p = 0.02$) (Fig. 7b) and in the EM lymphocytes ($r = 0.3$, $p = 0.008$) (Fig. 7d), but not in the TEMRA subset ($r = -0.2$, $p = 0.05$) (Fig. 7f). By contrast no correlation was found between the leptin concentration and the variables analyzed.

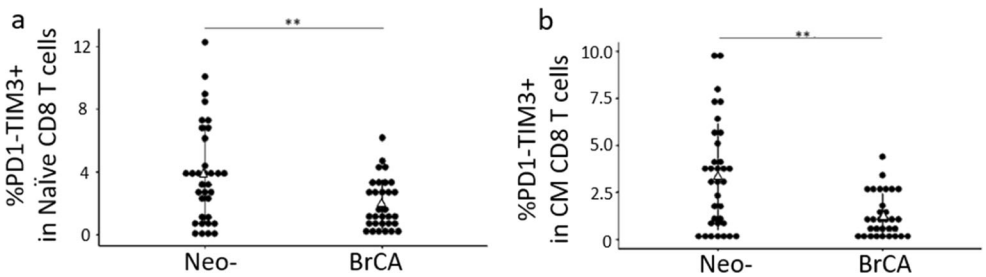

Fig. 3 PD1-TIM3+ CD8 T cells frequency is diminished in BrCA patients. Percentage of PD1-TIM3+ in Naïve CD8 T cells(**a**) and in CM CD8 T cells (**b**) in Neo- and BrCA. White triangle represents the mean percentage of PD1-TIM3+ on the CD8 T cells subsets. Vertical lines within the groups represents the range of Standard deviation (SD). **$p < 0.01$

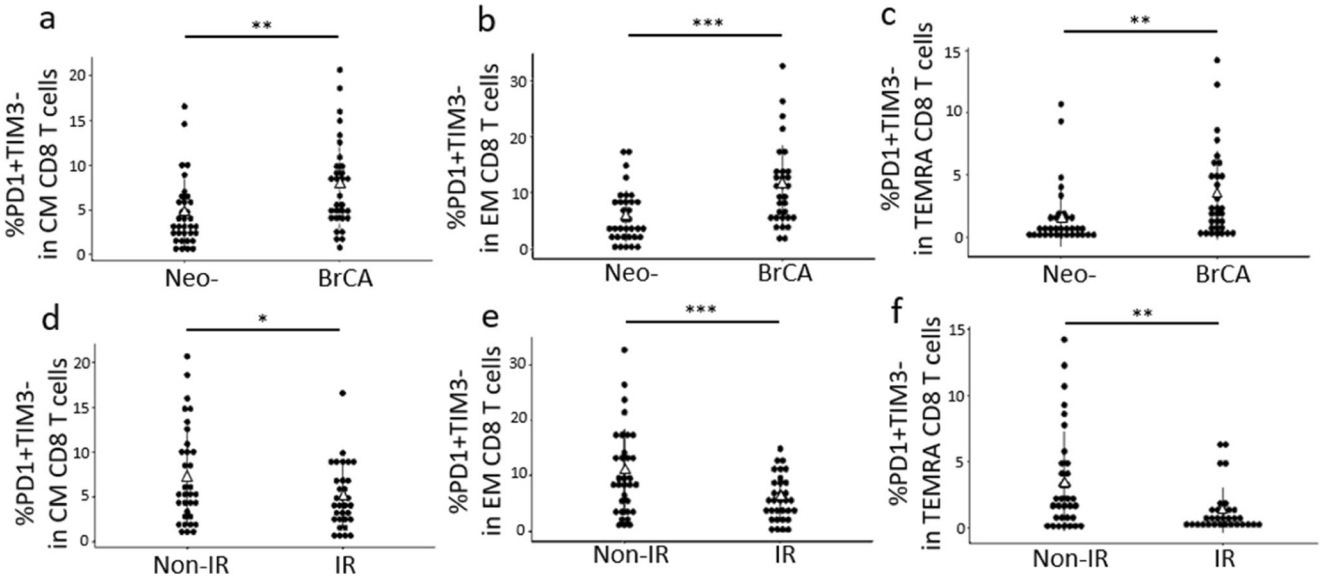

Fig. 4 PD1 + TIM3- CD8 T cells frequency is increased in BrCA patients and diminished in IR women. a-c Percentage of PD1 + TIM3- in CM (**a**), EM (**b**) and TEMRA CD8 T cells subsets(**c**) of BrCA and Neo-; (**d-f**) percentage of PD1 + TIM3 negative in CM (**d**), EM(**e**), TEMRA (**f**) CD8 T cells subsets of IR+ and Non-IR. White triangle represents the mean percentage of PD1 + TIM3- on the CD8 T cells subsets. Vertical lines within the groups represents the range of Standard deviation (SD). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

