Analysis of aluminium content and iron homeostasis in nipple aspirate fluids from healthy women and breast cancer-affected patients

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ABSTRACT: Aluminium is not a physiological component of the breast but has been measured recently in human breast tissues and breast cyst fluids at levels above those found in blood serum or milk. Since the presence of aluminium can lead to iron dyshomeostasis, levels of aluminium and iron-binding proteins (ferritin, transferrin) were measured in nipple aspirate fluid (NAF), a fluid present in the breast duct tree and mirroring the breast microenvironment. NAFs were collected noninvasively from healthy women (*NoCancer*; $n = 16$) and breast cancer-affected women (*Cancer*; $n = 19$), and compared with levels in serum ($n = 15$) and milk ($n = 45$) from healthy subjects. The mean level of aluminium, measured by ICP-mass spectrometry, was significantly higher in *Cancer* NAF (268.4 ± 28.1 µg l^{-1}; $n = 19$) than in *NoCancer* NAF (131.3 ± 9.6 µg l^{-1}; $n = 16$; $P < 0.0001$). The mean level of ferritin, measured through immunoassay, was also found to be higher in *Cancer* NAF (280.0 ± 32.3 µg l^{-1}) than in *NoCancer* NAF (55.5 ± 7.2 µg l^{-1}), and furthermore, a positive correlation was found between levels of aluminium and ferritin in the *Cancer* NAF (correlation coefficient $R = 0.94$, $P < 0.001$). These results may suggest a role for raised levels of aluminium and modulation of proteins that regulate iron homeostasis as biomarkers for identification of women at higher risk of developing breast cancer. The reasons for the high levels of aluminium in NAF remain unknown but possibilities include either exposure to aluminium-based antiperspirant salts in the adjacent underarm area and/or preferential accumulation of aluminium by breast tissues. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: aluminium; antiperspirant; breast cancer; cosmetics; iron; inflammation; metalloestrogen; nipple aspirate fluid

INTRODUCTION

Although aluminium (Al) compounds are abundant and make up about 8% of the earth's surface, Al is not a normal component of biological systems (Exley, 1998, 2001; Yokel and McNamara, 2001). However, humans are increasingly exposed to Al-based compounds through their use in industrial settings, pharmaceuticals, food additives, cosmetics and other household products (Krewski *et al.*, 2007), and inclusion of Al salts, including Al chlorhydrate, Al chloride and Al zirconium chlorhydrate glycine complexes, as the active ingredients of antiperspirant in underarm and bodycare cosmetics (Laden and Felger, 1988; Exley *et al.*, 2007) provides a main exposure route of Al to the human body (Exley, 1998). The Al salts act in antiperspirant to prevent perspiration by blocking the sweat ducts through the formation of a physical plug composed of a combination of metal–proteoglycan precipitate and damaged cells at the top of the duct (Laden and Felger, 1988; Burkhart and Burkhart, 2008; McGrath, 2009). In conjunction with shaving, which creates abrasions in the skin, the daily application of Al-based antiperspirant cosmetics could result in an undesired presence/accumulation of Al in the tissues of the underarm and surrounding areas, including breast tissue (Darbre, 2001, 2003, 2005a, 2006a and 2009; Darbre and Charles, 2010). Al has been demonstrated to be absorbed from topical application of Al antiperspirant salts to the underarm

(Flarend *et al.*, 2001) and Al has been measured at higher levels in breast tissue from outer than inner breast quadrants, which could be explained on the basis of antiperspirant use in the underarm area (Exley *et al.*, 2007). It is noteworthy that a disproportionately large number of breast cancers arise in the upper-outer quadrant of the breast, the local area to which antiperspirants are applied in largest quantities (Darbre, 2001, 2003, 2005b; Darbre and Charles, 2010) and that within a population of breast cancer patients, those who used more aluminium-based antiperspirant were diagnosed with breast cancer at a younger age (McGrath, 2003).

