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Original Article

Iron-binding proteins and C-reactive protein in Nipple Aspirate Fluids: role of Iron-driven inflammation in breast cancer microenvironment?

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Abstract: Breast cancer, a worldwide disease with increasing incidence, develops from ductal/lobular epithelium. Nipple aspirate fluid (NAF), secreted from the breast ducts and lobules, can be analyzed to assess metabolic activity in breast microenvironment. Premalignant and malignant cell alterations may produce biochemical signals that deliver inflammatory proteins to the site. C-reactive protein (CRP), acute-phase protein considered a prognostic marker of inflammation, is frequently over-expressed in invasive breast carcinomas. Starting from the evidence that soluble and cell-bound iron binding protein Ferritin (FTN) and Transferrin (TRF) are crucially involved in breast inflammation and cancer, the aim of the present study is to analyze in NAF (a ductal fluid mirroring the breast microenvironment noninvasively collected from healthy and proven breast cancer affected women, n=38), the concentrations of CRP, FTN and TRF through high sensitive immunoassays. We analysed also serum (n=35) and milk samples (n=20) from healthy subjects. The mean level of CRP in *Cancer* NAF was significantly higher than in No*Cancer* NAF ($P < 0.0001$), especially in postmenopausal patients. Moreover, in *Cancer* NAF we detected higher levels of TRF and FTN respect to *NoCancer* NAF ($P<0.001$). A highly significant positive correlation between FTN and CRP content ($Y= 2322x + 6.196$, $r^2 = 0.651$, $P<0.0001$) was found. These data may support the involvement of inflammation and deregulation of iron homeostasis in breast cancer etio-pathogenesis. The significant accumulation of CRP in NAF in conjunction to the disruption of iron homeostasis may help to identify women at higher breast cancer risk.

Keywords: Breast cancer, ferritin, iron, inflammation, nipple aspirate fluid, transferrin

Introduction

Iron (Fe) is an indispensable requirement for the vital biochemical activities, because Fe-containing proteins catalyze key reactions of many essential metabolic processes [1]. In healthy individuals, essentially all circulating plasma iron is bound to and transported by the transferrin protein (TFR), an 80 kDa glycoprotein that binds 2 atoms of Fe(III) with high affinity [2]; it delivers Fe to the cells via its binding to the transferrin receptor 1, internalizing it by a receptor-mediated endocytosis [3].

An excess of Fe in the intracellular pool is stored in ferritin (FTN), which is a 24-mer of 430–450 kDa composed of two subunits (H- and L-) storing about 4500 atoms of Fe [2, 3]. The sequestration of Fe within the shell of the FTN molecule prevents the formation of toxic free radical species, that otherwise may cause cellular damage; while FTN is mainly an intracellular protein, small amounts do occur in the serum, and this is usually proportional to the quantity of Fe in stores [4].

Some proteins involved in Fe metabolism may play a crucial role in the proliferation of human tumours [3, 4], in particular in breast cancer cells [5-7]. Generally, cancer cells have higher amounts of TFR receptors, as well as higher levels of TFR and FTN than their normal counterparts, taking up Fe at a higher rate [4]. Due to its Fe-binding properties, TFR is a growth fac-

tor required for all proliferating cells, both in healthy and neoplastic tissues [8]. The human breast cancer cell lines secrete TFR, and its secretion is enhanced by estrogens, suggesting that TFR may act as an autocrine growth factor by conferring a selective advantage to rapidly proliferating breast cancer cells and permitting tumour growth, expecially in poorly vascularised areas [4]. However, the mechanisms of the cancer-related TFR alterations remain largely unknown.

Some relationship may exist also between FTN and cancer; in fact, despite no increase in Fe stores, serum FTN is increased in patients suffering a number of neoplasms, including breast cancer [9, 10]. Generally, tumor cells contain lower quantities of FTN poor in Fe when compared to their normal counterparts [3]. Although there is evidence that neoplastic transformation may result in changes in the expression of molecules involved in cellular Fe metabolism (especially in breast cancer), only recently some studies have highlighted the hypothesis of a crucial role on iron in breast carcinogenesis, suggesting breast cancer as a "ferrotoxic disease", in which both iron overload and the deregulation of intracellular iron homeostasis (through the perturbations of both FTN and TFR expression) may be associated with the initiation and progression of breast cancer toward a more malignant phenotype [5-7].

The etiopathogenesis of human breast cancer (BC) is multi-factorial: in fact, many studies demonstrate the involvement of genetic, metabolic and environmental factors [11]. Even though a well known risk factor is represented by susceptibility genes (e.g., BRCA1 and 2), the main identified risk factors are hormonal and linked, in particular, to life time exposure to estrogens through variations in menarche, menopause, childbirth and personal choice (such as use of the contraceptive pill or hormone replacement therapy) [12]. On the other hand, the extensive research currently being done in stem cell biology, molecular biology, pathology, cancer genomics and proteomics are contributing to and will provide elucidation of all "breast microenvironmental factors" involved in breast cancer initiation and progression, giving insights into etiopathogenesis, early detection, and its potential prevention.

The possibility that iron-binding proteins and iron play a role in the development of breast cancer has received epidemiologically little attention, despite the fact that it has biological plausibility. In fact, it has been proposed that excess of body iron stores and/or elevated dietary intake of iron may increase the likelihood that free iron will be released from storage, inducing oxidative DNA damage and, thereby, contributing to increased risk of breast cancer by the enhancement of reactive oxygen species and related inflammation [5-7]. Moreover, increasing evidence highlighted the relationship between inflammation and cancer [13, 14], in particular in breast cancer [15]. Of particular interest, there are studies reporting in human cancer (including breast) an hypothetic link between inflammation and iron homeostasis [16, 17]; in fact, it has been suggested that an altered iron homeostasis may set the basis for an inflammatory microenvironment within the breast tissue, proning ductal/stromal cells to premalignant transformation [14] and BC initiation/progression [15], according to exciting but unresolved hypothesis [5, 6].