PD1 and TIM3 Expression on CD8 T Cells Subpopulations

We implemented a PCA to perform dimensional reduction of five immunological variables, the same that showed significant differences on one-factor ANOVA. Fig. 8a shows that the five principal components explain the complete variance and two principal components explain 80.6% of such variance. Online Resource 2 shows variable loading and correlation coefficients for principal components scores. We integrated these as follows: First component: Percentage of PD1 + TIM3- in CM, EM and TEMRA CD8 T cells. Second component: TIM3

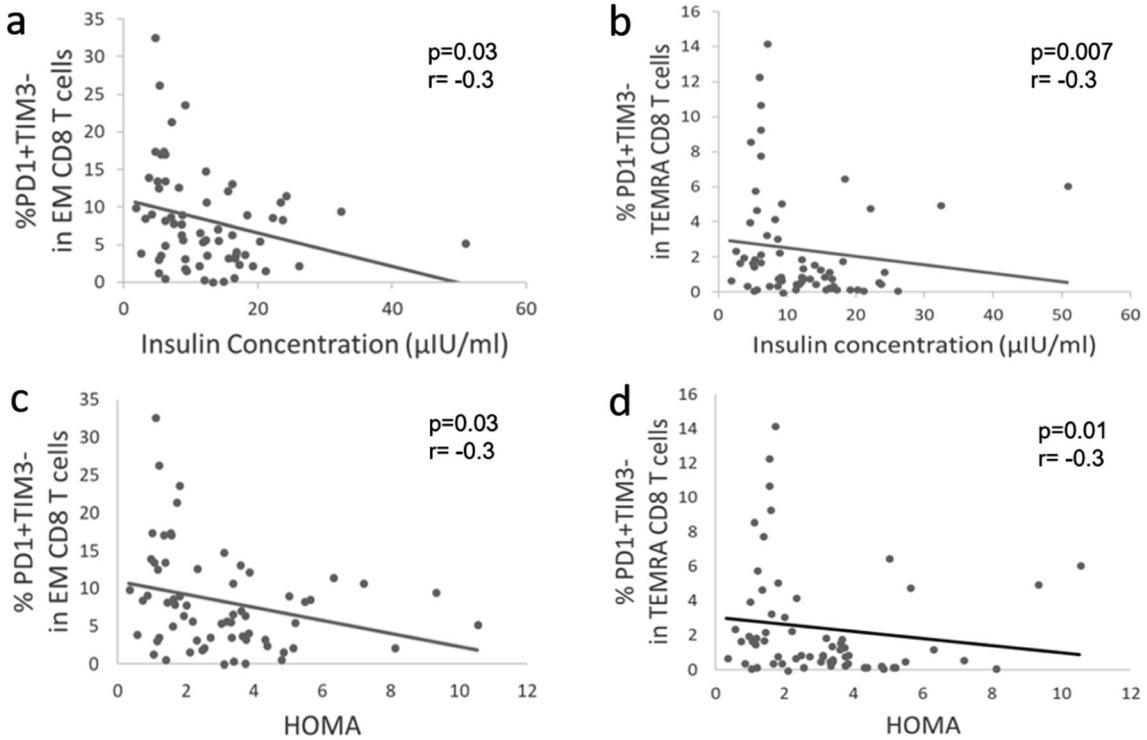

Fig. 5 HOMA-IR and Insulin correlate with PD1 + TIM3- EM and TEMRA CD8 T cells subsets. a-b Pearson correlation between insulin and PD1 + TIM3- EM (**a**) and TEMRA (**b**) CD8 T cell subsets; (**c-d**) Pearson correlation between HOMA and PD1 + TIM3- EM (**c**) and TEMRA (**d**) CD8 T cells subsets