Although risk factors have been identified, the cause of the rising incidence of breast cancer (Lipworth, 1995) remains unknown and it has been suggested that antiperspirant use could be a component factor (Darbre, 2001, 2003, 2005a, 2006a, 2009; Darbre and Charles, 2010). The involvement of

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oestrogen in the development of breast cancer is well established (Miller, 1996) and Al is a metalloestrogen (Darbre, 2006b). However, Al can bind to DNA (Karlik *et al.*, 1980; Ahmad *et al.*, 1996; Zhang *et al.*, 2002), modulating its topology (Latha *et al.*, 2002), is known to be genotoxic (Lukiw *et al.*, 1998; Exley, 1998) and has been shown to be carcinogenic in animal studies (Anghileri *et al.*, 2000). It also has pro-oxidant effects (Exley, 2004) and might contribute to oxidative stress through its interference with the action of transcription factors such as HIF-1 and NF-κB because of its ability to occupy key zinc binding sites essential for the function of zinc finger transcription factors (Exley, 2001). There is evidence that Al is involved in neuronal injury causing cholinergic dysfunctions (Silva *et al.*, 2007), but it may also affect the nonneuronal cholinergic system (Kaizer *et al.*, 2008), which may activate and stimulate breast tumor cell growth and neovascular response, promoting tumor progression (Fiszman *et al.*, 2007). Furthermore, Al can also disrupt iron (Fe) homeostasis. Chronic exposure to aluminium in rats has been shown to result in disturbed Fe homeostasis (Zhang *et al.*, 2010) and metal ion dyshomeostasis from Al exposure has been suggested to contribute to neurodegenerative diseases such as Parkinson's (Shiek *et al.*, 2010) and Alzheimer's (Zatta *et al.*, 2009) diseases. In the brain, Al is able to disrupt Fe homeostasis through mechanisms involving transferrin, ferritin and the non-transferrin iron transporter (Kim *et al.*, 2007), and these are also perturbed during breast carcinogenesis (Shpyleva *et al.*, 2010).

In view of the potential for Al to interfere with Fe homeostasis, it is important to evaluate whether accumulation of Al and Fe in the breast microenvironment could represent a risk factor in the development of breast cancer. The breast microenvironment may be easily studied through the analysis of nipple aspirate fluid (NAF), which is a biological fluid secreted from ductal/lobular cells and provides a useful tool for the early identification of biomarkers in women at higher risk of developing breast cancer (Mannello, 2008). NAF can be obtained easily and non-invasively through nipple aspiration from adult non-pregnant, non-lactating breasts. It can be obtained in more than 90% of pre- and post-menopausal women and is a low-cost procedure causing minimal or no discomfort (Sauter, 2005). NAF contains secreted proteins and cells shed from ductal and lobular epithelium (Mannello *et al.*, 2007), and several biomarkers (Petrakis, 1986, 1993; Mannello *et al.*, 2008a, 2008b) and proteinases (Mannello and Sebastiani, 2003; Qin *et al.*, 2003) of potential use as epidemiological and clinical research tools have been identified (Dua *et al.*, 2006). In addition, the intracrinology of NAF has provided a further panel of cancer biomarkers reflecting the hormonal status in the breast microenvironment (Mannello *et al.*, 2009a). On the basis of its composition, NAF has been classified into two types: (1) *NoCancer* NAF taken from healthy control women or patients diagnosed with benign breast diseases; and (2) *Cancer* NAF taken from women affected by breast carcinoma. The first type may represent a milieu resulting from a continuous exchange 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. The second type contains several biologically active compounds which may enhance the proliferation of epithelial cells and its composition mainly reflects the active metabolism of epithelial and stromal cells lining the duct (Dua

et al., 2006; Mannello *et al.*, 2009a). Because the majority of breast cancers develop from ductal and lobular epithelium, the analysis of NAF has attracted considerable interest as a way of assessing the metabolic activity within the mammary gland (Li *et al.*, 2005; Dua *et al.*, 2006; King and Love, 2006; Mannello *et al.*, 2009a). For these reasons, we sought to compare levels of Al, Fe, ferritin and transferrin in NAFs taken from healthy women (*NoCancer*) with those taken from women affected by breast cancer (*Cancer*).

MATERIALS AND METHODS

Patient Information and Sample Collection

Women were required to give written informed consent, and the present work was carried out in accordance with the ethical standards of the Helsinki Declaration and after the approval of the Ethics Committee of the University 'Carlo Bo' of Urbino (protocol 18/CE).