Starting from these evidences, it might be important to value if an alteration of iron-binding proteins and inflammation markers in the breast microenvironment may represent a risk factor in BC pathogenesis. The breast microenvironment may be easily studied through the analysis of noninvasively collected nipple aspirate fluid (NAF), which is a biological fluid secreted from ductal/lobular cells representing a useful tool for the early identification of women at high risk of developing BC [18]. NAF can be easily and noninvasively obtained through nipple aspiration from adult non-pregnant, non-lactating breasts; it can be obtained in more than 90% of pre- and post-menopausal subjects and is a low cost procedure causing minimal or no discomfort [19]. It consists of secreted proteins [20] and cells sloughed from ductal and lobular epithelium [21], and contains several biomarkers [22-25], and proteinases [26, 27] potentially useful as epidemiological and clinical research tools [28]. In addition, the intracrinology of NAF has provided a further panel of cancer biomarkers reflecting the hormonal status in breast microenvironment [29]. On the bases of its composition, it has been proposed to classify NAF in two types: 1) NoCancer NAF, found in healthy control women and in patients diagnosed with benign breast diseases, may represent a milieu resulting from a continuous ex-

change between ductal-alveolar components and plasma due to passage between adjacent, but not sealed, epithelial cells, that maintain their integrity and are not subject to proliferation and excessive exfoliation; 2) NAF, found in a high percentage of women affected by breast carcinoma, contains several biologically active compounds which may enhance the proliferation of epithelial cells its composition mainly reflecting the active metabolism of epithelial and stromal cells lining the duct [28, 30]. Because BC develops from ductal and lobular epithelium, the analysis of NAF has attracted considerable interest as a window to assess the metabolic activity within the mammary gland [20, 28, 29, 31].

Unexpectedly, only few data are available on NAF about inflammatory biomarkers, even though chronic subclinical inflammation may be associated as a pathogenic mediator with several human cancers [32], including breast [33]. This hypothesis is also supported by the observation of elevated levels of circulating biomarkers of inflammation (e.g., C-Reactive protein, CRP, a well-known acute phase protein produced by the liver in response to inflammatory stimuli [34]) in patients with prevalent cancer as well as in patients who develop cancer several years after the registration of an elevated CRP [35]. Although it has been recognized as prognostic marker for breast cancer survival [33], the association between plasma CRP and breast cancer is still controversial [36]. Recently, CRP has been detected at appreciable levels in NAF collected from women affected by benign breast diseases, suggesting that increased CRP levels in these breast ductal fluids may represent an early and noninvasive biomarker of inflammed and precarcinogenic breast microenvironment [37, 38].

Starting from the evidence that noninvasively collected nipple aspirate fluid (NAF) represents the breast microenvironment bathing ductal epithelial cells lining the terminal duct lobular unit in which may persist the proliferative growth conditions associated with carcinogenesis (including inflammation), we sought to evaluate in healthy and cancer NAF samples whether there may be a diverse homeostasis of iron-binding proteins in the breast microenvironment and whether an altered iron homeostasis in BC NAF may be related to inflammatory-related CRP levels, in order to study the possible role of iron-driven inflammation in breast cancer microenvironment and searching early inflammatory biomarkers related to early carcinogenic changes.

Materials and methods

Patients and sample collection

Among all subjects recruited for this study (n=60), we excluded 22 patients because of pregnancy within 3 years or who were medically treated during the previous year. Of the remaining 38 subjects, NAF was successfully collected from 35 women (92%), using a modified breast pump, in calibrated capillary tubes as described elsewhere [19, 21]. Women were required to give written informed consent; the present work was carried out in accordance with the ethical standards of the Helsinki Declaration and after the approvals of the Ethics Committee of the University 'Carlo Bo' of Urbino (protocol 18/CE).

The median volume of NAF collected was 300 μL (range 100–1500 μL). Without pooling, samples were snap-frozen and stored at –80°C until use. NAFs were centrifuged at 15,000 *g* for 15 min at 4°C, and the supernatants analyzed. To avoid possible interference of the age differences between cases and controls, we performed the age-adjustment based on the gamma distribution [39]. NAF samples were analyzed for total protein and then for iron-binding protein content. All samples were assayed at least in duplicate.

On the basis of whether the enrolled subjects had or did not have biopsy proven newly diagnosed BC, all the 38 recruited patients were classified into two categories: *Cancer*, if there was evidence of biopsy-proven carcinoma (n=19) and *NoCancer*, without evidence of breast malignancy (n=19). NAF samples were analyzed from only one breast. As stated previously, NAFs were successfully collected from 35 non-lactating women (92%) (ages ranged from 31 to 77 years): 16 out 19 (84.2%) healthy women without evidence of pre-cancer or cancer (*NoCancer,* median age of 40), and 19 (100 %) of patients with biopsy proven BC (*Cancer,* median age of 56). NAF samples from *Cancer* patients were always collected before the biopsy and/or the surgical treatment in the breast with the disease. For subjects with cancer, NAF was analyzed from the breast with the disease, and routinely visualized by ecographic

and mammography procedures. Cancer patients did not show any abnormal nipple discharge. Clinico-pathological characteristics (e.g., disease stage, tumour size, nodal status, or distant disease spread) were determined according to the American Joint Committee on Cancer TNM staging system for breast cancer [40].

Thirty-five blood samples were also collected from healthy subjects without any evidence of pre-cancer or cancer syndrome; after clotting, serum was prepared by centrifugation at 2000xg for 5 min and stored at -20°C until use.

Finally, twenty milk samples were obtained aseptically from healthy lactating women and stored at -30°C until analysis (within three weeks). The intermediate milk specimens (collected at 8-13 days after birth), thawed and centrifuged at 12,000xg for 15 min at 4°C and, after the top lipid layer was removed, the clear supernatants were analysed.