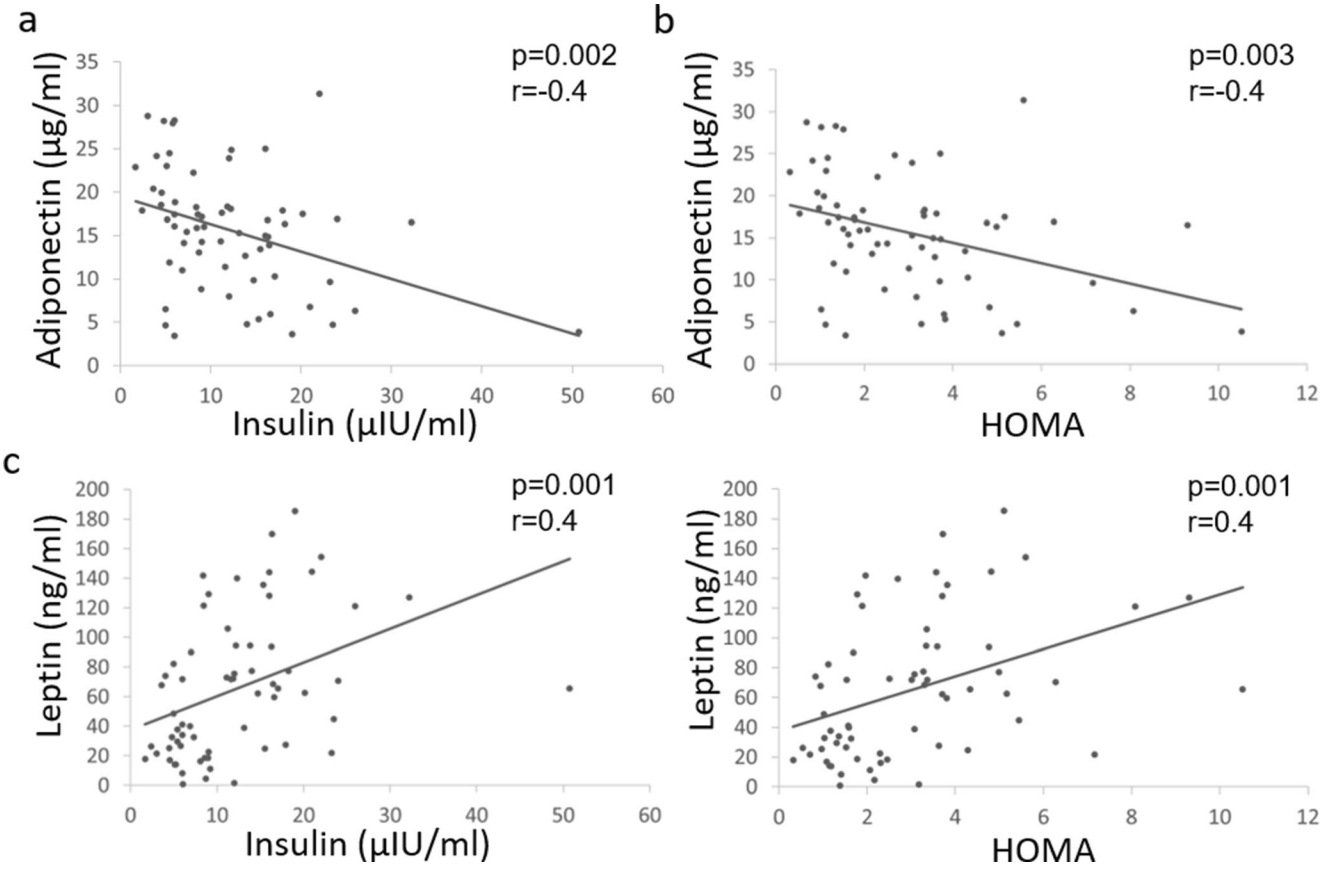

Fig. 6 HOMA-IR and Insulin correlate with adipocytokines. a-b Pearson correlation between insulin (**a**), HOMA(**b**) and adiponectin; (**c-d**) Pearson correlation between insulin (**c**), HOMA (**d**) and leptin

expression on Naïve and CM PD1-TIM3+ T CD8 cells. A FCM clustering algorithm was computed to the first two scores of principal components, in order to form clusters according their immunophenotypic profiles. The statistical clustering analysis yielded six different clusters. The Fig. 8b shows that 86% of BrCA/Non-IR are on cluster 6. Then, we applied correspondence analysis (CA) to associate the immunological clusters with the groups of Neo- and BrCA, Non-IR or IR+. The total Chi-squared value of CA was 40.69 (d.f. = 15, $p < 0.001$), and the first two dimensions explain 83.7% of the total inertia. According to this, visual analysis on Fig. 8c shows that clusters 2, 3 and 6 are associated with Neo-/IR, Neo-/Non-IR and BrCA/Non-IR, respectively. Finally, subjects in cluster 3 have overexpressed TIM3 on CM T CD8 cells that clusters 2 and 6, and in cluster 3 have higher percentage of PD1 negative TIM3 positive in Naïve and CM CD8 T cells that cluster 6 as shown in the table (Online Resource 3).

Discussion

In this study we found that both cancer and IR either together or separately have an effect on the different CD8 T subsets. We found that BrCA patients have a higher percentage of TEMRA and EM cells and a lower percentage of Naïve cells in peripheral blood; these are in accordance with results of previous studies made in early stages of BrCA [21] as in head and neck cancer [27]. Interestingly, Poshke et al. (2012) reported that the subset composition observed in peripheral blood of BrCa partially reflected the situation in tumor and that the pronounced tumor-induced T cell differentiation may be due to cytokine-driven expansion of the memory subsets in view of the low tumor antigen-specific T cell frequencies observed [21]. Furthermore, studies in head and neck cancer patients suggest that lower thymic output combined with rapid turnover of Naïve CD8 T cells account for altered lymphocyte homeostasis [27]. On the other hand, we found in IR+ no differences in CD8 T subsets, as reported by Fischer et al., probably because they are mostly Naïve and do not express the insulin receptor, but they start to upregulate it briefly after stimulation [12].