Among all subjects recruited for this study ($n = 60$), we excluded 22 patients because of pregnancy within 3 years or medical treatment during the previous year. Of the remaining 38 subjects, NAF was successfully collected from 35 women (92%) using a modified breast pump (Sartorius) as described elsewhere (Sauter, 2005; Mannello *et al.*, 2007). The HALO NAF Collection System (Neomatrix, Irvine, CA, USA) utilizes plastic devices to aspirate samples and calibrated capillary glass tubes to collect NAF droplets that appear on the tip of the nipple. The median volume of NAF collected was 800 μL (range 130–1500 μL). The nipple was first cleansed and then the depression caused by the breast pump device was used to collect the intraductal fluid which reached the surface of the nipple directly into the glass capillary tube without spreading along the skin of the nipple. Although contact with the skin surface cannot be excluded, the NAF was collected as directly as possible.

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, which contained the intraductal fluid, were analysed without any associated cellular components. To avoid possible interference of the age differences between cases and controls, we performed the age-adjustment based on the γ distribution (Fay and Feuer, 1998). NAF samples were analysed for total protein and then for Al 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 breast cancer, all the 38 recruited patients were classified into two categories: *Cancer*, if there was evidence of biopsy-proven carcinoma ($n = 19$) and *NoCancer* where there was no evidence of breast malignancy ($n = 19$). NAF samples were analysed 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 of 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 analysed 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 (Singletary and Connolly, 2006).

Fifteen 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 2000 *g* for 5 min and stored at −20°C until use.

Forty-five milk samples were obtained aseptically from healthy lactating women and stored at −30°C until analysis (within 3 weeks). The specimens were collected at each of three stages of lactation: colostrum (3–5 days after birth, *n* = 14), intermediate milk (8–13 days after birth, *n* = 19) and mature milk (3–6 weeks after birth, *n* = 12). Milk samples were thawed and centrifuged at 12 000 *g* 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 samples were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce). Al analyses were performed by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7500ce ICP-MS (McMillan, TX, USA), equipped with a Shield Torch System and an inert PFA sample introduction system. The 7500ce uses collision reaction cell technology in the form of the Octopole Reaction System to remove polyatomic interferences, allowing detection of Al in one Multi-Tune analysis. The inert PFA sample introduction system consists of a PFA-100- self-aspirating microflow nebulizer, 35 mm PFA end cup, 35 mm PFA spray chamber, 2 mm O-ring free platinum injector and quartz torch. All samples were analysed directly without dilution, thus avoiding sensitivity loss due to dilution and potential contamination. Samples were analysed using a Multi-Tune method consisting of H_2, He, normal and cool plasma condition. Since this method features fully automated switching from one analysis mode to the next, all elements are analysed in a single acquisition, reducing the risk of contamination from further sample handling. All samples were treated with Optima Grade HNO_3 and heated at 95°C (±5°C) for 1 h. After cooling, the samples were analysed in triplicate by Agilent 7500ce ICP-MS, with aspiration at an uptake rate of 100 µl min^{-1}. Calibration standards were prepared with 2% Optima Grade HNO_3, 2% Optima Grade HCl and trace Optima Grade HF, at concentrations ranging from 250 to 10 000 ppt. The content of Al in serum and NAF samples was reported as µg l^{-1}. Optima Grade HNO3 and Optima Grade HCl diluted in double-deionized water were used as blank samples. The mean blank level was deducted from all the measured results, and so corrected values of Al levels are presented in this work. Both instrument and method detection limits were calculated from calibration data generated during this study. Method detection limit (MDL) is defined by the EPA as 'an estimate of the measured concentration at which there is 99% confidence that Al is present in a given sample matrix'. The MDL in the present study was 290.35 ppt. In all standard samples the RSD was <4%. Averaged acid-cleaned flow-sensor metal results show the deionized-water baselines of <0.1 ppb contaminants/flow-sensor, proving that acid leaching with Optima Grade HNO_3, HF and HCl is effective in removing contaminants.

Transferrin (TRF) concentration was assayed by a turbidimetric method, using the Synchron® System (Beckman Coulter) according to the manufacturer's instructions. In the reaction, TRF combines with a specific monoclonal antibody to form insoluble antigen–antibody complexes. The system monitors the change in absorbance at 340 nm, which is proportional to the concentration of transferrin in the sample and is calculated and expressed based upon a single-point calibration. TRF concentrations in serum and NAF samples were calculated according to the standard curves, generated using serum and NAF samples spiked with recombinant TRF (0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 g l^{-1} added) to assess the assay recovery function. The sensitivity limit of the assay is 0.3 g l^{-1} with a range of 1.0–10 g l^{-1}. 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 and 150 µg l^{-1} 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^{-1} with a range of 5–600 µg l^{-1}. The intra-assay and inter-assay variability were 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. Significance was set at $P < 0.05$. Data were analysed with Prism software for windows, version 3.1 (Graph-Pad, San Diego, CA, USA).

