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Increased levels of erythropoietin in nipple aspirate fluid and in ductal cells from breast cancer patients

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Abstract. *Background*: Erythropoietin (Epo) is an important regulator of erythropoiesis, and controls proliferation and differentiation of both erythroid and non-erythroid tissues. Epo is actively synthesized by breast cells during lactation, and also plays a role in breast tissues promoting hypoxia-induced cancer initiation. Our aims are to perform an exploratory investigation on the Epo accumulation in breast secretions from healthy and cancer patients and its localization in breast cancer cells. *Methods*: Epo was determined by ELISA, immunoprecipitation, western blot and immunocytochemical analyses in 130 Nipple Aspirate Fluids (NAF) from 102 *NoCancer* and 28 *Breast Cancer* (BC) patients, comparing results with those found in 10 milk, 45 serum samples and breast cancer cell lines. *Results*: Epo levels in NAFs were significantly higher than those in milk and serum. No difference in Epo electrophoretic mobility was found among NAF, milk and serum samples, and conditioned cell culture medium. Immunolocalization of intracellular Epo in ductal cells floating in BC NAFs was similar to those of cancer cell lines. No significant correlation between TNM classification and Epo in NAFs from BC patients was found. Significantly higher Epo concentration was found in NAF from BC patients compared to NoCancer. *Conclusion*: We demonstrate that breast epithelial cells are a source of Epo in breast microenvironment, suggesting the presence of a paracrine/autocrine Epo function in NAFs, triggering off intracellular signaling cascade with subsequent BC initiation.

Keywords: Erythropoietin, breast cancer, nipple aspirate fluid, benign breast disease, milk, serum, immunocytochemistry

1. Introduction

Breast cancer (BC) is the most common malignancy affecting women in developed countries. Despite earlier diagnosis by means of the application of screening programs and therapies, 30–50% of women with resected BC will fatally relapse [21]. The discovery of molecular aberrations contributing to cellular proliferation, invasion and metastases is highly recommended in the selection of subgroups of patients with higher BC risk and to identify bio-molecules, possible targets of specific drugs [50].

Erythropoietin (Epo) is a glycoprotein hormone constituted by a single polypeptide chain of 165 aminoacids with a predicted molecular mass of 21 kDa and apparent glycosylated molecular mass of about 30 kDa; it is the primary regulator of erythropoiesis stimulating growth, preventing apoptosis, and inducing differentiation of red blood cell precursors [16]. Epo, normally produced by the foetal liver and the adult kidney, acts physiologically in several tissues, via erythropoietin receptors, members of the type I cytokine receptor family. During pathological processes (such as cancer development, invasion and metastases), this binding results in cell growth and proliferation [15].

Recently, the expression of Epo and its receptor has been demonstrated in several nonhematopoietic tissues, including breast [15,19], showing several pleiotropic effects and acting as an endogenous mediator (through paracrine/autocrine pathways) of adaptive tissue response to metabolic stress, and finally protecting tissues from injuries [13,17,32]. In the last five years, Epo and its receptor have been identified in the human breast (i.e., milk, neoplastic cell lines, healthy and diseased tissues) suggesting that the

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Epo/Epo receptor system may be involved in proliferation, growth, viability and angiogenesis [15,19]. Epo has been characterized as a physiologic constituent of human milk [24] produced/secreted by duct epithelial cells of lactating women, but found in low amount in nonlactating healthy breast tissue [22], suggesting that Epo may have diverse physiological actions [25]. On the other hand, Epo and Epo receptor (at transcript and protein levels) have been found at highly enhanced concentrations in BC compared to normal and benign breast disease tissues [1–5,8].

Although the function of Epo in BC is not completely understood [19], the enhanced autocrine/paracrine Epo signalling may be involved in BC promotion, linked to the activation of both hypoxia-inducible factor-1 and mitogen-activated protein kinase dependent pathways, contributing to increased aggressiveness and therapy resistance [2,34]. BC cells of cancer patients may be exposed to Epo not only due to local breast synthesis but also as a results of Epo therapy [19], used to treat anaemia resulting from chemo/radiation therapy [42]. The beneficial/detrimental roles of Epo are a matter of recent debate [47]; in fact, problems associated with Epo therapy in BC were highlighted by a recent clinical trial terminated before time [35]. These data suggest its potential harmful effects [7], even though recent evidences show that recombinant epoetins do not stimulate tumor growth in Epo receptor positive BC models [31] and fail to interfere with the antitumor drug effects [18–20].

The recent debate about Epo/EpoR axis in cancer (in particular in BC patients treated with Epo for cancer related anaemia) [33] has raised the need, on one side to understand the presence and function of EpoR on cancer cells [30], and on the other to take into consideration that Epo may impair, not improve, BC survival [10]. A hypothetical bio-molecular mechanism regulating Epo/EpoR axis in BC has been theorized [41], trying to explain the diverse biological availability of activated or cleaved EpoR with endogenous/exogenous Epo in breast microenvironment, which may in such condition either promote tumor proliferation or patient survival in BC. Until such time as the mechanisms of the contrasting literature data are resolved, the use and risks of Epo therapy should be carefully weighed, balancing the potential beneficial effects ameliorating anaemia against the detrimental tumor promoting activity.