To our knowledge, the interaction between cancer and IR on CD8 T cells has not been fully explored. Remarkably, we found that TIM3 expression on Naïve CD8 T cells is lower in Neo-/IR+ and in BrCA Non-IR or IR+ than in Neo-/Non-IR and that the effect of IR on TIM3 expression on CM PD1-TIM3+ CD8 T cells may be inverse, depending on the presence or absence of BrCA. Although TIM3 is one of the most important molecules that mediate T cell exhaustion [28] and

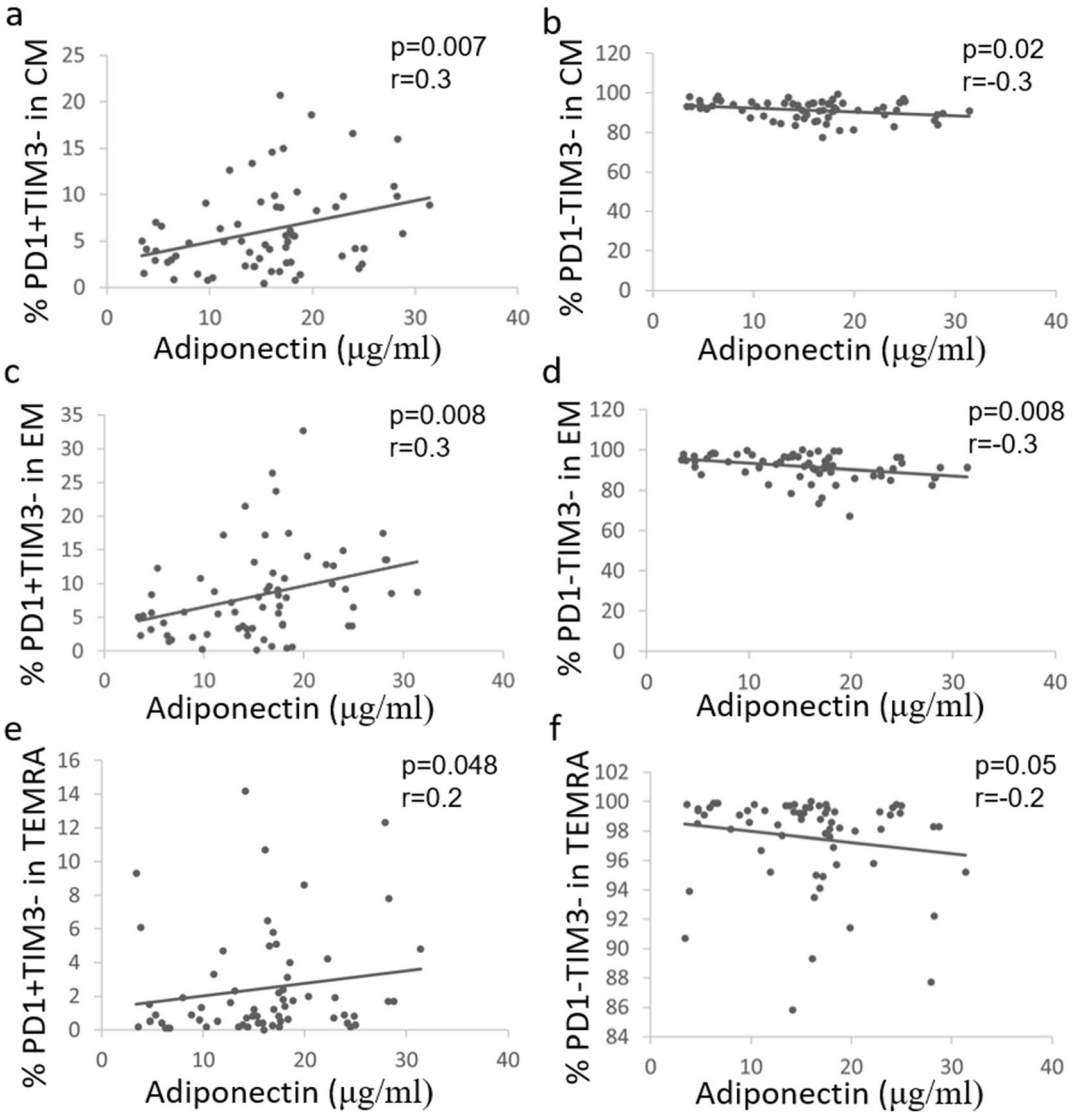

Fig. 7 Adiponectin correlates with PD1 + TIM3- and PD1-TIM3- in CD8 T cell subsets. **a,c,e** Pearson correlation between adiponectin and PD1 + TIM3- CM(**a**), EM(**c**) and TEMRA(**e**) CD8 T cell subsets; (**b,d,f**) Pearson correlation between adiponectin and PD1-TIM3- CM(**b**) EM(**d**) and TEMRA(**f**) CD8 T cells subsets

