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Steroid Biochemistry and Categorization of Breast Cyst Fluid: Relation to Breast Cancer Risk

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Patients bearing macrocysts of the breast are at higher risk of later developing cancer. The fluid filling the cysts (breast cysts fluid, BCF) contains unusual amounts of steroid conjugates, first androgen and estrogen sulfates. Measuring BCF cations (K^+, Na^+) allows categorization of cysts into two major subsets (type I and type II) that are associated with a different degree and/or turnover of apocrine metaplastic cells in the lining epithelium. Type I cysts (high K^+/Na^+ ratio) accumulate huge amounts of dehydroepiandrosterone sulfate, estrone sulfate, androstane-3α,17β-diol glucuronide, androsterone glucuronide and contain more testosterone and dihydrotestosterone than type II. Conversely, type II cysts (low K^+/Na^+ ratio) contain more progesterone and pregnenolone. A cohort study was started in 1983 at the Cancer Prevention Center, Ravenna, Italy, with the aim of evaluating the relationships between the biochemistry of BCF and the incidence of breast cancer in women with gross cystic disease (GCD) of the breast. The bimodal distribution of the cationic pattern has been confirmed from data obtained in 798 patients aspirated. The risk of cyst relapse was significantly higher among women with type I cysts or with multiple cysts at presentation. Twelve incident cases of breast cancer have been diagnosed among women whose BCF was categorized. Eleven out of 12 cases had type I or multiple cysts. The cumulative incidence of breast cancer among patients bearing type I cysts was 2.5%. We conclude that women with GCD bearing type I cysts have an increased breast cancer risk when compared with the counterpart bearing type II cysts or the general population.

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INTRODUCTION

Gross cystic disease (GCD) is the most common benign breast pathology. It has been reported that about 7% of women in Western countries develop a palpable cyst [1]. Review of the autopsy findings [2] and studies of women undergoing breast biopsy [3] suggest an even higher frequency.

Clinically evident macrocysts develop mainly in the premenopausal decade [4]; their appearance and local pain depend on intracystic tension and pericystic stromal fibrosis. Two pictures can be distinguished, possibly subserved by different mechanisms of cyst formation. A cyst may present as a clearly isolated lesion, and it is viewed as a consequence of duct obstruction by fibrosis, which in turn leads to retention of secretory material (obstructive or transudative cyst); this kind of cyst usually does not recur after aspiration. Alternatively, cysts may present as multiple, often bilateral; in this case they conceivably depend on continuous ductular-alveolar secretion, which in turn leads to cyst formation due to impaired drainage of the supervening fluid (secretive cysts); they frequently recur after aspiration [5, 6].

Cysts are not considered premalignant lesions. Several reports, however, have indicated that bearers of cysts are at 2- to 4-fold higher risk of later developing cancer [1, 7–11] (Table 1). This assumption has been

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Table 1. Epidemiological studies consistent with increased risk of breast cancer in women with GCD of the breast

Author	Ref.	Year	Number of women	Years of follow-up	Number of cancers	Relative risk
Haagensen	1	1971	1693	5–30	72	4.0
Harrington *et al.*	7	1980	696	5.6	19	3.5
Jones and Bradbeer	8	1980	322	5	7	2.5
Roberts *et al.*	9	1984	428	10–15	17	3.5
Devitt	10	1988	451	5–10	20	NS
Bundred *et al.*	11	1988	352	5–12	14	4.4

From Ref. [11] modified. NS, not stated.

questioned by Dupont and Page [3], who reported that the presence of cysts only doubled the risk in women with a family history of breast cancer. They demonstrated that the major determinants of the risk were hyperplastic lesions, although they could not exclude that these lesions were specifically associated with particular subsets of cysts, not separately designated in their retrospective study based on biopsy specimens.

Studies on the composition of the aspirated fluid (breast cyst fluid, BCF) have demonstrated that this "biochemical space" has a distinctive composition with unusual profiles of many constituents, including electrolytes, steroid and peptide hormones, growth factors, enzymes, immunoglobulins, cytokines and other proteins [12–16]. It has been stated that mammary cysts are complex endocrine entities capable of accumulating, metabolizing and probably synthesizing certain hormones [12].

An interesting phenomenon is that many components show not only a large range of levels in BCF, but a bimodal distribution of values which is consistent with the presence of distinct subpopulations of cysts. The list includes electrolytes such as Na^+, K^+ and Cl^- [17–20], hormones [19–22] and growth factors [23]. Measuring homovalent ion concentrations is a useful tool for segregating cyst fluids into relatively homogeneous subsets (Table 2) [17–19].