The adult nonpregnant, nonlactating breast secretes fluid into the breast ductal system that can be easily and noninvasively obtained through nipple aspiration. Nipple aspirate fluid (NAF) contains cellular and metabolic biomarkers that may be useful in epidemiological and clinical research [43,45]. Because BC develops from ductal and lobular epithelium, the analysis of NAF has attracted much interest as a noninvasive method to assess the metabolic activity within the mammary gland [36]. Biosynthetically active apocrine cells [37] sloughed from ducts and floating in NAFs [9], are associated with exogenous/endogenous bio-compounds and proteinases involved in early malignant transformation [14,39,40, 43].

To noninvasively investigate the capability of breast ductal/lobular epithelial cells to produce and secrete Epo in the ductal tree (thus accumulating in NAF), and to evaluate the modulation of its expression in breast microenvironment, we analyzed the quantitative and qualitative expression of Epo in NAFs, as well as in breast cells floating in NAFs collected from *Cancer* and *NonCancer* patients. To confirm if the ductal/lobular epithelial cells are able to produce and secrete/accumulate Epo during physiologic condition, we also analyzed milk samples from healthy subjects during the first trimester of lactation and the Epo expression/secretion in breast cancer cell lines.

2. Materials and methods

2.1. Subjects

The present work was carried out in accordance with the ethical standards of the Helsinki Declaration of 1975 (as revised in 1983) and after the approval of the Ethics Committee of University "Carlo Bo" of Urbino (protocol 18/CE). All subjects analyzed signed informed consent prior to enrollment. One hundred and eighty women were recruited and enrolled as unselected consecutive patient populations attending the Center of Senology of Pesaro-Urbino (1998–2005). Demographic data for analyzed subjects are reported in Table 1. We excluded 30 patients reporting pregnancy within 3 years before the study or who were medically/hormonally treated during the previous year; in 20 women we were not able to collect NAF sample (89% success percentage of nipple aspiration). All NAF samples from the remaining 130 non-lactating women (age range 31–67 years) were aspirated noninvasively from one breast only using the

Table 1
Demographic data for women analyzed in the present study ($N = 130$)

NAF samples	NoCancer ($N = 102$)	Cancer + BBD/Ca ($N = 28$)	P value
No. (% detectable)	88 (86.3)	28 (100)	<0.001
Median age (Range)	41 (31–57)	52 (47–67)	<0.0001
Premenopausal (%)	46 (45.1)	7 (25.0)	<0.0001
Age of menarche (±SD)	12 (±1.6)	11 (±1.3)	0.88
Use of BCP (%)	20 (19.6)	1 (3.6)	<0.0001
Parity (±SD)	2.4 (±1.3)	2.1 (±1.4)	0.08

NAF: nipple aspirate fluid; BCP: birth control pills.

Sartorius breast pump [39,45]. One hundred and two specimens were collected from healthy women without evidence of cancer in the analyzed breast (*NoCancer* subjects). Twenty-one NAFs were obtained from patients who had biopsy proven confirmed ductal carcinoma *in situ* ($n = 13$) and invasive breast cancer ($n = 8$) (*Cancer* subjects). NAFs were collected prior to biopsy from women who underwent an invasive procedure (needle or surgical biopsy and mastectomy). NAFs from seven women, histologically categorized at the time of NAF collection as affected by benign breast diseases (e.g., hyperplasia with atypia, papilloma with atypia), who within three-five years developed BC, were also collected (*BBD/Ca* subjects). For women with breast cancer, all specimens were collected before definitive treatment. Clinico-pathological characteristics (e.g., disease stage, tumor size, nodal status, or distant disease spread) were determined according to the American Joint Committee on Cancer TNM staging system for breast cancer [46].

2.2. Sample processing

NAF fluid in the form of droplets was noninvasively collected in calibrated capillary tubes, the volume recorded and the ends sealed with clay. The median volume of NAF collected was 15 μl (range 5–400 μl), with no statistically significant difference between NAF volume and presence of cancer (data not shown). The high variability of NAF volume may be related to complex mechanisms of active secretion by ductal cells, passive filtration from plasma, and physiologic fluid reabsorption [37,43]. Without pooling, after collection NAF specimens were immediately snap-frozen at −80°C until use. NAFs were centrifuged at 15,000g for 15 min at 4°C, and the supernatants analyzed. We also collected milk and matched serum samples from 10 healthy women during the first month of lactation and, after centrifugation, the clear supernatants were stored at −80°C. The median age of the lactating women did not significantly differ from that of the other healthy patients recruited. To avoid possible interference of the age differences between cases and controls, we performed the age-adjustment based on the gamma distribution [28]. Since serum specimens were not available for all the women recruited, we analyzed matched serum and NAF samples only from *NoCancer* ($n = 50$) and *Cancer* ($n = 25$) women. NAF, milk and serum samples were firstly diluted and then analyzed for total protein and Epo content. All samples were assayed at least in duplicate.