its expression on circulating CD8 T cells has been associated with advanced tumor stage, metastasis, and high tumor grade [29–31], TIM3 function on CD8 T cells is still controversial. Actually, in murine model TIM3 may enhance activation of antigen-specific T cells both during acute stimulation and within the memory compartment. Moreover, in the setting of TIM3-KO mice, there are fewer T cells getting initially activated, with poor memory recall response in both the acute and chronic setting [32]. Several studies have demonstrated that TIM3 function varies depending on the circumstance [25], and TIM3 can also promote T cell responses [33–35]. Furthermore, the crossover interaction observed in this study between BrCA and IR on TIM3 expression on CM CD8 T cells, strengthens the theory that the role of TIM3 varies according to the context. Taken together, we suggest that this decrease in TIM3 expression is related with increased risk of developing BrCA in insulin resistant women probably by two potential mechanisms: i) CD8 T cells couldn't respond adequately to activation, or reactivation due to the diminution of TIM3 ii) or TIM3 reduction in these subsets could be associated with the development of chronic inflammation. In accordance with the second mechanism, it is known that CD8+ T cells are crucially involved in initiating inflammatory cascades in obese adipose tissue [36]. In addition, it is known that chronic inflammation has been associated with cancer risk and development [37, 38].

Unexpectedly, our study in circulating CD8 T cells revealed a decrease in TIM3 expression in Naïve subpopulations, and a reduction in the frequency of PD1-TIM3+ populations within Naïve and CM subsets in BrCA patients compare with Neo-.

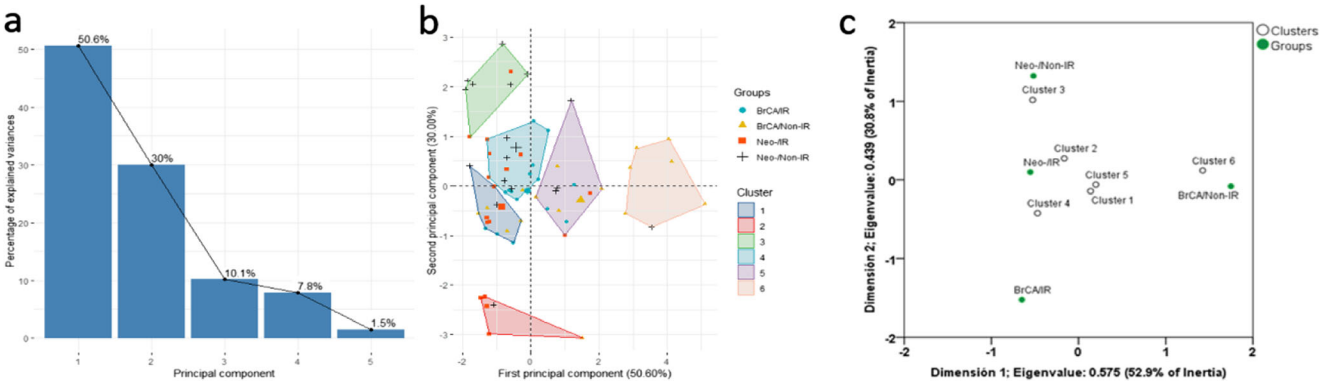

Fig. 8 Conglomerate analysis of PD1 and TIM3 on different subpopulations of CD8 T cells from Neo- and BrCa, Non-IR or IR+ shows that the first two components explains most of the variance. a Contribution of principal components to total variance of the immunophenotypes variables. The first two components explains 80.60% of total variance. **b** The biplot shows the distribution of the subjects according to two first principal components and analysis by clusters. Cluster 2 has 67% of Neo-/IR+, cluster 3 has 75% of Neo-/Non-IR and cluster 6 has 86% of BrCA/Non-IR. **c** The ordination diagrams shows the associations between the computed immunological clusters and the groups of Neo- and BrCA, IR+ or Non-IR. The dimensions 1 and 2 explain 52.9% and 30.8% of the inertia, respectively. PCA and Fuzzy C-Means clustering were applied with the R package FactoMineR, Factoextra and Ppclust

Usually cancer patients express more TIM3 than controls [29–31], maybe the decline of TIM3 expression could be attributed to the higher frequency of Naïve T cells in controls since its expression correlates positively with this percentage. In addition, it is important to considerate that in the studies where they found an increased percentage of TIM3+ CD8 T cells in cancer patients, they evaluated TIM3 on circulating CD8 T lymphocytes in cancer patients surgically treated or with a higher frequency of positive drinking history than controls [29–31]. While in our study, we evaluated treatment-naive cancer patients without a reported drinking history.