Biochemical determinations

Total protein concentrations in NAF, milk and serum samples were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce). Samples were centrifuged immediately after collection, and stored in microtubes at –70°C. C-Reactive protein (CRP) was assessed with a standard CRP ELISA kit (KMI Diagnostics). The protocol was adapted for clinical laboratory use by modifying it to address the small volumes of samples (in particular for the NAF samples) and to achieve significant quantitative detection of low levels (<1 μg/mL) of CRP in the NAF samples. Briefly, these modifications include diluting the samples and determining the protein concentration. The protein concentration was then used to normalize sample loading. In addition, the standard curve for the CRP ELISA was modified to better differentiate values in the low CRP ranges, as were expected in the NAF samples. For what concerns CRP assay in this study, the eligibility criteria were no known cancer and no apparent acute or chronic infections at blood and NAF sampling, thus excluding subjects with CRP ≥ 10 μg/mL and those in whom CRP was missing due to logistic reasons [32].

Transferrin (TFR) concentration was assayed by a turbidimetric method, using the SYNCHRON® System (Beckman Coulter) according to the manufacturer's instructions. In the reaction, TFR combines with specific monoclonal antibody to form insoluble antigen-antibody complexes. The system monitors the change in absorbance at 340 nanometers, which is proportional to the concentration of TFR in the sample and is calculated and expressed based upon a single-point calibration. TFR concentrations in serum and NAF samples were calculated according to the standard curves, generated using samples "spiked" with recombinant TFR (0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 g/L added) to assess the assay recovery function. The sensitivity limit of the assay is 0.3 g/L with a range of 1.0-10 g/L. The intra-assay and inter-assay variability were 3.2% and 4.5%, respectively.

Ferritin (FTN) was assayed by the ADVIA Centaur® Ferritin assay (Bayer Diagnostics), according to the manufacturer's instructions; it is a two -site sandwich immunoassay using direct chemiluminometric technology, which uses constant amounts of two anti-ferritin monoclonal antibodies. FTN concentrations in serum and NAF samples were calculated against a standard curve generated using serum and NAF samples "spiked" with recombinant FTN (2.5, 5, 10, 25, 50, 75, 150 μg/L added) to assess the immunoassay recovery function. The commercial kit utilizes two monospecific monoclonal antibodies raised against human FTN, that bind to non-overlapping epitopes on the FTN polypeptide, showing high-affinity binding to both native and recombinant FTN. The sensitivity limit of the assay is 0.5 μg/L with a range of 5-600 μg/L. The intra-assay and inter-assay variability are 5.4% and 4.8%, respectively.

To exclude in both assays the possible NAF "matrix" artefacts caused by interference substances (*e.g.,* lipids, hormones and peculiar proteins), we serially diluted randomly selected samples, reanalyzing them for the response linearity.

Statistical Analysis

Median values of continuous variables were computed for the various groups of subjects. Owing to the potential non-normality of the data, ranking procedures were used for all analyses with continuous variables. The Wilcoxon rank sum test was used to compare independent groups; the Wilcoxon signed ranks test was used to make within-group comparisons. Signifi-

Table 1. Demographic data for women analyzed in the present study (n=38)

Demographic data	NoCancer (n=19)	Cancer (n=19)	*P* value
No. NAF samples (% detectable)	16 (84.2)	19 (100)	-
Median age, years (Range)	40 (31-58)	56 (48-77)	-
Premenopausal, n (%)	9 (56.3)	5 (26.3)	<0.005
Use of BCP, n (%)	7 (43.8)	1 (5.3)	-
Age of menarche (±SE)	13 (±2)	11 (±3)	0.87
Parity (±SE)	2.5 (±2)	2 (±1.5)	0.0
Use of HRT	2.5 (±2)	*nonusers*	-

NAF= nipple aspirate fluid; BCP= birth control pills; HRT= hormone replacement therapy.

Table 2. Ferritin, Transferrin and C-Reactive protein concentrations in human nipple aspirate fluid and matched serum samples collected from healthy subjects and breast cancer patients (mean±ES)

	Serum (n=35)	Milk (n=20)	NAF (n=35)	
			NoCancer (n=16)	*Cancer* (n=19)
CRP (μg/L)	1.36 ± 0.17	0.018± 0.008	0.054 ± 0.008	0.12 ± 0.011
FTN (μg/L)	41.55 ± 7.36	57.24 ± 9.16	55.50 ± 7.21	280.25 ± 32.32
TFR (g/L)	2.90 ± 0.35	3.02 ± 0.11	2.82 ± 0.24	8.51 ± 0.46

cance was set at $P < 0.05$. Data were analysed with Prism software for windows, version 3.1 (Graph-Pad, San Diego).

Results

We were able to collect NAF from 35 (aged from 31 to 77 years) out of the 38 women studied (about 92% of successful collection). Median age of the subjects ranged from 40 years in the *NoCancer* to 56 in the *Cancer* group. In the *NoCancer* group most subjects were in premenopausal status (≈56%), while in the *Cancer* group the majority of patients were postmenopausal (≈74%); among the clinical/demographic variables compared, only age was related to cancer status. In fact, women with biopsy proven BC (*Cancer* patients) tended to be older than healthy counterparts (*NoCancer* subjects) (**Table 1**). Although the age was different between cases and controls, no statistically significant correlation between aluminium, iron-linked proteins levels and women's age was found; the age-adjustment did not affect metal and protein concentrations (data not shown).

After dilutions (ranging from 8- to 80-fold) of NAFs containing high FTN, TFR and CRP levels, a significant linearity of dilution and a correlation with proteins was found (Y= -0.15+73.18 x, r^2=0.975). The mean (± SE) of analytical recovery percentage of recombinant proteins added to the samples was 97(7)%. Intra- and inter-assay CVs with NAF samples were 5% and 6%, respectively. The curve generated using spiked samples paralleled the standard curve (data not shown). These data suggest that the NAF "matrix" (*i.e.* proteins, hormones, and lipids present in breast secretions) did not affect the TFR, FTN and CRP assay performance, originally developed for plasma/serum specimens.