2.3. Cell culture and reagents

Breast cancer cell lines (MCF-7 and BT-549) were purchased from ATCC (American type culture collection, Rockville, MD) and maintained in cell culture at 37°C in a humidified 5% CO_2 atmosphere in accordance with the instruction from ATCC. Cultured cells were grown to 80% confluence in 6 cm tissue culture dishes, washed with phosphate buffered saline, and then lysed as described below. All unspecified reagents were purchased from Sigma-Aldrich (Milan, Italy). To prepare whole cell lysates, monolayer cultures of human breast cancer cells grown to confluence in 10 cm tissue culture plates were washed twice with ice-cold phosphate buffered saline and lysed in ice-cold buffer containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 10 mM NaF, and 1% Triton X-100 supplemented with 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin followed by centrifugation at 13,000g for 20 min at 4°C.

2.4. Protein determination and ELISA assay

Protein concentrations were determined by commercially available protein assay kit (Bio-Rad, Milan, Italy). Epo concentrations in milk, serum and NAF samples were determined by ELISA kit (StemCell Technologies Inc., Vancouver, Canada). Standard curves were generated using serum, milk and NAF samples "spiked" with recombinant Epo (2.5, 5, 10, 25, 50, 75, 100 mU/ml added) to asses the ELISA assay recovery function. ELISA kit utilizes two monospecific monoclonal antibodies raised against human urinary Epo that bind to non-overlapping epitopes on the Epo polypeptide, showing high-affinity binding to

both native and recombinant Epo [51]. The sensitivity limit of the assay is 0.6 mU/ml with a range of 1.6–100 mU/ml. The intra-assay and inter-assay variability are 5.2% and 4.8%, respectively. To exclude milk and 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. Thermal stability was studied treating aliquots of samples at different temperatures (fresh samples, specimens frozen at −20°C and thawed at 25°C, and samples heated at 60°C for 10 min).

2.5. Immunoprecipitation and western blotting

In order to preliminarily characterize Epo protein in milk, NAF and cell lysates, randomly selected samples with high Epo concentrations were analysed by immunoprecipitation and Western Blot, performed using monoclonal mouse IgE anti-human Epo antibody (clone 26, 26G9C10) (StemCell Technologies), that can also bind denatured Epo. Immunoprecipitation was performed through IPeX columns by GeBa (Gene Bio-Applications Ltd., Milan, Italy) according to the manufacturer's instructions. Briefly, serum, milk and NAF samples, containing 50 μg of protein, were loaded into agarose beads affinity columns; after several washings, the immunocomplex was obtained using mouse IgE anti-human Epo antibody, while the non-bound material was removed from the affinity column. Subsequently, the elution buffer dissociated the specifically bound antigen from the complex, without dissociation of the antibody from the agarose beads. The eluted protein was mixed with standard Laemmli sample buffer, resolved by 12% SDS-polyacrylamide gel electrophoresis, and finally stained with standard Coomassie blue R-250. Western Blots were finally carried out according to Towbin's standard method and the proteins transferred to the membrane filter were immunologically recognized by monoclonal anti-Epo antibody through the amplified alkaline phosphatase kit (Bio-Rad).

2.6. Immunoelectron microscopic analysis

After observing several samples, we chose five well-preserved and representative samples of NAF cells. Each sample was processed according to previously described method [35]. Briefly, Immediately after collection NAF samples were mixed 1:1 (v:v) with the fixative solution (0.1 M Soerensen phosphate buffered 8% para-formaldehyde and 1% glutaraldehyde, pH 7.4). Fixation was performed for 1 h at 4°C after which the cell suspensions were centrifuged at 340g at 4°C for 15 min. The supernatants were removed and the cell pellets washed in Soerensen buffer and embedded in 1.5% agar–agar. After washing in PBS, free aldehydes were blocked in 0.5 M NH_4Cl in PBS at 4°C for 45 min. Following washing in PBS, the specimens were dehydrated through graded concentrations of ethanol and embedded in LRWhite resin. Resin polymerisation was carried out with UV-light. Ultrathin sections were placed on nickel grids coated with a Formwar carbon layer and then processed for immunocytochemistry as follows: the sections were floated for 3 min on normal goat serum (NGS) diluted 1:100 (v:v) in PBS and then incubated with mouse anti-human Epo antibody diluted in PBS containing 0.05% Tween-20 and 0.1% albumin. After rinsing, the section was reacted for 30 min with the secondary gold-conjugated antibody diluted in PBS. Finally, after the last incubation, the sections were rinsed, air-dried and then stained with lead citrate. As controls, some grids were treated with the incubation mixture without the primary antibody and then processed as described above. The specimens were observed in a Zeiss EM 902 Electron Microscope operating at 80 kV.

2.7. Statistical analyses

The demographic characteristics of patients and controls were compared using the Student's t-test. In view of the non-Gaussian distribution of data for protein and Epo levels, the differences between *NoCancer* and *Cancer* NAFs, and among breast cancer patients with different clinico-pathological features were compared using nonparametric Man–Whitney rank U-test. The data are presented as median and range (minimum and maximum), and expressed as mU/ml. The association studies were estimated using the nonparametric Spearman correlation coefficients method. The age-adjustment was based on the gamma distribution [28]. In all instances, significance was indicated if the two-side P value was <0.05. All the data were analyzed with Prism software version 3 (Graph-Pad Inc., San Diego, CA).