We also observed that PD1 + TIM3- CD8 T cells within CM, EM and TEMRA subsets were lower in IR+ and that there was a negative correlation between HOMA, insulin concentration and PD1 + TIM3- EM and TEMRA frequencies. These results suggest that high insulin levels can be related with an inflammatory state. According to results of a study performed in a murine model of diet-induced obesity with hyperinsulinemia and IR, Tregs had a significant reduction in IL-10 mRNA, a diminution of IL-10 production and an increase in IFNγ expression compared with normal mice [13]. Moreover, in a murine model, silencing of insulin receptor caused decrease of CD8 T cell cytotoxicity and reduced proliferation, suggesting that insulin receptor was necessary for full T cell activity in a physiological situation [12]. This impaired function could be related with a higher expression of PD1 as in our study when insulin levels are reduced, PD1+ populations are elevated. Meanwhile, children with type one diabetes failed to upregulate PD1 upon circulating CD8 T cell receptor stimulation and 4–6 months after insulin therapy PD1 normalizes [14]. Interestingly, PD1 alters T cell metabolic reprograming by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation [39]. In contrast, insulin promotes glycolysis and inhibits lipolysis and fatty acid oxidation. Based on this premise the negative correlation that we found between insulin concentration and PD1 + TIM3- CD8 T cells could result from the contrary effects of PD1 and insulin. It is interesting to note that a correlation was observed between HOMA and PD1 + TIM3- CD8 T cells frequency, although no relationship was observed between glucose levels and this subpopulation frequency.

In addition, we found a negative correlation between insulin levels, HOMA and adiponectin concentration and a positive correlation between insulin levels, HOMA and leptin concentration. These outcomes are in accordance with results of previous studies made in diet-induced insulin resistance mice [40] in the case of adiponectin and in accordance with results of previous studies made in Healthy North Indian Population [41] as in morbid obesity women [42] in the case of leptin. Furthermore, we detected a positive correlation between adiponectin levels and the frequency PD1 + TIM3- in the CM, EM and TEMRA T CD8 cells and a negative correlation between adiponectin levels and the frequency of PD1-TIM3- in the CM and EM lymphocytes. Suggesting that insulin could be related with PD1 + TIM3- and PD1-TIM3- frequencies by modulating the levels of some adipocytokines. It is known that adiponectin is implicated in the regulation of immune responses. Adiponectin has anti-inflammatory properties and several data suggest that adiponectin is a negative T cell regulator [43]. Notably, adiponectin treated dendric cells showed an increase in the expression of PD-L1 compared to untreated cells. In co-culture experiments of T cells and adiponectin-treated dendritic cells, a reduction in T cells proliferation and IL-2 production and a higher percentage of CD4 + CD25 + Foxp3+ Treg cells was observed [43, 44].

By contrast, although insulin stimulates leptin synthesis and secretion and this adipocytokine is able to regulate the

secretion of several cytokines from peripheral CD8 T cells [45], we didn't find any correlation between leptin levels and the percentage of PD1 + TIM3- nor PD1-TIM3-. Maybe this lack of association is related to a state of leptin resistance associated with obesity.

Taking together, it is reasonable to think that PD1 diminution within populations could be associated with BrCA progression, perhaps by promoting chronic inflammation, which is associated with cancer risk and development. In addition, was notable that most of BrCA/Non-IR were well differentiated by PCA but this conglomerated immunoprofile is lost when IR is present.

Further studies evaluating the function of CD8 T cells of each group used, as well as studies examining the possible mechanisms that leads to PD1 diminution in IR individuals, will be necessary to fully characterize changes in CD8 T cells of BrCA patients and/or IR.

In conclusion, our results show that both BrCA and IR, independently or in conjunction can affect co-inhibitory receptors on circulating CD8 T cells. The decrement in PD1+ and TIM3+ CD8 T cells populations in IR+ could be related with BrCA progression by participating in chronic inflammation or by CD8 T cell activation impairment in case of TIM3. Furthermore, our study suggests that insulin has an important role in CD8 T cell modulation that could be related with its regulation of adipocytokines. Finally, this study provides a novel association among BrCA, IR and immune system that needs further research for a better understanding. New therapeutic approaches like anti-inflammatory therapy could be developed to treat BrCA patients with IR.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval The study was approved by the ethical and research committees of the General ospital of Mexico "Dr. Eduardo Liceaga" (DI/15/UME/03/47 and DI/12/III/4/30). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