RESULTS

Patient Information and Assay Controls

NAF were collected from 35 (aged from 31 to 77 years) out of the 38 women studied (92% collection). Median age of the subjects was 40 years in the *NoCancer* group and 56 years in the *Cancer* group (Table 1). In the *NoCancer* group, more subjects were pre-menopausal than in the *Cancer* group, where more patients were post-menopausal (Table 1). Among the clinical/demographic variables compared, only age was correlated with cancer status, and women with biopsy-proven breast cancer (*Cancer* patients) tended to be older than non-affected counterparts (*NoCancer* subjects; Table 1). Although the age was different between cases and controls, no statistically significant correlations between aluminium or iron-linked protein levels and age were 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 and TRF levels, a significant linearity of dilution, and a

Table 1. Demographic data for women analyzed in the present study ($n = 35$)

Demographic data	NoCancer ($n = 16$)	Cancer ($n = 19$)	P-value
Number of NAF samples	16	19	—
Median age in 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)	0.06
Use of HRT	Nonusers	Nonusers	—

NAF, nipple aspirate fluid; HRT, hormone replacement therapy.

correlation with proteins was found ($Y = -0.15 + 73.18x$, $r = 0.975$). The mean (± SE) of analytical recovery percentage of recombinant proteins added to the samples was 98.6%. 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 NAF 'matrix' (i.e. proteins, hormones, and lipids present in breast secretions) did not affect the TRF and FTN immunoassay performance, originally developed for plasma/serum specimens.

Measurement of Aluminium in NAF

Al was detected in all serum samples analysed, and the mean value in total serum ($n = 15$) was 5.6 ± 0.5 µg l^{-1} (range 3–9 µg l^{-1}; Table 2). Al measurements in human milk samples ($n = 45$) gave a mean content of 24.8 ± 0.8 µg l^{-1} (range 11–36 µg l^{-1}; Table 2). When milk samples were subdivided according to the stage of lactation, mean levels of Al were 23.4 ± 2.0 µg l^{-1} in colostrum ($n = 14$), 25.5 ± 1.2 µg l^{-1} in intermediate milk ($n = 19$), and 25.0 ± 1.0 µg l^{-1} in mature milk ($n = 12$); no statistically significant differences in Al concentrations were found between the stages of lactation. However, the mean level of Al was higher in the milk samples than in the serum samples (24.8 ± 0.8 vs 5.6 ± 0.5 µg l^{-1}, $P < 0.001$).

Al was detected in all NAF samples and the values are shown in Table 3. Mean levels of Al were higher in Cancer NAFs ($n = 19$) than in NoCancer NAFs ($n = 16$; 268.4 ± 28.1 vs 131.3 ± 9.6 µg l^{-1}, $P < 0.0001$; Table 2, Fig. 1). The mean levels of Al were significantly higher in all NAF samples than in human milk ($n = 45$; 205.7 ± 19.4 µg l^{-1} vs 24.8 ± 0.8, $P < 0.0001$) or in human serum ($n = 15$; 205.7 ± 19.4 vs 5.6 ± 0.5 µg l^{-1}, $P < 0.0001$; Table 2). Subdivision of NAFs into Cancer and NoCancer NAFs showed higher Al content in both subgroups with respect to serum (268.4 ± 28.1 µg l^{-1} and 131.3 ± 9.6 µg l^{-1} vs 5.6 ± 0.5 µg l^{-1} respectively; $P < 0.0001$) and to milk (268.4 ± 28.1 µg l^{-1} and 131.3 ± 9.6 µg l^{-1} vs 24.8 ± 0.8 µg l^{-1} respectively; $P < 0.0001$).