Type I cysts, often referred to as secretory cysts, contain high K^+ and low Na^+, as in the intracellular compartment; **type II** cysts, also referred to as transudative cysts, have an opposite pattern with low K^+ and high Na^+. A third group of cysts with intermediate features is often identified (**type III**).

It has been suggested that most cysts originate from apocrine lobules [24]. The relationship between apocrine metaplasia of breast epithelial cells and the whole organ propensity for malignant changes is still a matter of controversy [25].

Some observations suggest that women with **type I** cysts are at increased risk of breast cancer [11, 13, 26]. These cysts have indeed been associated with major apocrine expression of the lining epithelium [27]. This association has been subsequently questioned [28]. The issue still remains of the significance of apocrine metaplasia in the wall and in the tissue surrounding the cysts.

STEROID BIOCHEMISTRY OF BCF

A peculiar feature of BCF biochemistry refers to conjugate steroids. Several reports have clearly demonstrated that androgen sulfates, notably dehydroepiandrosterone sulfate (DHAS) and androsterone sulfate are accumulated in wide ranging amounts up to concentrations 100-fold higher than in plasma [19, 21, 22]. Elevated values for estrogen sulfates have also been reported, in particular for estriol sulfate [29] and estrone sulfate [22, 30]. Studies that aimed to compare levels of these steroid conjugates in subgroups of cysts were consistent in indicating significantly higher values in type I than in type II cysts [19, 21, 22, 30]. Differences between the two subgroups are thought to reflect different activity of the lining epithelium [31]. Among the above mentioned molecules, DHAS is considered a marker of apocrine activity because it is present, sometimes in very high concentrations, in extramammary apocrine secretions such as axillary sweat glands, anogenital sweat glands, cerumen producing glands, etc. [32].

With regard to non-conjugated steroids, more recent reports point to an important androgenic milieu inside type I cysts.

Belanger *et al.* [33] have reported the intracystic concentrations of 18 non-conjugated and conjugated steroids: C19 steroids were selectively elevated in type I BCF. Another interesting finding was the presence of

Table 2. Cut-off values used for segregating cysts in different subsets, according to the cationic composition

	Dogliotti *et al.* [19]	Bradlow *et al.* [17]	Miller *et al.* [18]
Type I (high K^+, low Na^+)	$K^+/Na^+ > 1.5$	$K^+ > 100$ mmol/l, $Na^+ < 50$ mmol/l	$K^+/Na^+ > 1$
Type II (low K^+, high Na^+)	$K^+/Na^+ < 0.66$	$K^+ < 50$ mmol/l, $Na^+ > 100$ mmol/l	$K^+/Na^+ < 0.25$
Type III (intermediate)	K^+/Na^+ 0.66–1.5	$K^+ < 100$ mmol/l, $Na^+ > 50$ mmol/l	K^+/Na^+ 0.25–1

high concentrations of androsterone glucuronide (ADT-G) and androstane-3α,17β-diol glucuronide (3α-17β DIOL-G) and the relationship between these steroids and their C19 steroid precursors, namely DHAS and dihydrotestosterone (DHT).

This observation was confirmed by Secreto *et al.* [31] who found higher amounts of testosterone and DHT in type I than in type II cysts as well as a significant correlation of these androgens with DHAS in type I cysts. The concept that DHAS is converted into potent androgens by the breast tissue surrounding type I cysts does not contrast with the widely accepted influence of androgens upon apocrine epithelium. To summarize, a hyperandrogenic microenvironment could characterize the terminal duct lobular units (TDLU) of breasts bearing type I cysts. This could account, on the one hand, for the accumulation of precursors and active hormones in BCF and, on the other hand, for the higher expression of apocrine changes.

The presence in BCF of C21 steroids at levels comparable to those found in plasma was reported by Bradlow *et al.* [22]. More recently, it was found that progesterone and pregnenolone are selectively accumulated in type II cysts [33]. This pattern was putatively attributed to the presence in these fluids of specific progestin components. Rosner *et al.* [34] demonstrated, in fact, that steroid binding proteins are more elevated in fluids with high concentrations of Na^+. Three major proteins are present in BCF and account for approx. 70% of the total protein content of this medium [35]. These three proteins have been isolated, characterized and termed gross cystic disease fluid protein-15 (GCDFP-15), -24, and -44 for GCDFP of 15,000, 24,000, and 44,000 monomer molecular size [35, 36]. GCDFP-24 has been described as a glycoprotein which shows a high degree of binding specificity for the C17 and C21 regions of progesterone; it accounts for about half of the total protein present in BCF and is identical to the sequence of human apolipoprotein D [37]. The issue of differential concentrations of GCDFP-24 as a function of the cyst type needs to be clarified before any conclusion on the mechanisms accounting for progesterone and pregnenolone accumulation in type II BCFs.