As reported in **Table 2**, CRP was detected in all sera samples analyzed (n=35), and the mean value was 1.36±0.17 μg/mL (range 0.2-4.3 μg/mL).

For what concerns iron-binding proteins, as shown in **Table 2**, their concentrations in 35 human serum samples matched collected from our series of subjects revealed a mean content within the normal reference ranges. When serum samples were subdivided according to the breast diseases, no statistically significant differences were found among the mean levels (data not shown).

The mean levels of CRP in human serum were

significantly higher respect to the levels in all NAF samples (1.36±0.17 vs 0.089±0.009 μg/mL , $P < 0.0001$) (**Table 2**), even though no correlation was found between serum and NAF CRP values (Y=0.0007x+0.0877; r^2=0.0002; P=0.939, data not shown). According to previous studies [41], we found that human milk collected from healthy women contained significantly lower levels of CRP respect to serum samples (0.018±0.008 vs 1.36±0.17 μg/L, respectively; $P < 0.0001$); moreover, no correlation was found between serum and milk CRP values (data not shown). These data suggest that the differential presence of CRP in NAFs and milks did not merely reflect serum CRP levels and that is not simply transported in the breast gland via blood circulation (in particular during physiologic conditions like during lactation). In fact, according to previous studies [37, 38], CRP may be specifically accumulated in the breast microenvironment through mechanisms which remain, however, largely unknown. It is noteworthy that CRP may be selectively accumulated in milk during lactation in women affected by mastitis [41], suggesting that a specific uptake, secretion and transport of CRP in human breast gland may occur.

To evaluate if a peculiar CRP accumulation may occur in physio-pathological breast conditions (e.g., healthy, benign breast diseases and cancer), we have analysed nipple aspirate fluid (NAF), a noninvasively collected breast fluid mirroring the breast microenvironment and the pathways of production, secretion and accumulation of biocompounds, fluid and cells directly from the breast ductal tree [19, 28].

From the 38 women studied, we were able to collect NAF in 35 subjects (aged from 31 to 77 years) with about 92% of successful collection. CRP was detected at appreciable levels in all NAF samples examined; in *Cancer* NAFs (n=19) we found the highest value (0.236 μg/mL) with mean levels significantly higher than those found in *NoCancer* NAFs (n=16) (0.12±0.011 vs 0.054 ± 0.008 μg/mL, respectively; $P < 0.0001$) (**Table 2**).

Starting from the evidence that NAF samples from both *NoCancer* and *Cancer* women contained higher levels of CRP respect to milk specimens (0.054±0.008 and 0.12±0.011 μg/L vs 0.018±0.008 μg/L, respectively; $P<0.0001$), we evaluated if there were differences in CRP accumulation among different subclasses of NAF, according to pathologies, age and cancer characteristics.

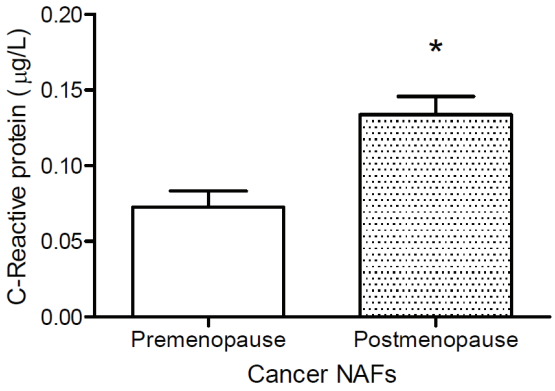

Figure 1. C-Reactive Protein concentrations in Nipple Aspirate Fluids according to menopausal status. In *Cancer* NAFs (n=19) we found significantly higher mean levels of CRP in post-menopausal (n=14) respect to those found in pre-menopausal women (n=5) (0.134±0.012 vs 0.073±0.011 μg/L , respectively; P=0.011).

NoCancer NAFs (n=16) were further subdivided in samples collected from healthy subjects (n=10) and women with benign hyperplasic lesions (e.g., atypical duct hyperplasia, n=6), finding no statistically significant differences between CRP mean levels (0.052±0.009 vs 0.057±0.017 μg/L; data not shown). In *NoCancer* subjects (n=16) CRP concentrations did not show any significant difference between pre- and post-menopausal status (0.054±0.011 vs 0.054±0.015 μg/L, respectively; data not shown), whereas in *Cancer* NAFs (n=19) we found significantly higher mean levels of CRP in post-menopausal (n=14) respect to those found in pre-menopausal women (n=5) (0.134±0.012 vs 0.073±0.011 μg/L , respectively; P=0.011) (**Figure 1**). Furthermore, even though in a limited number of women with *in situ* ductal breast carcinoma (n=4), we showed that in their NAF samples there were significantly lower median levels of CRP respect to those found in patients bearing invasive breast carcinoma (n= 15) (0.067±0.012 vs 0.131±0.012 μg/L , P=0.014) (**Figure 2**).

In order to evaluate the iron-binding protein homeostasis, we assayed the content of both ferritin (FTN) and transferrin (TRF) in all NAF, milk

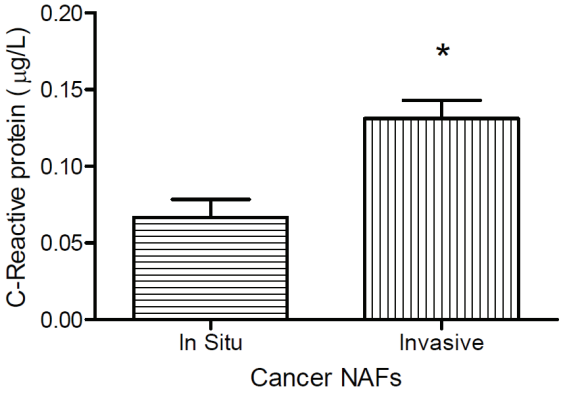

Figure 2. C-Reactive Protein concentrations in Nipple Aspirate Fluids noninvasively collected from Cancer patients. Women with *in situ* ductal breast carcinoma (n=4) showed that in their NAF samples significantly lower mean levels of CRP respect to those found in patients bearing proven invasive breast carcinoma (n= 15) (0.067±0.012 vs 0.131±0.012 µg/L, *P*=0.014).