NoCancer NAFs ($n = 16$) were further subdivided into samples collected from healthy subjects ($n = 10$) and women with benign hyperplastic lesions ($n = 6$) but no statistically significant differences between mean levels were found (131 ± 11 vs 132 ± 12 µg l^{-1}). In NoCancer subjects, Al concentrations did not show any significant difference between pre- and post-menopausal status (130 ± 9 vs 133 ± 15 µg l^{-1}), whereas in Cancer NAFs ($n = 19$), higher mean levels of Al were found in post-menopausal than in pre-menopausal women (275 ± 33 vs 210 ± 57 µg l^{-1}, $P < 0.01$). In a limited number of women with in situ ductal breast carcinoma ($n = 4$), NAF samples had lower median levels of Al than in patients bearing invasive breast carcinoma ($n = 13$; 207.5 ± 21.8 vs 297.7 ± 38 µg l^{-1}, $P < 0.001$).

Measurement of Iron-binding Proteins in NAF

In order to evaluate the iron-binding protein homeostasis, the levels of FTN and TRF were assayed in all NAF, milk and serum samples. FTN was detectable in all NAFs ($n = 35$) and values are shown in Table 3. Mean FTN values were higher in total NAFs than in serum ($n = 15$; 177.2 ± 26.0 vs 41.0 ± 7.3 µg l^{-1}, $P < 0.0001$; Table 2). However, following subdivision into Cancer and NoCancer NAFs, the mean level of FTN was significantly higher in Cancer NAF ($n = 19$) compared with serum ($n = 15$; 280 ± 32 vs 41 ± 7 µg l^{-1}, $P < 0.0001$), but no significant difference was noted in NAF samples from NoCancer women ($n = 16$) compared with serum ($n = 15$; 41.0 ± 7.3 vs 55.5 ± 7.2, respectively; Table 2). The mean level of FTN in Cancer NAFs ($n = 19$) was significantly higher than in NoCancer NAFs ($n = 16$; 280 ± 32 µg l^{-1} vs 56 ± 7

Table 2. Aluminium, ferritin and transferrin concentrations in human nipple aspirate fluid (NAF) and matched serum samples of women with (Cancer) and without (NoCancer) breast cancer (mean ± SE)

	Serum ($n = 15$)	Milk ($n = 45$)	NAF NoCancer ($n = 16$)	NAF Cancer ($n = 19$)	P-value
Aluminium (µg l^{-1})	5.6 ± 0.5	24.8 ± 0.8	131.3 ± 9.6	268.4 ± 28.1	a, b, c, d
Ferritin (µg l^{-1})	41.0 ± 7.3	25.2 ± 4.6	55.5 ± 7.2	280.0 ± 32.3	e, f, g, h
Transferrin (g l^{-1})	2.9 ± 0.3	2.9 ± 0.8	2.8 ± 0.2	8.3 ± 0.5	i, j, k, l

a, serum vs milk, $P < 0.001$; b, Cancer vs NoCancer NAF, $P < 0.0001$; c, NAF vs serum, $P < 0.0001$;
d, NAF vs milk, $P < 0.0001$; e, NoCancer NAF vs serum, NS; f, Cancer NAF vs serum, $P < 0.0001$; g, NoCancer vs Cancer NAF, $P < 0.0001$;
h, NoCancer NAF vs milk, $P < 0.001$; i, NoCancer vs Cancer NAF, $P < 0.001$; j, Cancer NAF vs serum, $P < 0.001$; k, NoCancer NAF vs milk, NS; l, Cancer NAF vs milk, $P < 0.001$.
NS, not significant.

Table 3. Concentrations of aluminium, ferritin and transferrin in nipple aspirate fluids (NAF) collected from women with (*Cancer*) and without (*NoCancer*) breast cancer

Cancer NAF (n = 19)			NoCancer NAF (n = 16)		
Al (μg l^{-1})	Ferritin (μg l^{-1})	Transferrin (g l^{-1})	Al (μg l^{-1})	Ferritin (μg l^{-1})	Transferrin (g l^{-1})
210	275.3	8.2	120	42.2	3.9
150	126.9	8.6	110	71.4	1.2
230	292.5	9.6	150	44.8	1.4
470	408.7	10.4	120	35.8	1.8
190	189.6	6.8	110	89.6	3.5
150	126.4	7.8	160	57.5	3.2
200	249.9	6.9	140	31.4	3.8
230	252.8	9.7	90	17.9	4.2
170	137.5	6.0	170	112.2	3.7
500	567.4	11.8	160	115.1	3.2
320	343.8	9.8	160	44.8	2.7
250	289.6	9.7	90	52.9	2.4
200	206.3	5.6	80	39.3	3.6
450	374.9	10.9	120	59.9	2.1
200	204.4	8.5	130	21.1	1.6
190	148.6	5.0	190	52.8	2.9
520	635.2	11.6			
280	306.6	8.2			
190	175.8	6.6			