As far as C18 steroids are concerned, intracystic estrogen levels of estrone (E1) and estradiol (E2) have been found comparable to plasma, despite the marked accumulation of the sulfo-conjugated molecules [22]. No significant differences have been found in E1 and E2 levels between the two major types of fluid [33]. We should conclude that, in the face of high intracystic levels of both androgen and estrogen sulfates, the non-conjugated molecules display a different behavior. While the concentrations of the non-conjugated active androgens parallel those of androgen sulfates, thus making conceivable the occurrence of conversion processes, this relationship does not hold for estrogens.

Table 3. Non-conjugated and conjugated steroids assayable at significantly different concentrations in type I and in type II cysts

Higher in type I	Higher in type II
—Testosterone	—Pregnenolone
—Dihydrotestosterone	—Progesterone
—Androsterone	
—Androstane-3α,17β-diol	
—Androst-5-ene-3β,17β-diol	
—Dehydroepiandrosterone-sulfate	
—Androsterone-glucuronide	
—Androstan-3α,17β-diol-glucuronide	
—Estrone-3-sulfate	
—Estriol-3-sulfate	

From Refs [19, 29–31, 33].

Table 3 shows steroids measurable at significantly different concentrations in the two opposite cyst types.

As regards the mechanisms of steroid sulfate accumulation in BCF, it is of interest that radiolabeled DHAS is the only steroid that shows appreciable intracystic accumulation after exogenous administration [22]. It cannot be excluded that the other sulfates found in large amounts in BCF are synthesized *in situ* from DHAS. The presence of sulfatase and aromatase activity in the surrounding tissue could account for the intracystic levels of unconjugated steroids. The relatively lower concentrations of estrogens may be viewed as compatible with low aromatase activity, as suggested by Belanger *et al.* [33].

GCDFP-15: IS IT ONLY A HISTOCHEMICAL OR ALSO A BIOCHEMICAL MARKER OF APOCRINE METAPLASIA IN THE BREAST?

It has been stressed that cysts originate from apocrine lobules and apocrine metaplasia of the breast epithelium is the primary lesion that allows cysts to develop [24]. The elevated intracystic levels of androgens raise interest when considering that the secretory activity of apocrine cells is under the influence of androgenic molecules [38].

GCDFP-15 is a glycoprotein with a M_w of 15,000, extracted and purified from BCF; this medium, in fact, contains huge amounts of the protein [35, 36]. GCDFP-15 has been recognized as a highly predictable immunocytochemical marker for cells with apocrine changes including those of breast cancers with apocrine differentiation [39, 40]. *In vitro* studies have demonstrated that the production of this protein can be modulated at the gene level by various hormones, with androgens appearing to increase the rate of gene transcription and estrogens decreasing this rate [36]. It could be hypothesized that intracystic concentrations of the protein may be affected by the androgen/estrogen ratio in BCF or in the apocrine cells lining the cysts. Also the concentrations of GCDFP-15 in the peripheral plasma of women with GCD deserve interest in

Table 4. Plasma levels of GCDFP-15 in women with GCD, breast cancer (BC) and controls

	n°	Mean ± SE	Median	Range
GCD (premenopausal)	200	60.13 ± 5.0	42.5	10–721
Breast cancer				
Total	68	45.20 ± 5.6	30.8	7.4–232
Premenopausal	24	44.84 ± 6.3	40.1	7.4–120
Menopausal	44	45.40 ± 8.1	27.5	9.3–232
Controls				
Total	75	22.10 ± 2.0	18.6	5.1–75.6
Premenopausal	35	27.74 ± 2.8	25.4	5.4–75.6
Menopausal	40	17.14 ± 1.7	13.8	5.1–47.9

Premenopausal GCD vs premenopausal controls, $P < 0.0001$; premenopausal BC vs premenopausal controls, $P < 0.05$; menopausal BC vs menopausal controls, $P < 0.0001$.

that they could reflect the extent of the apocrine changes of the mammary tissue as a consequence of the leakage phenomenon from metaplastic cells in the breast [36].

Little amounts of radioimmunoassayable GCDFP-15 have been detected in the peripheral plasma of normal women, whereas higher amounts are measurable in patients with advanced breast cancer and notably in those with GCD [36]. At present, the function and the biological significance of the secreted GCDFP-15 is unknown [25].