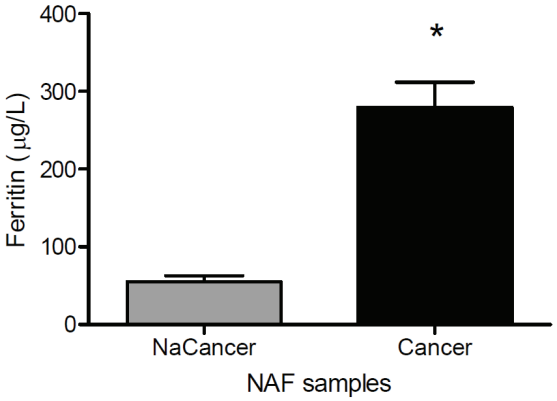

Figure 3. Ferritin concentrations in Nipple Aspirate Fluids from healthy subject and cancer women. FTN mean value in *NoCancer* NAFs (detectable in all healthy women without BC evidence, n=16) was significantly lower than that in *Cancer* NAFs (detectable in all BC bearing patients, n=19) (55.50±7.21 vs 280.25±32.32µg/L, *P*<0.0001, respectively).

and serum samples. As reported in **Table 2**, we found that all serum and NAF samples contained appreciable levels of iron homeostasis-linked proteins. FTN was detectable in all NAFs (n=35) with a total mean level significantly higher compared to total serum level (n=35) (177.0±26.0 vs 41.55±7.36 µg/L, *P*<0.001). We found no significant difference in FTN mean level between serum (n=35) and NAF samples from *NoCancer* women (n=16) (41.55±7.36 vs 55.50±7.21, respectively), whereas a significant higher FTN mean level was found in *Cancer* NAF (n=19) compared to serum (280.25±32.32 vs 41.55±7.36 µg/L, *P*<0.0001). FTN mean value in *NoCancer* NAFs (detectable in all healthy women without BC evidence, n=16) was significantly lower than that in *Cancer* NAFs (detectable in all BC bearing patients, n=19) (55.50±7.21 vs 280.25±32.32µg/L, *P*<0.0001, respectively) (**Figure 3**). Related to menopausal status, in post-menopausal subjects we found a significantly higher FTN mean level in *Cancer* (n=14 out of 19) compared to *NoCancer* (n=7 out of 16) NAFs (324.00±37.00 vs 58.20±10.50 µg/L, *P*<0.0001), as well as in pre-menopausal NAFs we detected significant difference in FTN mean levels between *NoCancer* (n=9 out of 16) and *Cancer* (n=5 out of 19) (53.50±10.30 vs 156.00±11.80 µg/L, *P*<0.0001) (**Figure 4**). Interestingly, NAF collected in Cancer condition (n=19) showed FTN mean levels significantly different between pre-

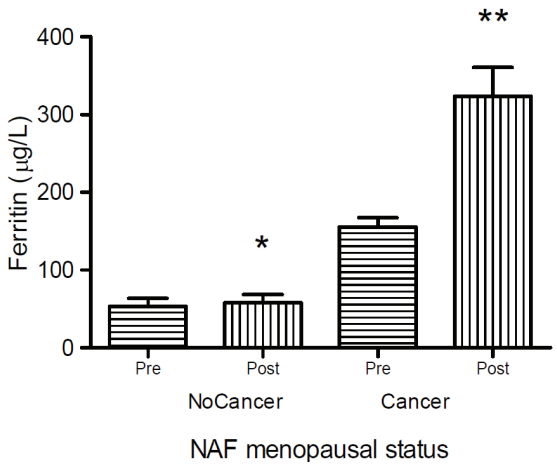

Figure 4. Ferritin levels in Nipple Aspirate Fluids from NoCancer and Cancer women grouped according to the menopausal status. In post-menopausal subjects we found a significantly higher FTN mean level in *Cancer* (n=14 out of 19) compared to *NoCancer* (n=7 out of 16) NAFs (324.00±37.00 vs 58.20 ± 10.50 µg/L, *P*<0.0001), as well as in pre-menopausal NAFs we detected significant difference in FTN mean levels between *NoCancer* (n=9 out of 16) and *Cancer* (n=5 out of 19) (53.50±10.30 vs 156.00±11.80 µg/L, *P*<0.0001). NAF collected in Cancer condition (n=19) showed FTN mean levels significantly different between pre- and post-menopause patients (156.00±11.80 vs 324.00 ± 37.00 µg/L, *P*=0.0167), whereas in NoCancer NAF no significant difference of FTN mean levels were found according to menopausal status (53.46 ± 10.34 vs 58.21±10.55 µg/L).

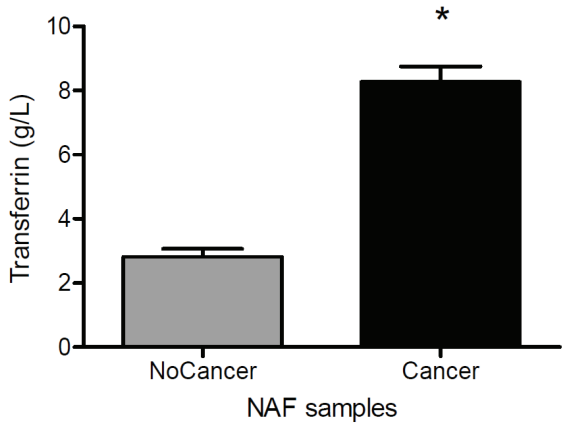

Figure 5. Transferrin levels in Nipple Aspirate Fluids noninvasively collected from NoCancer and Cancer women. TRF mean value in *NoCancer* NAFs (detectable in all healthy women without evidence of BC, n=16) was significantly lower than that in *Cancer* NAFs (detectable in all BC bearing patients, n=19) (2.82±0.24 vs 8.51±0.46 g/L, respectively; *P*<0.0001).