respectively, $P < 0.0001$; Table 2, Fig. 1). Subdivision according to menopausal status gave mean FTN levels higher in *Cancer* NAFs than in *NoCancer* NAFs for both postmenopausal (327 ± 36 vs 52 ± 9 μg l^{-1}, $P < 0.0001$) and pre-menopausal NAF samples (146 ± 12 vs 58 ± 11, $P < 0.001$).

TRF was detectable in all NAFs ($n = 35$; Table 3) with a higher total mean level than in serum ($n = 15$; 5.9 ± 0.6 vs 2.9 ± 0.3 g l^{-1}, $P < 0.001$; Table 2). No significant difference in the mean level of TRF was found between serum ($n = 15$) and NAF samples from *NoCancer* women ($n = 16$; 2.9 ± 0.3 vs 2.8 ± 0.2 g l^{-1}, respectively), whereas a significant higher TRF mean level was found in *Cancer* NAF ($n = 19$) compared with serum (8.3 ± 0.5 vs 2.9 ± 0.3 g l^{-1}, $P < 0.001$; Table 2). The mean value of TRF in *Cancer* NAFs

($n = 19$) was significantly higher than in *NoCancer* NAFs ($n = 16$; 8.3 ± 0.5 g l^{-1} vs 2.8 ± 0.2 respectively, $P < 0.001$; Table 2, Fig. 1). No significant difference in the median level of TRF was found between *Cancer* and *NoCancer* either in pre- or in post-menopausal NAFs (data not shown).

Correlation Between Levels of Aluminium and Iron-binding Proteins

Investigation into the possible relationship between Al content/accumulation and expression of iron-binding proteins revealed that only in NAF collected from cancer patients were there positive and significant correlations between Al and FTN
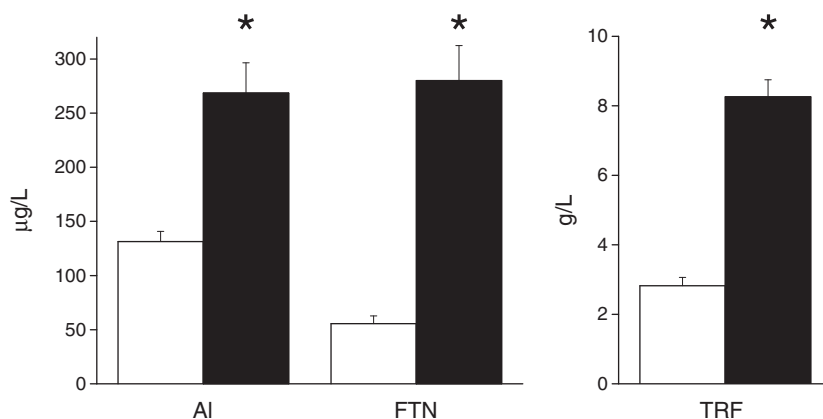

Figure 1. Mean levels of aluminium (Al), ferritin (FTN) and transferrin (TRF) in nipple aspirate fluid (NAF) samples obtained from women with (*Cancer*) (black bars) or without (*NoCancer*) (white bars) breast cancer. * $P < 0.001$ for *Cancer* vs *NoCancer*.

levels (Fig. 2A) and between Al and TRF levels (Fig. 2B), whereas neither levels of FTN (Fig. 2C) nor TRF (Fig. 2D) were significantly correlated with Al levels in NAF collected from healthy subjects.

DISCUSSION

The data presented here showing higher levels of Al in *Cancer* NAFs than in *NoCancer* NAFs opens the possibility that increased levels of this metalloestrogen in the breast microenvironment could contribute to the development of breast cancer. Previous studies have also demonstrated the unexpected presence of Al in the human breast, both in breast tissue from cancer patients (Mulay *et al.*, 1971; Ng *et al.*, 1997; Exley *et al.*, 2007) and in human breast cyst fluids (Mannello *et al.*, 2009b), particularly in fluid from type 1 secretory cysts (Mannello *et al.*, 2009b), which can be associated with increased breast cancer risk (Mannello *et al.*, 2006). Although the source of the aluminium cannot be identified, this does demonstrate that Al is entering the breast microenvironment in the modern world and this justifies further investigation given the known genotoxic and carcinogenic properties of Al (see introduction).