We have measured the circulating levels of the protein in patients with GCD and breast cancer using a new two stage solid phase enzyme-linked immunosorbent assay (ELISA), kindly supplied by Signet Laboratory (Dedham, MA, U.S.A.). After validation of the method (sensitivity 5 ng/ml; intra- and interassay variations 3.7 and 12.1%, respectively) we applied it to plasma samples drawn from pre- and menopausal controls (No. 75), premenopausal patients with GCD (No. 200) and pre- and menopausal patients with advanced breast cancer (No. 68). In addition, we measured GCDFP-15 in 47 BCFs [41]. A summary of results is presented in Table 4. Plasma GCDFP-15 levels were significantly higher in premenopausal than in menopausal controls ($P < 0.0001$). Consistently higher values were found in patients with GCD than in premenopausal controls ($P < 0.0001$), whereas no significant difference was apparent between the group of GCD patients and that of premenopausal cancer patients. Higher levels than controls ($P < 0.0001$) were found in cancer patients only in menopausal age. Huge amounts of GCDFP-15 were obviously detected in BCF (mean 6.9 mg/ml); unexpectedly significant differences were not apparent between type I and type II fluids.

Methodological biasing relevant to working dilutions of this particular medium and the small series of our specimens as well may explain this result. It is noteworthy, however, that we did not find significant differences in plasma GCDFP-15 levels among patients bearing type I or type II cysts. From the body of our data, we could provisionally state that the postulated significance of immunoassayable GCDFP-15 as a marker of the intensity of apocrine metaplasia in a given benign or malignant breast condition is not apparent. The supranormal levels observed in the peripheral blood of premenopausal women with GCD and of menopausal patients with advanced breast cancer, on the other hand, call for large scale protocols aimed to assess the value of longitudinally measuring plasma GCDFP-15 in relation to cancer risk and to diagnosed cancer disease.

Table 5. Number of patients enrolled in the cohort study and cationic categorization of cysts

Total number	1038
With BCF evaluated	798
With solitary cysts	
—Type I (K^+/Na^+) > 1.5	362 (45.3%)
—Type II + III (K^+/Na^+) < 1.5	311 (39%)
With multiple cysts	
2	98 (12.4%)
3	24 (3.0%)
4	1 (0.1%)
5	2 (0.2%)

CATEGORIZATION OF CYSTS AND CANCER RISK: A COHORT STUDY

A vast body of observations is consistent with the view that gross cysts of the breast can be classified into two main subpopulations according to their electrolyte profile. As discussed previously several hormones are present in different amounts in type I and type II cysts.

The cationic pattern of BCF appears predictable of a natural history of GCD. Patients with type I cysts have more commonly multiple or relapsing cysts than patients with type II cysts [26, 42]. Interestingly, a higher risk of breast cancer has been found in the classical studies of Haagensen *et al.* [1] for patients with relapsing cysts as compared to women who had a single aspiration.

An important issue is whether cationic typing of BCF is predictive of breast cancer risk in individual patients. It has been reported that patients with type I "apocrine" cysts were significantly more likely to have "*in situ*" carcinoma or epithelial hyperplasia in their breasts than matched controls [43]. Preliminary observations by Bradlow *et al.* [13] and Bundred *et al.* [11] suggest that an increased risk of breast cancer may be pertinent to women who have had one or more type I aspirated cysts. Conversely, others have questioned the assumption that cationic categorization of cysts is of value in predicting the natural history of GCD or in assessing cancer risk [44, 45].

In order to evaluate the relationship between the risk of breast cancer and some biochemical features of BCF in women affected by GCD, in 1983 a cohort prospec-