3. Results

3.1. Demographic results

Among the clinical/demographic variables compared, only age was significantly related to BC status;

women with BC tended to be significantly older than their *NoCancer* counterparts (Table 1). Although the age was different between cases and controls, no statistically significant correlation between Epo levels and women age was found; the age-adjustment did not affect Epo concentrations (data not shown).

3.2. Accuracy and recovery studies

After dilutions (ranging from 8- to 80-fold) of NAFs containing high Epo levels, a significant linearity of dilution, and a correlation with Epo was found ($Y = -0.14 + 72.12x$, $r = 0.98$). The mean (SEM) of analytical recovery percentage of recombinant Epo added to the samples was 97 (5)%. Intra- and inter-assay CVs with NAF samples were 4% and 7%, respectively. The curve generated using spiked samples paralleled the standard curve (data not shown). These data suggest that the milk and NAF "matrix" (i.e. proteins, hormones, and lipids present in breast secretions) did not affect the Epo immunoassay performance, originally developed for plasma/serum specimens.

3.3. Erythropoietin levels in milk and sera

As shown in Table 2, Epo was detected in all milk and serum samples. Median Epo level in milk was significantly higher compared to matched serum samples ($P = 0.003$). Aqueous and lipid phases of milk collected during the first trimester showed no difference in Epo concentration compared to matched whole milk sample ($P = 0.41$). We found a significant negative nonlinear correlation between matched milk and serum Epo levels ($n = 10$, $r = -0.56$, $P < 0.007$). Serum Epo levels were not related to the presence of BC, with no significant difference between *NoCancer* and *Cancer* subgroups (Table 3).

Table 2
Median of EPO levels in milk, serum and nipple aspirate fluid samples

Samples	No. (% detectable)	Epo (mU/ml)	P value
Milk ($N = 10$)	10 (100)	25.0 (4.9–45.2)	a, b
Serum ($N = 85$)	85 (100)	8.8 (2.5–31.6)	c
NAF ($N = 130$)	116 (89.2)	13.6 (2.5–91.4)	

NAF: nipple aspirate fluid; Epo: erythropoietin. a: milk vs. serum, $P = 0.003$; b: milk vs. NAF, $P = 0.44$; c: serum vs. NAF, $P < 0.0001$.

3.4. Erythropoietin expression in NAF from NoCancer and Cancer patients

Epo was detectable in 116 out of 130 NAFs with a total median level significantly higher compared to total serum level ($n = 85$) ($P < 0.0001$) (Table 2). We found no significant difference in Epo median level between matched serum ($n = 50$) and NAF ($n = 88$) samples from *NoCancer* women, whereas a significant higher Epo median level was found in *Cancer* NAF ($n = 28$) compared to matched serum ($n = 25$) ($P < 0.0001$) (Table 3). No statistically significant difference was found between the median level of total NAFs ($n = 116$) and milk ($n = 10$) samples (Table 2). Epo concentration in *NonCancer* NAFs (detectable in 88 out of 102 healthy women without BC evidence) was significantly lower compared to milk samples collected from women during the first trimester of lactation ($P = 0.017$), whereas *Cancer* NAFs ($n = 28$) contained statistically higher Epo level than that found in milk ($P < 0.0001$) (Table 3). Epo median value in *NoCancer* NAFs was significantly lower than that in *Cancer* NAFs (detectable in all BC bearing patients, $n = 28$) ($P < 0.0001$) (Fig. 1). *Cancer* NAFs showed significantly higher Epo expression compared to total serum ($n = 85$), serum from BC patients ($n = 25$) and milk ($n = 10$) ($P < 0.0001$).

Notably, in *BBD/Ca* patients (women originally diagnosed with benign breast diseases who subsequently developed breast cancer, $n = 7$), the median NAF

Table 3
Erythropoietin levels in nipple aspirate fluid and sera based on the cancer presence

Samples	Epo (mU/ml) (median, range)	P value
Serum ($N = 85$)		
NoCancer ($N = 50$)	9.2 (2.5–27.5)	a, b,
Cancer ($N = 25$)	11.8 (3.1–31.6)	c
Milk ($N = 10$)	8.2 (2.5–15.9)	d, e, f
NAF ($N = 130$)		
NoCancer ($N = 88$)	11.7 (2.5–33.5)	g, h
Cancer ($N = 28$)	72.5 (30.8–91.4)	i
Milk ($N = 10$)	25.0 (4.9–45.2)	

NAF: nipple aspirate fluid; Epo: erythropoietin. a: serum NoCancer vs. serum Cancer, $P = 0.354$; b: serum NoCancer vs. NAF NoCancer, $P = 0.089$; c: serum Cancer vs. NAF Cancer, $P < 0.0001$; d: serum NoCancer vs. serum milk, $P = 0.235$; e: serum Cancer vs. serum milk, $P = 0.052$; f: serum milk vs. milk, $P = 0.004$; g: NAF NoCancer vs. NAF Cancer, $P < 0.0001$; h: NAF NoCancer vs. milk, $P = 0.017$; i: NAF Cancer vs. milk, $P < 0.0001$.