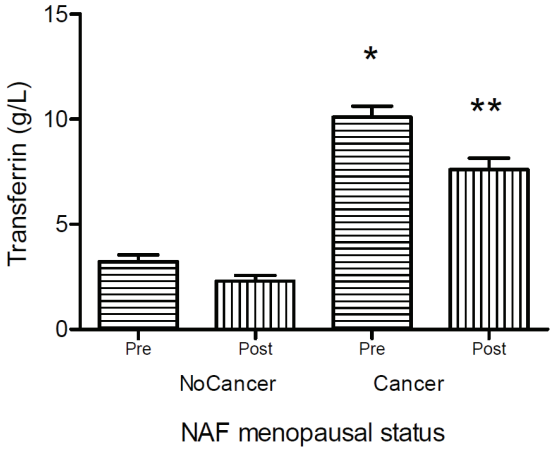

Figure 6. Transferrin concentrations in Nipple Aspirate Fluids noninvasively collected from NoCancer and Cancer women according to menopausal status. In post-menopausal subjects a significantly higher TRF mean level in *Cancer* (n=14 out of 19) compared to *NoCancer* (n=7 out of 16) NAFs (7.61±0.54 vs 2.3±0.27 μg/L, *P*<0.0001) was found, as well as in pre-menopausal NAFs we detected significant difference in TRF mean levels between *NoCancer* (n=9 out of 16) and *Cancer* (n=5 out of 19) (3.23±0.32 vs 10.09±0.53 μg/L, *P*<0.0001). NAF collected in Cancer condition (n=19) showed TRF mean levels significantly different between pre and post-menopause patients (10.09±0.53 vs 7.61±0.54 μg/L, *P*=0.0201), whereas in NoCancer NAF no significant difference of TRF mean levels were found according to menopausal status (3.23±0.32 vs 2.30±0.27 μg/L).

and post-menopause patients (156.00±11.80 vs 324.00±37.00 μg/L, *P*=0.0167), whereas in NoCancer NAF no significant difference of FTN mean levels were found according to menopausal status (53.46±10.34 vs 58.21±10.55 μg/L).

Finally, we found no significant difference in FTN mean level between serum (n=35), milk specimens (n= 20) and NAF samples from *NoCancer* women (n=16) (41.55±7.36, 57.24± 9.16 and 55.50±7.21 μg/L, respectively).

For what concerns TRF, we found that it was detectable in all NAFs (n=35) with a total mean level significantly higher compared to total serum level (n=35) (5.91±0.56 vs 2.90±0.35 g/L, *P*<0.001) (data not shown). We found no significant difference in TRF mean level between serum (n=35) and NAF samples from *NoCancer* women (n=16) (2.90±0.35 vs 2.82±0.24 g/L, respectively), whereas a significant higher TRF mean level was found in *Cancer* NAF (n=19) compared to serum (8.51±0.46 vs 2.90±0.35 g/L, *P*<0.0001) (**Table 2**). TRF mean value in *NoCancer* NAFs (detectable in all healthy women without evidence of BC, n=16) was significantly lower than that in *Cancer* NAFs (detectable in all BC bearing patients, n=19) (2.82±0.24 vs 8.51±0.46 g/L, respectively; *P*<0.0001) (**Figure 5**). Related to menopausal status (**Figure 6**), in post-menopausal subjects we found a significantly higher TRF mean level in *Cancer* (n=14 out of 19) compared to *NoCancer* (n=7 out of 16) NAFs (7.61±0.54 vs 2.3±0.27 μg/L, *P*<0.0001), as well as in pre-menopausal NAFs we detected significant difference in TRF mean levels between *NoCancer* (n=9 out of 16) and *Cancer* (n=5 out of 19) (3.23±0.32 vs 10.09±0.53 μg/L, *P*<0.0001). Interestingly, NAF collected in Cancer condition (n=19) showed TRF mean levels significantly different between pre and post-menopause patients (10.09±0.53 vs 7.61±0.54 μg/L, *P*=0.0201), whereas in NoCancer NAF no significant difference of TRF mean levels were found according to menopausal status (3.23±0.32 vs 2.30±0.27 μg/L). Moreover, we found no significant difference in TRF mean level between serum (n=35), milk specimens (n= 20) and NAF samples from *NoCancer* women (n=16)

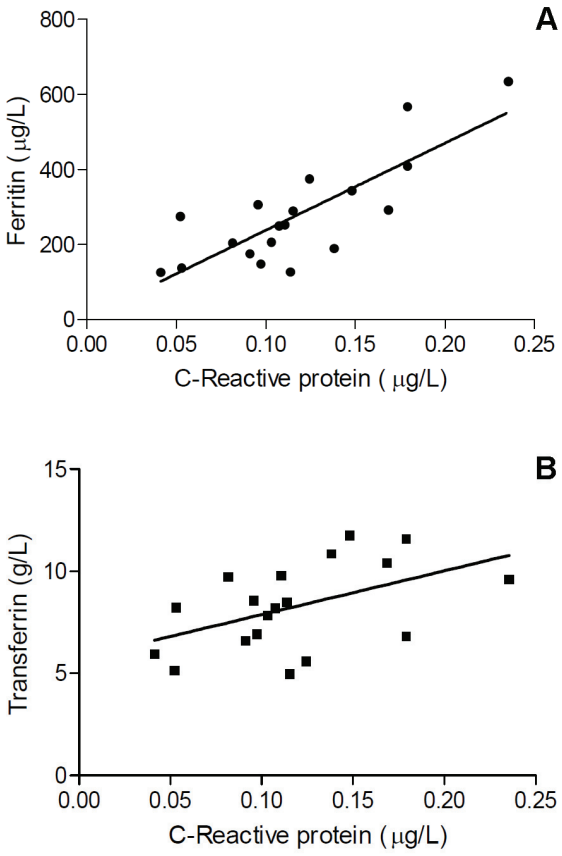

Figure 7. Correlation and regression analysis among the concentrations of C-reactive protein, Ferritin and Transferrin in Nipple Aspirate Fluids collected from Cancer patients. (**A**) A highly significant correlation between CRP and FTN levels (Y= 2322x + 6.196, r^2 = 0.651, *P*<0.0001) was obtained , whereas in (**B**), a positive and slightly significant correlation between CRP and TRF amounts (Y= 21.42x + 5.742, r^2 = 0.2434, *P*=0.0318) was shown.