References

1. Sun Y-S, Zhao Z, Yang Z-N, Xu F, Lu HJ, Zhu ZY, Shi W, Jiang J, Yao PP, Zhu HP (2017) Risk Factors and Preventions of Breast Cancer. Int J Biol Sci 13:1387–1397
2. Sun W, Lu J, Wu S, Bi Y, Mu Y, Zhao J, Liu C, Chen L, Shi L, Li Q, Yang T, Yan L, Wan Q, Liu Y, Wang G, Luo Z, Tang X, Chen G, Huo Y, Gao Z, Su Q, Ye Z, Wang Y, Qin G, Deng H, Yu X, Shen F, Chen L, Zhao L, Wang T, Sun J, Xu M, Xu Y, Chen Y, Dai M, Zhang J, Zhang D, Lai S, Li D, Ning G, Wang W (2016) Association of insulin resistance with breast, ovarian, endometrial and cervical cancers in non-diabetic women. Am J Cancer Res 6: 2334–2344
3. Gunter MJ, Xie X, Xue X, Kabat GC, Rohan TE, Wassertheil-Smoller S, Ho GYF, Wylie-Rosett J, Greco T, Yu H, Beasley J, Strickler HD (2015) Breast cancer risk in metabolically healthy but overweight postmenopausal women. Cancer Res 75:270–274, Breast Cancer Risk in Metabolically Healthy but Overweight Postmenopausal Women
4. Nam S, Park S, Park HS, Kim S, Kim JY, Kim SI (2016) Association between insulin resistance and luminal B subtype breast cancer in postmenopausal women. Medicine (Baltimore) 95:e2825
5. Shanik MH, Xu Y, Skrha J, Dankner R, Zick Y, Roth J (2008) Insulin resistance and hyperinsulinemia: is hyperinsulinemia the cart or the horse? Diabetes Care Suppl 2:S262-S268
6. Arcidiacono B, Iiritano S, Nocera A et al (2012) Insulin resistance and cancer risk: An Overview of the pathogenetic mechanisms. Exp Diabetes Res 2012:789174
7. Yadav A, Kataria MA, Saini V, Yadav A (2013) Role of leptin and adiponectin in insulin resistance. Clin Chim Acta 18(417):80–84
8. Paz-Filho G, Mastronardi C, Wong ML, Licinio J (2012) Leptin therapy, insulin sensitivity, and glucose homeostasis. Indian J Endocrinol Metab 16(Suppl 3):S549–S555
9. Paz-Filho G, Lim EL, Wong ML, Licinio J (2011) Associations between adipokines and obesity-related cancer. Front Biosci (Landmark Ed) 16:1634–1650
10. Ali HR, Provenzano E, Dawson SJ, Blows FM, Liu B, Shah M, Earl HM, Poole CJ, Hiller L, Dunn JA, Bowden SJ, Twelves C, Bartlett JMS, Mahmoud SMA, Rakha E, Ellis IO, Liu S, Gao D, Nielsen TO, Pharoah PDP, Caldas C (2014) Association between CD8+ infiltration and breast cancer survival in 12, 439 patients. Ann Oncol 25:1536–1543
11. Matsumot H, Thike AA, Li H et al (2016) Increased CD4 and CD8-positive T cell infiltrate signifies good prognosis in a subset of triple-negative breast cancer. Breast Cancer Res Treat 156:237–247
12. Fischer HJ, Sie C, Schumnn E et al (2017) The insulin receptor plays a critical role in T cell function and adaptive immunity. J Immunol 198:1910–1920
13. Han JM, Patterson SJ, Speck M, Ehses JA, Levings MK (2014) Insulin inhibits IL-10-mediated regulatory T cell function: implications for obesity. J Immunol 192:623–629