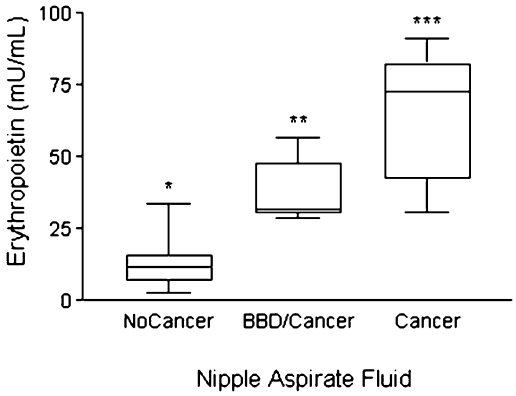

Fig. 1. Median levels of erythropoietin (Epo) in NAF from *NoCancer* women ($N = 88$), patients affected by *Cancer* ($N = 21$), and patients originally diagnosed with benign breast diseases who subsequently developed breast cancer (BBD/Ca, $N = 7$). (* = *NoCancer* vs. *Cancer*, $P < 0.0001$; ** = BBD/Ca vs. *Cancer*, $P = 0.004$; *** = BBD/Ca vs. *NoCancer*, $P < 0.0001$.)

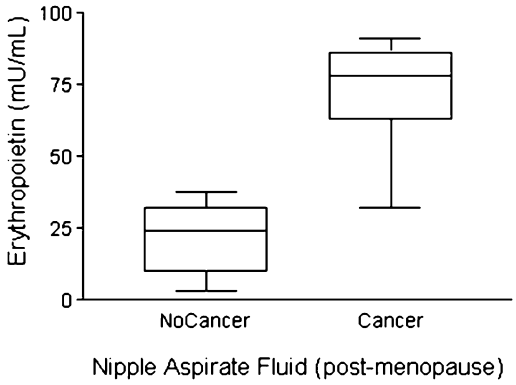

Fig. 2. Median erythropoietin levels in NAF of post-menopausal breast cancer patients ($N = 21$) and *NoCancer* ($N = 42$) women ($P < 0.0001$).

Epo concentration was significantly higher compared to the *NoCancer* patients (31.7 vs. 11.7 mU/ml, $P < 0.0001$). Epo found in *Cancer* NAFs was 2.3-fold higher than that found in *BBD/Ca* NAFs (72.5 vs. 31.7 mU/ml, $P = 0.0044$) (Fig. 1).

Related to the menopausal status, in pre-menopausal subjects we found no difference in Epo median level between NAFs from *NoCancer* ($n = 46$) and *Cancer* ($n = 7$) (24.8 vs. 31.5 mU/ml, respectively; $P = 0.083$; data not shown), whereas in post-menopausal conditions we detected a significantly higher Epo median level in *Cancer* ($n = 21$) compared to *NoCancer* ($n = 42$) NAFs (24.5 vs. 78.2 mU/ml, respectively; $P < 0.0001$) (Fig. 2).

To assess whether Epo expression may be related to BC development and/or progression, we examined Epo expression in *Cancer* NAFs, collected from women with ductal carcinoma *in situ* and invasive BC of increasing tumour staging. Median concentration of Epo in NAF from women with ductal carcinoma *in situ* was not statistically different from that found in NAF of patients with invasive breast cancer (72.5 vs. 73.2 mU/ml, respectively). No significant correlation was found between Epo median levels and clinico-pathological features; no difference in Epo expression among subgroups of primary tumor sizes, tumour stages, lymph node status and distant disease spread was also found (data not shown).

3.5. Erythropoietin immunoprecipitation and western blotting

It is well known that Epo is a highly glycosylated protein [16,19], containing a signal prepeptide suggesting its role in secretory mechanism(s) [29]. To analyze the variation in the Epo molecular mass due to a possible difference in glycosylation, we preliminarily carried out immuno-precipitation and subsequent western blotting analyses of Epo polypeptide found in NAF compared to that present in serum, and in milk collected during the first trimester of lactation. No variation (in both electrophoretic mobility and molecular mass) among all samples was revealed (Fig. 3). Moreover, Epo protein found in both NAF and milk samples was stable after five freeze/thaw cycles and after heating at 60°C for 10 min ($96 \pm 6\%$ of original concentration). On the contrary, after the same treatments, recombinant Epo was almost completely degraded ($11 \pm 7\%$ of original content) (data not shown). In serum and milk samples no degraded Epo protein was immunologically revealed, whereas, especially in NAFs collected from BC patients, some faint bands at lower M_r compared to constitutive Epo protein were occasionally identified (Fig. 3, lane 4). Moreover, both MCF-7 and BT-549 breast cancer cell lines showed constitutively high amounts of intracellular Epo, immunologically recognized as 30 kDa protein with no apparent difference in molecular mass and electrophoretic mobility compared to serum, milk and NAF Epo (Fig. 3).