(2.90±0.35, 3.02±0.11 and 2.82±0.24 μg/L, respectively).

Finally, evaluating the possible relationships between CRP content/accumulation and expression of iron-binding proteins in breast microenvironment, we found that only in NAF collected from *Cancer* patients there were positive and significant correlations. In fact, as shown in **Figure 7A** and **B**, in *Cancer* NAF there was a significant correlation between CRP and FTN levels (Y= 2322x + 6.196, r^2 = 0.651, *P*<0.0001) (**Figure 7A**) as well as CRP and TRF amounts (Y= 21.42x + 5.742, r^2 = 0.2434, *P*=0.0318) (**Figure 7B**), whereas neither FTN nor TRF levels were significantly related to CRP accumulation in NAF collected from healthy subjects (*NoCancer*) (Y= 105x + 49.85, r^2 = 0.014, *P*=0.6583 and Y=2.676x +2.678, r^2 = 0.008, *P*=0.737 respectively; data not shown).

Discussion

Nipple aspirate fluid (NAF) holds promise as a clinical diagnostic tool to detect precarcinogenic states in epithelial cells lining the terminal duct lobular unit (TDLU); NAF operates as a "protein factory" [42], synthesizing proteins and delivering them into the breast fluids that subsequently bathe the ductal lining influencing cell microenvironment [18, 20, 23].

As the ductal epithelial cells persist in the proliferative growth associated with carcinogenesis [21, 24], NAF proteins (and potentially inflammatory proteins) become concentrated in this mammary ductal fluid [19, 20, 23, 25]. Furthermore, at the interepithelial cell junctions, protective serum proteins, such as immunoglobulins, and toxic and mutagenic substances move into the fluid, resulting in a bioactive, pooled substrates reflective of and interacting with the breast microenvironment [18, 21, 24]. At this cell surface, the origin of most breast carcinomas, molecular and cellular changes in the microenvironment stimulate immune processes, resulting in a local inflammatory response [43].

Breast epithelial-cell carcinogenesis typically follows the transformation of the cell by carcinogenic stimuli; once a cell has deviated from its normal function, it emits signals recognized as foreign by the inflammatory-response detection mechanisms [44, 45]. Although it has been hypothesized that benign cellular changes in the breast intralumen may result "from inflammatory or traumatic conditions, notably in connective tissues" [46], several studies have further demonstrated that tumor cells in the breast microenvironment can induce the inflammatory cascade [47], demonstrating that inflammation may be related to early carcinogenic changes and that markers of the inflammatory response, then, may be indicative of breast cancer risk [13, 33].

Breast cancer (BC) is the third most common worldwide malignancy among women, and is continuously increasing in incidence [48]. The

high frequency of human BC and the observations that it develops from ductal and lobular epithelium [49], emphasizes the urgent need both to understand the mechanisms involved in breast tumorigenesis and to find alternative methods to invasive biopsy for early diagnosis of BC [19]. An innovative method to assess the metabolic activity within the mammary gland is the multidisciplinary analysis of NAF, which represents the mirror of the breast tissue to noninvasively analyze the breast microenvironment [18, 19, 25, 28, 31, 49]. NAF, as mammary microenvironment, is thus of particular interest in the search for early biomarkers of malignancy in the breast [28, 31, 49].

In the frame to searching reliable NAF protein as early biomarkers of breast malignancy [18, 20] and according to the hypothesis linking inflammation with altered iron-homeostasis in BC [15, 36], in the present study we have evaluated in NAF collected from healthy and BC patients the amounts CRP , FTN and TRF, in order to find possible correlations among inflammatory and iron-bound proteins.

The findings from our study suggest that CRP represent a noninvasive biomarker that may help to detect a carcinogenic breast ductal microenvironment. In fact, although CRP in serum was at higher concentration respect to NAF levels, Cancer NAF contained significantly increased amount of CRP (about two-fold higher) than that found in NAF collected from healthy women. The presence of CRP in the NAF from the breast ductal microenvironment is a significant finding in that this acute-phase protein is not believed to be generated and released until after activation of the inflammatory cascade; in this respect, we found that CRP was found at appreciable/detectable levels in both healthy NAF and normal milk without any inflammatory condition. Furthermore, this protein's presence in NAF did not simply reflect its presence systemically in this sample, as serum CRP was not significantly related to NAF CRP. C-reactive protein (CRP) is an acute-phase protein synthesized in the liver and circulating routinely in the vasculature at low levels, although standard normal levels in biological fluids remain poorly defined. CRP is considered a classic marker for inflammation acting as a surveillance molecule for altered body cells, rapidly elevates 1,000-fold within hours after tissue damage, and remains elevated in the presence of continued inflammation [32, 50, 51]. The baseline plasma protein, a biomarker in some human cancers and an indicator of cancer disease prognosis in some sites [32], has not been associated with the increased risk of BC in apparently healthy women [36], but this is a matter of more recent debate. In fact, increased levels of serum CRP have been detected in more advanced stages of breast malignancy [33, 52]. These finding have led to the proposed use of CRP as a biomarker of BC prognostics [32, 53]. According to previous studies performed in pooled NAF and suggesting that CRP may indicate local inflammation and contribute to the diagnosis of BC early when progress is favourable [37, 38], the results obtained with the present study support the hypothesis that the proliferative changes occurring in the ductal epithelium after carcinoma are associated with the possible activation of the inflammatory cascade, resulting in differential levels of CRP in NAF; in fact, we found that NAF collected from patients with invasive BC contained higher CRP levels respect to NAF samples collected from both healthy women and patients affected by in situ carcinoma. Moreover, we have found that Cancer NAF from post-menopausal women contained higher amounts of CRP respect to premenopausal cancer condition, suggesting a potential role of CRP accumulation in breast microenvironment during BC evolution/progression. Therefore, if CRP is quantitatively responsive to the magnitude of early cellular or tissue damage of malignancy, it may be a candidate biomarker of the activation of the inflammatory process in premalignant stages. Studies are in progress in more wide cohorts evaluating if CRP is differentially accumulated in NAF collected from benign breast diseases, proliferatice hyperplastic lesions, in situ carcinoma, lobular and ductal invasive BC with different anatomo-pathologic characteristics.