14. Granados HM, Draghi A 2nd, Tsurutani N et al (2017) Programmed cell death-1, PD-1, is dysregulated in T cells from children with new onset type 1 diabetes. PLoS One 12:e0183887
15. Buchbinder EI, Desai A (2016) CTLA-4 and PD-1 pathways: similarities, differences, and implications of their inhibition. Am J Clin Oncol 39:98–106
16. Jin HT, Ahmed R, Okazaki T (2011) Role of PD-1 in regulating T-cell immunity. Curr Top Microbiol Immunol 350:17–37
17. Keir ME, Butte MJ, Freeman GJ, Sharpe AH (2008) PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 26:677–704
18. Sharpe AH, Pauken KE (2018) The diverse functions of the PD1 inhibitory pathway. Nat Rev Immunol 18:153–167
19. Muenst S, Soysal SD, Gao F, Obermann EC, Oertli D, Gillanders WE (2013) The presence of programmed death 1 (pd-1) positive tumor-infiltrating lymphocytes is associated with poor prognosis in human breast cancer. Breast Cancer Res Treat 139:667–676
20. Ghebeh H, Mohammed S, Al-Omair A et al (2006) The B7-H1 (PD-L1) T lymphocyte-inhibitory molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors. Neoplasia 8:190–198
21. Poschke I, De Boniface J, Mao Y, Kiessling R (2012) Tumor-induced changes in the phenotype of blood-derived and tumor-associated T cells of early stage breast cancer patients. Int J Cancer 131:1611–1620
22. Wherry EJ, Kurachi M (2015) Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol 15:486–499
23. Okoye IS, Houghton M, Tyrrell L, Barakat K, Elahi S (2017) Coinhibitory Receptor Expression and Immune Checkpoint Blockade: Maintaining a Balance in $CD8^+$ T Cell Responses to Chronic Viral Infections and Cancer. Front Immunol 8:1215
24. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, Kirkwood JM, Kuchroo V, Zarour HM (2010) Upregulation of TIM3 and PD-1 expression is associated with tumor antigen-specific CD8+T cell dysfunction in melanoma patients. J Exp Med 207:2175–2186
25. Gorman JV, Colgan JD (2014) Regulation of T cell responses by the receptor molecule Tim-3. Immunol Res 59:56–65
26. Fuertes-Marraco SA, Neubert NJ, Verdeil G, Speiser DE (2015) Inhibitory receptors beyond T cell exhaustion. Front Immunol 6:310
27. Kuss I, Schaefer C, Godfrey TE, Ferris RL, Harris JM, Gooding W, Whiteside TL (2005) Recent thymic emigrants and subsets of naïve and memory T cells in the circulation of patients with head and neck cancer. Clin Immunol 116:27–36
28. Anderson AC (2012) Tim-3, a negative regulator of anti-tumor immunity. Curr Opin Immunol 24:213–216
29. Zhang Y, Cai P, Li L, Shi L, Chang P, Liang T, Yang Q, Liu Y, Wang L, Hu L (2017) Co-expression of TIM-3 and CEACAM1 promotes T cell exhaustion in colorectal cancer patients. Int Immunopharmacol 43:210–218
30. Liu H, Zhi L, Duan N, Su P (2016) Abnormal expression of Tim-3 antigen on peripheral blood T cells is associated with progressive disease in osteosarcoma patients. FEBS Open Bio 6:807–815
31. Wu J, Liu C, Qin S, Hou H (2013) The expression of Tim3 in peripheral blood of ovarian cancer. DNA Cell Biol 32:648–653
32. Avery L, Filderman J, Szymczak-Worman AL, Kane LP (2018) Tim-3 co-stimulation promotes short-term effector T cells, restricts memory precursors and is dispensable for T cell exhaustion. Proc Natl Acad Sci U S A 115(10):2455–2460
33. Qiu Y, Chen J, Liao H et al (2012) Tim-3-expressing CD4(+) and CD8(+) T cells in human tuberculosis (TB) exhibit polarized effector memory phenotypes and stronger anti-TB effector functions. PLoS Pathog 8:e1002984
34. Gorman JV, Starbeck-Miller G, Pham NL et al (2014) Tim-3 directly enhances CD8 T cell responses to acute *Listeria monocytogenes* infection. J Immunol 192:3133–3142
35. Meggyes M, Miko E, Polgar B, Bogar B, Farkas B, Illes Z, Szereday L (2014) Peripheral blood TIM-3 positive NK and CD8+ T cells throughout pregnancy: TIM3/galectin-9 interaction and its possible role during pregnancy. PLoS One 9:e92371
36. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, Otsu M, Hara K, Ueki K, Sugiura S, Yoshimura K, Kadowaki T, Nagai R (2009) CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nat Med 15:914–920
37. DeNardo DG, Coussens LM (2007) Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. Breast Cancer Res 9:212
38. Pierce BL, Ballard-Barbash R, Bernstein L, Baumgartner RN, Neuhouser ML, Wener MH, Baumgartner KB, Gilliland FD, Sorensen BE, McTiernan A, Ulrich CM (2009) Elevated biomarkers of inflammation are associated with reduced survival among breast cancer patients. J Clin Oncol 27:3437–3444
39. Patsoukis N, Bardhan K, Chatterjee P, Sari D, Liu B, Bell LN, Karoly ED, Freeman GJ, Petkova V, Seth P, Li L, Boussiotis VA (2015) PD-1 alters T-cell metabolic reprogramming by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation. Nat Commun 6:6692
40. Maeda N, Shimomura I, Kishida K et al (2002) Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. Nat Med 8(7): 731–737
41. Yadav A, Jyoti P, Jain SK, Bhattacharjee J (2011) Correlation of adiponectin and leptin with insulin resistance: a pilot study in healthy north Indian population. Indian J Clin Biochem 26(2): 193–196
42. Osegbe I, Okpara H, Azinge E (2016) Relationship between serum leptin and insulin resistance among obese Nigerian women. Ann Afr Med 15(1):14–9
43. Carbone F, La Rocca C, Matarese G (2012) Immunological functions of leptin and adiponectin. Biochimie 94(10):2082–8, 2088
44. Tsang JY, Li D, Ho D, Peng J et al (2011) Novel immunomodulatory effects of adiponectin on dendritic cell functions. Int Immunopharmacol 11(5):604–609
45. Rodríguez L, Graniel J, Ortiz R (2007) Effect of leptin on activation and cytokine synthesis in peripheral blood lymphocytes of malnourished infected children. Clin Exp Immunol 148(3):478–485