3.6. Immunolocalization of erythropoietin in human breast cells

Ultrastructural observations of immunolabelled samples revealed that the epithelial cells floating in NAFs collected from *Cancer* displayed a cytoplasmic signal, mainly localized along the cell surface and on free ribosomes, while Golgi complexes were only

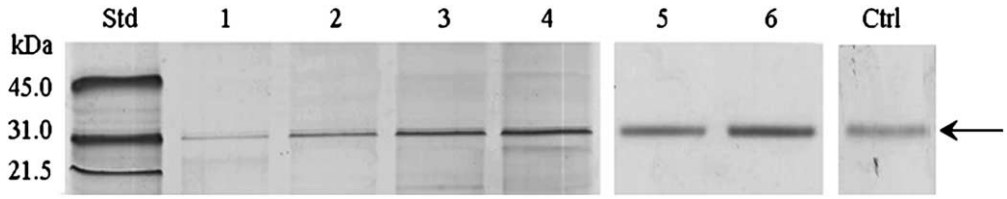

Fig. 3. Erythropoietin protein, immunoprecipitated and subsequently immunologically detected by Western blot, in representative serum (lane 1), milk (lane 2), *NoCancer* and *Cancer* NAF samples (lane 3 and 4, respectively), MCF-7 and BT-549 cancer cells (lane 5 and 6, respectively). In lanes Std and Ctrl, the standard proteins with their molecular masses expressed in kDa and a loading control of Epo, are respectively visualized. The arrow indicates the position of Epo of molecular mass of about 30 kDa.

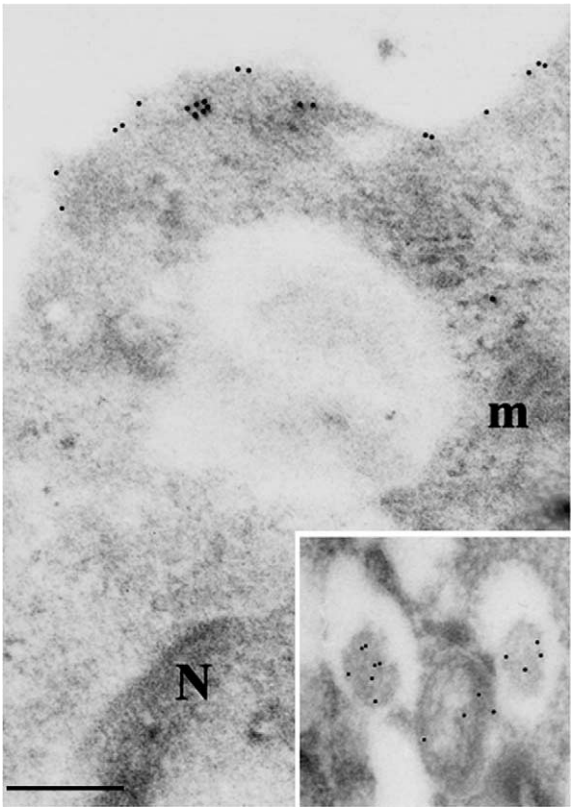

Fig. 4. Breast epithelial cells floating in *Cancer* NAF immunelabelled with anti-human Erythropoietin antibody. The labelling occurs on free ribosomes, dispersed in the cytosol and along the cell surface, whereas the cell nucleus (N) and the mitochondria (m) are devoid of gold grains. In the inset, the Epo labelling concentrated in vesicular structure containing homogeneous slightly electron-dense material is visible. Bar 0.5 μm.

some times labeled (Fig. 4). Moreover, the signal was concentrated in small vesicular structures containing homogeneous slightly electron-dense material (Fig. 4, inset). Cell nuclei and mitochondria never showed any labeling. Control samples showed only a negligible signal (data not shown).

4. Discussion

Epo is a glycoprotein belonging to the class I cytokine family that initiates cell signalling by forming a complex with Epo receptor molecules [16]. Epo binding induces a conformational change in the Epo receptor, leading to the activation of extracellular signal-regulated kinases [12,19]. In adults, Epo is mainly produced by the kidney, even though diverse cells in multiple organs also express it [13,15,17,19, 32]. Epo is well characterized for its ability to inhibit apoptosis and promote proliferation/differentiation of erythroid progenitor cells [16,32]. However, this glycoprotein is active outside the hematopoietic system as well [13,15,17,19].

Although Epo and its receptor are expressed by several human tumor types and cell lines, their complex and pleiotropic functions in cancer biology are not clearly defined (as excellently reviewed in [16]).

Epo has been characterized as a physiologic constituent of human milk [22,24,25], and together with its receptor (at transcript and protein levels) has been found at higher levels in BC tissue compared to normal and benign breast disease tissues [1–5,8,19]. The activation of both hypoxia-inducible factor-1 and mitogen-activated protein kinase-dependent pathway triggers Epo expression during BC initiation/evolution, enhancing autocrine/paracrine Epo signalling and contributing to increased BC aggressiveness and therapy resistance [2,34]. In this respect, Epo may be viewed as orchestrating the response to hypoxia [16]. In addition to its effects on erythroid development, Epo has a pro-angiogenic activity [44] that may be disadvantageous in cancer, promoting via autocrine/paracrine pathway cell survival, neovascularization of tumours, proliferation and migration of cancer cells, including human breast [16].