It is notable that Al levels were higher not only in *Cancer* versus *NoCancer* but also in all NAF compared with either blood serum or milk. The underlying molecular mechanisms remain unknown, but one explanation might be linked to the metabolically active ductal epithelial cells lining the breast duct,

which may allow for raised Al levels in all NAF but may differ between *Cancer* and *NoCancer*. Previous studies indicate that NAF composition depends on epithelial cell morphology and metabolic activity and can lead to differences between NAF composition in *NoCancer* and *Cancer* samples (Mannello *et al.*, 2009a). In *No-Cancer* patients, the junctional complexes between epithelial cells may be leaky, allowing components of the interstitial space and plasma to pass unimpeded into the duct, and *vice versa* for substances in NAF to freely enter the plasma. This leakiness is essential for normal physiological functions, allowing secretion products to leave the breast, inflammatory cells to enter, and products of apoptosis, for example during involution after pregnancy and lactation, to be cleared from the breast. Therefore, NAF from normal women is a milieu resulting from a continuous exchange 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 (Malatesta *et al.*, 2000). By contrast, clustered epithelial cells in NAF from *Cancer* patients have shown the presence of zonulae occludens as tight and gap junctions which can seal epithelial cells. This can result in prolonged exposure of the ductal epithelial cells to bio-active substances such as growth factors, hormones, and proteinases (Petrakis, 1986; Mannello *et al.*, 1999; Mannello and Sebastiani, 2003; Qin *et al.*, 2003), which could lead to an increase in the likelihood of malignant transformation (Petrakis, 1986; Malatesta *et al.*, 2000).
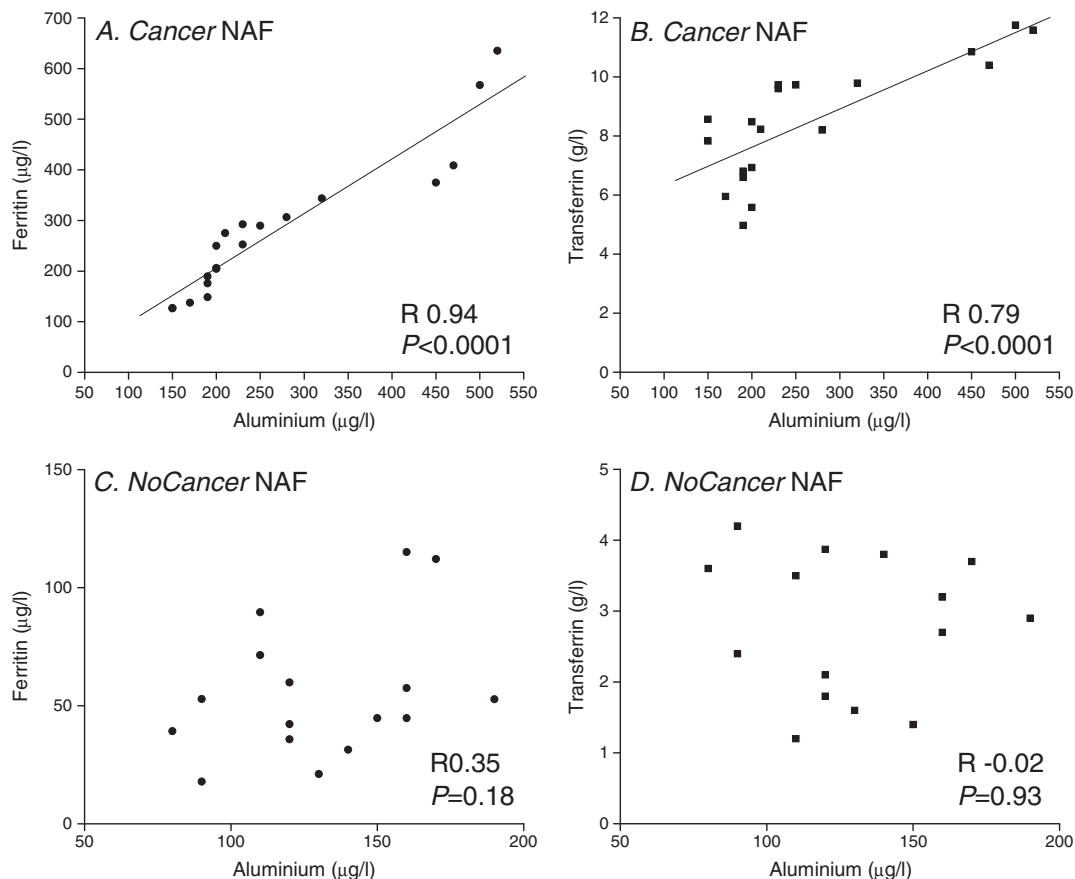