For what concern the iron homeostasis during BC initiation and progression, it is well known the hypothesis that alterations of the iron metabolism (in particular FTN and TRF proteins) in neoplastic cells may facilitate their growth and proliferation [3], suggesting an association between iron overload and cancer risk [4]; moreover, studies have highlighted the identification of iron as carcinogenic metal suggesting BC as a "ferrotoxic disease" [4, 7]. Through significantly different molecular mechanisms of the metabolism and transport of iron [5], normal

and neoplastic breast cells may peculiarly transport/accumulate/metabolize iron molecules both in pre- and post-menopause conditions, leading to different BC recurrence, incidence and overall survival [6].

Starting from these evidences, we have found for the first time that both iron-binding proteins FTN and TRF are present in appreciable levels in all breast ductal fluids; moreover, NAF collected from healthy women contained no significant different amounts of both proteins from that found in normal serum and human milk. On the contrary, we found in Cancer NAF (especially in post-menopausal condition) significantly increased levels of both iron-bound proteins respect to NoCancer NAF, milk and serum. Our present findings are in accordance to the evidence that both FTN and TRF proteins are much less saturated in premenopausal than in postmenopausal women [54], supporting the "iron-linked breast cancer" hypothesis that in young premenopausal women systemic oestrogen concentrations are high and iron concentrations are low [6]. On the other hand, in Cancer NAF (in particular in post-menopausal women) there is the reverse biological situation of that in premenopause (i.e., low systemic oestrogen concentrations due to cessation of menstruation, and high iron concentrations) [6]. Starting from the evidence that the way in which an overall low concentration of estrogen contributes to a high BC-incidence in post-menopausal women is not completely understood, the Huang's hypothesis [6] suggests that increased iron concentrations after the menopause could be an important etiological factor in the development of BC in this population, through reactive oxygen species formation and lipid peroxidation. Furthermore, superoxide anions may cause iron release from ferritin, enhancing the onco-promoter effects of ROS; this profile is in accordance to our previous data reporting down-regulation of SOD-1 in Cancer NAF [55]. Finally, according to the "iron-linked BC" hypothesis (suggesting the role of iron load in the incidence of BC in postmenopausal women)[6], our findings of both FTN and TRF increased levels in Cancer NAF may help the understanding of the role of iron imbalance in BC, supporting the possible association between iron and BC recurrence in post-menopausal women. In this respect, it is noteworthy to highlight the recent observation that perturbations in ferritin levels and deregulation of iron homeostasis (including TRF protein alteration) in several human breast cancer cell lines are associated with the progression of BC toward a more advanced malignant phenotype [10].

Finally, it is well established through epidemiological studies that chronic inflammation predisposes to different forms of human cancer [13], and that an inflammatory component is present in the microenvironment of most neoplastic tissues, including those not causally related to an obvious inflammatory process (like breast cancer) [15]. According to the role of soluble and cell-bound iron-binding proteins in conditions of both inflammation and tumor (especially in human breast gland) [16, 17], for the first time our data demonstrate that in breast microenvironment of only cancer conditions a significant and positive relationship between CRP and enhanced accumulation of both FTN and TRF levels exists, in particular in NAF collected from malignant breast cancers. In particular, our findings in Cancer NAF of highly statistical significant correlation between acute phase-protein CRP and iron storage protein FTN ($Y= 2322x + 6.196$, $r^2 = 0.651$, $P<0.0001$) may represent an interesting topic for future translational research in breast microenvironment, on the basis of the crucial link between enhanced local inflammation [38] and a deregulation of breast cell iron homeostasis [10]. Although our study is based on a limited sample size (and then needing confirm on a wide number of healthy and breast cancer patients), our data may open new translational research about important aspects that has not fully been investigated, i.e., the considerable difference in soluble and cell-bound iron binding protein concentrations before and after menopause in conjunction to the crucial role of local chronic inflammation in breast tissue. Both C-reactive protein and ferritin accumulation in NAF collected from BC patients (especially in post-menopause condition) may actively activate cellular signalling through oxidative-stress pathways and promote cell proliferation/growth through particular ferritin chains in metabolically active epithelial cells bathing in breast cancer microenvironment; our results suggest that measuring soluble CRP, FTN and TRF in NAF may improve the early identification of women with increased breast cancer risk.

Although the hypothesis of CRP-dependent influence of iron-regulatory proteins perturbation is

under further investigations, but if the association among deregulation of iron homeostasis, enhancement of inflammation and BC initiation/progression will be definitely proven, a potential clinical solution to BC recurrence could be provided proposing iron-chelation and anti-inflammatory treatment for postmenopausal cancer patients.

Finally, understanding the role of iron imbalance and inflammation in BC could lead to new translational researches on biomolecular alterations of breast microenvironment, opening a new strategies for an innovative approach to both breast cancer early prevention and possible adjuvant therapeutic treatment.

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