Here, we report for the first time that Epo protein is constitutively localized in breast ductal cells floating in

NAFs from healthy women and constitutively found in NAF collected from *NoCancer* women at higher levels compared to serum. Our data are in agreement with the findings of Epo synthesis and secretion by lactating breast cells and naturally accumulated in milk during lactation [22,24,25]. Breast milk from healthy women contains higher levels of Epo than matched plasma, thus excluding plasma filtration, and suggesting an active secretion by mammary duct epithelia as the main source. Similarly, epithelial cells lining the breast ductal tree (sloughed in NAF) synthesize Epo and may secrete it in breast microenvironment. Moreover, similarly to what occurs in human milk [22,24,25,27], we found no fragmented or deglycosylated Epo protein in *NoCancer* NAF, suggesting that Epo may be protected from digestion and degradation by proteolytic and glycolytic enzymes [37,39,43,45].

We found significantly higher levels of Epo in NAFs compared to serum. Our results argue against passive blood filtration as the source of Epo in NAF, and are consistent with previous immunohistochemical findings demonstrating that Epo protein is accumulated in the cytoplasm, at the apical part of epithelial cells, particularly in the lobules that show secretory changes [3]. Accordingly, in NAF we found floating breast epithelial cells with ultrastructural morphological feature typical of synthesizing/secreting cells (Epo immunolocalized on free ribosomes and along the cell surface).

The higher levels of Epo detected in NAF samples from *BBD/Ca* women compared to *NoCancer* NAFs provide evidence that the highly metabolizing apocrine/ductal cells floating in NAF [9,37] may be able to actively synthesize and thus secrete Epo at enhanced levels in the breast microenvironment. In fact, in breast epithelial cells floating in *Cancer* NAF, we found Epo protein immunolocalized in secreting vesicular structures. Our findings of Epo synthesis and its secretion in the NAF of women affected by benign breast disease that subsequently developed breast cancer are consistent with previous studies, suggesting that breast tissues and human cell lines with enhanced Epo expression may be related to tumour progression [1–3,5,8, 15,19,34]. Moreover, we found that MCF-7 and BT-549 breast cancer cell lines constitutively contain intracellular Epo protein, with molecular mass and electrophoretic mobility similar to Epo protein found in *Cancer* NAF.

Although the role and function of Epo in BC aggressiveness and therapy resistance are controversial [19,42,47], Epo and Epo receptor expression in BC tissues [1,3–5] suggests a crucial role in stimulating the proliferation of human breast cells through autocrine/paracrine mechanism [2,19,34]. The Epo/Epo receptor system may promote the hypoxic survival of breast tumors through hypoxia-inducible factor-1 [1, 2], the tumor cell proliferation by mitogen-activated protein kinase dependent pathways [5,20,34], and may enhance tumor growth by its angiogenetic effects [15, 19] triggering matrix metalloproteinases [44], key extracellular matrix proteolytic enzymes found at higher concentrations in *Cancer* NAF [39].

According to the immunohistochemical findings showing that Epo immunostaining is similar in invasive and in *in situ* ductal and lobular carcinoma [1,3], we found no significant differences of Epo protein levels in NAF collected from *in situ* and invasive biopsy-proven *Cancer* patients (data not shown); similarly, breast epithelial cancer cells floating in NAF from both *in situ* and invasive BC showed no different immunolocalization of Epo protein (data not shown). Moreover, we found no correlation between Epo levels and tumor size, tumor grade, and lymph-node status, in accordance to previously described absence of any association between Epo immunostaining and TNM clinicopathological features, including also hormone receptor status and HER2/neu expression [3,15,19].

Our results, describing in breast epithelial cells floating in NAFs an evident Epo protein localization and its subsequent secretion/accumulation in NAF from *Cancer* patients, may highlight in NAF microenvironment the role of this glycoprotein hormone in mechanism(s) leading to breast tumorigenesis [15,19]. The noninvasive method of NAF sampling allows the direct analysis of the breast ductal microenvironment, which represents the mirror of biochemical and cellular pathway modifications during physiologic, premalignant and cancerous breast conditions. In agreement with previous immunohistochemical evidence reporting that normal breast tissue was minimally reactive to Epo [1,3], we describe for the first time low levels of Epo in NAF collected from *NoCancer* women, not statistically different from those in matched sera; accordingly, we found only faint Epo immunolabelling in few breast ductal cells floating in NAF from *NoCancer* women.