Figure 2. Correlations between levels of aluminium and ferritin (A, C) and between levels of aluminium and transferrin (B, D) in nipple aspirate fluid from women with (*Cancer*) (A, B) or without (*NoCancer*) (C, D) breast cancer. *R* is the correlation coefficient of the fit. *P* is the probability that *R* is zero.

The presence of increased levels of several compounds in *Cancer* NAF is therefore a reflection of the dynamic process which is taking place in the breast epithelial cells as part of their transformation (Petrakis, 1986; Lee *et al.*, 1994). Therefore it could be that the increased aluminium content in NAF collected from women affected by breast cancer may result from the increased uptake of Al by metabolically active epithelial cells present in the cancer condition. In this context, we have also previously reported higher levels of Al in breast cyst fluids collected from type 1 secretory breast cysts than from type II transudative cysts and have suggested that this might also be explained by the presence of metabolically active apocrine epithelial cells lining the type I cysts (Mannello *et al.*, 2009b). On the other hand, it might also be simply that those with breast cancer have been exposed to higher levels of the Al, including possibly through greater use of Al salts in cosmetic products (McGrath, 2003), and/or that the biochemistry of the tumour tissue is linked to preferential accumulation of Al. Malignant breast tissue is known to overexpress the calcium binding protein osteopontin (Rangaswami *et al.*, 2006) which coincidentally forms stable complexes with aluminium (Rowatt *et al.*, 1997). Malignant breast tissue can also contain calcium phosphate deposits in the form of microcalcifications (Baker *et al.*, 2010) and aluminium is known to combine readily with phosphate to form aluminium phosphate and aluminium has also been noted along lines of calcification in bone (Exley, 2001).

The finding here of a positive correlation between Al levels and levels of iron-binding proteins, in particular ferritin, suggests that another possible consequence of the increased concentrations of Al in the breast cancer microenvironment may be to disrupt iron homeostasis. In rats, chronic oral exposure to Al has been shown to disrupt iron homeostasis with alterations to serum levels of transferrin and total iron-binding capacity (Zhang *et al.*, 2010). In erythroleukemia, hepatoma and glioma cells, exposure to aluminium has been shown to disrupt iron homeostasis through alterations to ferritin, transferrin, transferrin receptors and the nontransferrin iron transporter (Kim *et al.*, 2007). Since perturbations to iron-regulatory proteins are also found during progression of breast cancer (Shpyleva *et al.*, 2010), it is possible that the observed correlation here between Al levels and levels of ferritin and transferrin could be of physiological significance. Although our study is based on a limited sample size, to our knowledge, this is the first time that a significant and positive relationship has been described between increased Al levels and enhanced levels of both FTN and TRF specifically in *Cancer* NAFs and not in *NoCancer* NAFs.

If aluminium does play a role in breast cancer development through multiple actions including not only toxic/carcinogenic mechanisms but also perturbation of iron-binding protein levels, then further investigations are required to understand the biomolecular mechanisms by which Al can influence iron-regulatory proteins and the effects of long-term exposure of the human population to aluminium-based xenocompounds on iron homeostasis. Furthermore, it might also then be possible to exploit the raised aluminium levels in the *Cancer* NAFs as a biomarker to identify women at increased risk of developing breast cancer. The genotoxic profile of aluminium, together with its reported oestrogenic properties and possible effects on biomolecular alterations to the breast microenvironment, should be a reason for future research into the potential involvement of aluminium in the development and progression of human breast cancer, opening a new strategy for an innovative approach to breast cancer prevention (Darbre, 2010).

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