Although administration of Epo does not seem to modulate chemosensitivity, and to interfere with antiproliferative and cytotoxic effects of antitumor drugs [7,19,42,47], the roles and functions of Epo in mammary carcinogenesis are conflicting, in particular linked to signaling mechanisms/pathways (involving p38, c-Jun-NH(2)-kinase, Akt, STAT5, p44/42

mitogen-activated protein kinase, Erk1, Erk2) [18–20, 31,34]. However, we show that MCF-7 and BT-549 breast carcinoma cell lines contain significant amounts of Epo similar to that previously described in other breast cancer cell lines and tissues (expressing both Epo and Epo receptor mRNA and protein) [1,2]; moreover, the highest levels of Epo and Epo receptor were found under hypoxic conditions suggesting a role for autocrine-paracrine Epo signaling in the hypoxic survival and neovascularization of breast tumor [15,19]. Further biomolecular studies are in progress to evaluate the association of Epo with matrix metalloproteinases and the hypoxia inducible factor-1 expression in NAF and breast cells floating in NAF from *Cancer* patients. These data may then add crucial details to the function of Epo signalling during early breast cancer development, making noninvasive NAF a possible reliable tool to identify women with altered bio-cellular pathways proning to BC evolution [14,45].

Epo protein was characterized as intracellular glycoprotein in BC cell lines and tissues [1,3,4] additionally found in extracellular breast fluids [22,24,25]. Furthermore, studies report that EpoR mRNA and protein were identified in BC cell lines and tumor tissues [1,2,4,5,31] while immunohistochemical studies localized EpoR more frequently in the cell cytoplasm [1, 3,4] and only in tumors as membrane-bound protein [3]. Interestingly, it has been demonstrated that BC cell lines may secrete the soluble fragment of EpoR peptide in conditioned medium, which may compete with membrane-bound receptor for ligand binding [5].

The "scattered" pieces of Epo/EpoR puzzle in breast may involve, after Epo binding, different mechanisms regarding internalization, ubiquitination, degradation or recycling of both Epo protein and its receptor [41]. In fact, after Epo binding, EpoR may be internalized without both the JAK2 trans-phosphorylation of and the ubiquitination process, thus not involving protein kinase cascade/network [6,48]. The JAK2-independent mechanism is in agreement with several literature data on breast tissues and cell lines: the concurrent localization of both intracellular and membrane-bound EpoR [1–5,31] the lack of Akt, and STAT5 activation after Epo binding [18,31] the intracellular localization of Epo protein [1,3–5,8,52] and its secretion in extracellular fluids, as in milk [22,24,25] and breast ductal fluids (present work), the identification of Epo as protein not undergoing proteolytic/glycolytic degradation [3,4, 19,22,24,25].

On the other hand, Epo binding lead to the JAK2-activation and EpoR ubiquitination, driving to the lysosomal degradation of the complex. The JAK2-dependent processes do not allow EpoR to recycle back to the cell surface, leading to a transient down-regulation of EpoR due to Epo binding [48,49]. This JAK2-dependent mechanism is in agreement with several literature data on breast tissues and cell lines: Epo strongly enhances intracellular phosphotyrosine levels [1]; Epo binding activates EpoR phosphorylation [34]; Epo induces phosphorylation of Akt, MAPK and ERK [20]. Ubiquitinated and proteosomal-degraded EpoR maintains the Epo binding capability, but loses the ability to activate further intracellular signal transduction: in fact, in EpoR positive BC cell lines (1) Epo binding did not activate the MAPK, Akt or STAT5 signaling [18,31]; (2) proteosomal-cleaved EpoR may be secreted in extracellular milieu as 26 kDa soluble form of EpoR, able to compete with membrane-bound receptor for ligand binding decreasing receptor-mediated signal generation [5]. Although these mechanisms need to be fully demonstrated in breast cells, they represent a model to explain the apparently contradictory results reported in literature [41].

Emerging evidences strongly support the importance of an intraductal approach to breast cancer biomarker discovery, highlightening the usefulness of noninvasive collection of NAF as a mirror of the metabolic activity of ductal/lobular microenvironment [11,23]. Being NAF a rich source of proteins and hormones with possible role as breast cancer biomarkers [14], the identification of novel endogenous bio-compound in NAF may help to understand the cellular and biochemical mechanisms occurring in breast microenvironment that may cause BC initiation/progression. Further studies are in progress to estimate the sensitivity and specificity of Epo in a wider number of subjects both in pre- and in post-menopause, as well as to evaluate the possible Epo differences in high risk compared to low risk benign breast diseases (e.g., hyperplasia with atypia vs. hyperplasia with no atypia or with apocrine cysts), in order to ascertain whether Epo may be of value for predicting BC risk.

Finally, in agreement with Epo histochemical immunoreactivity in lobules with secretory changes and in ductal breast cancer cells [3], we conclude that our data report for the first time the ultrastructural localization of Epo in breast epithelial cells sloughed in NAF from ducts and a significant accumulation of Epo in *Cancer* NAFs. These data may represent the mirror of increased secretory activity of apocrine epithelial cells [9,37,40], which may be triggered to prema-

lignant transformation during hypoxic conditions [2], suggesting a paracrine/autocrine function of Epo in breast microenvironment. The intracellular Epo synthesis and its extracellular active secretion may be related to Epo/Epo receptor signaling cascade, influencing the fate of apocrine cells lining the breast duct.

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