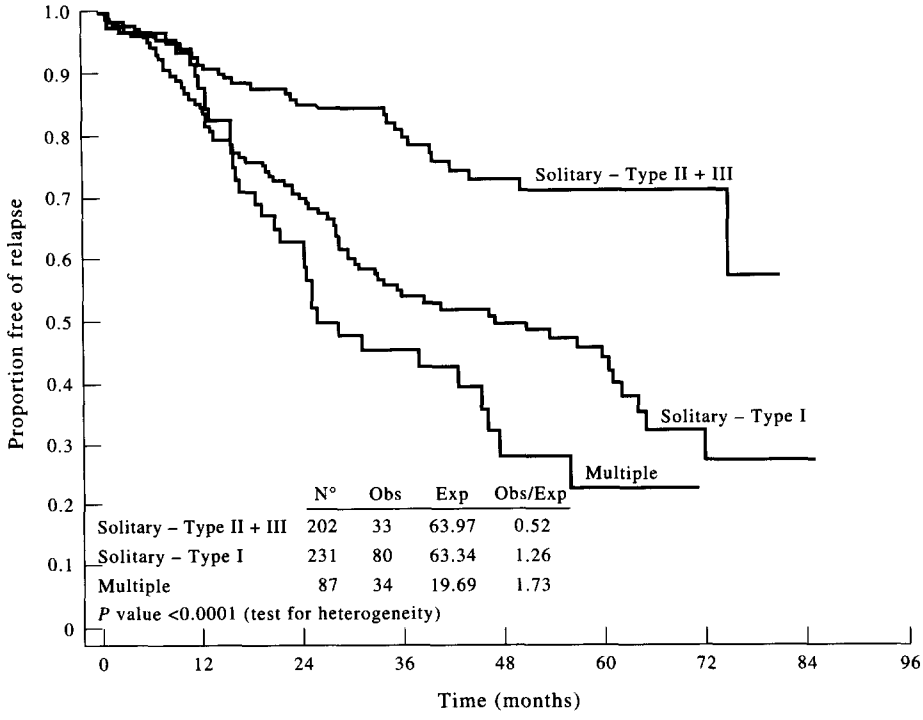

Fig. 1. Relapse-free survival among 520 patients by number of cysts at presentation and, among patients with solitary cysts, by cationic type of cyst.

tive study was undertaken at the Cancer Prevention Center of Ravenna and the study is still in progress.

From February 1983 to June 1992, 1038 consecutive women aged 30–69 years, with no previous or concomitant malignancy at any site including breast, underwent aspiration of a breast cyst >1 ml and were enrolled in a cohort study. The follow up program included physical examination and ultrasound scan every year and mammography every 2 years. The proportion of women lost to follow up has been very low (<1%).

BCF was assayed for biochemical components in 798 women. The bimodal distribution of the electrolyte composition was confirmed; the scatter plot of log K^+ vs log Na^+ of BCF suggested that fluids could be

Table 6. Some details of the 18 incident breast cancer cases

Patients	Date of enrollment	Age at enrollment	Date of diagnosis of breast cancer	K^+/Na^+ ratio	Cyst type
1.*	02.09.83	50	11.22.93	0.60	III
				5.19	I
2.	03.02.83	37	07.22.88	2.69	I
3.	03.18.83	42	05.11.88	Unknown	Unknown
4.	03.26.83	41	01.19.84	Unknown	Unknown
5.	12.01.83	45	09.05.86	3.96	I
6.	04.19.84	50	06.28.88	1.85	I
7.	05.21.84	45	12.31.87	3.68	I
8.	06.04.85	47	02.18.88	4.45	I
9.	07.10.85	42	09.17.85	5.44	I
10.	07.31.85	48	09.17.87	3.66	I
11.	06.03.86	47	06.16.92	Unknown	Unknown
12.	07.30.86	38	10.29.92	Unknown	Unknown
13.	02.28.87	48	06.29.92	1.45	II
14.	08.10.88	54	03.11.92	2.51	I
15.	02.03.89	30	02.08.91	Unknown	Unknown
16.	09.26.89	44	06.21.90	1.98	I
17.	02.26.90	46	05.02.92	Unknown	Unknown
18.**	08.13.91	38	03.02.92	1.58	I
				2.17	I
				2.21	I

*Patient with 2 cysts at enrollment; **patient with 3 cysts at enrollment.

separated in three subgroups: **type I** cysts, with $K^+/Na^+ > 1.5$; **type II** cysts, with $K^+/Na^+ < 0.25$; and **type III** cysts, with K^+/Na^+ 0.25–1.5. Table 5 shows the general composition of the cohort study.

Type I cysts were found to be more frequent in women with 0 or 1 birth, in women with a history of previously aspirated "apocrine" cysts, in current smokers and in those not drinking coffee.

The risk of cyst relapse was significantly higher among women with **type I** cysts or with multiple cysts at presentation. In fact, a 2-fold increase in the relapse rate was observed when patients with K^+/Na^+ in BCF > 1.5 were compared with women with $K^+/Na^+ < 1.5$. The highest relapse rate was observed among women with multiple cysts ($P < 0.05$) [26], as reported in Fig. 1. No correlation between BCF composition and Wolf's mammographic parenchymal patterns was observed.

In the whole cohort, 18 breast cancers have been so far diagnosed, in apparent excess (2-fold increase) than expected on the basis of the incidence reported in the area (Cancer Registry of Romagna region, Italy). Eleven out of 12 cases of breast cancer diagnosed among the 798 women with categorized BCF occurred among women with type I cysts or multiple cysts at presentation (Table 6). This observation suggests that the increase in cancer risk is associated with a peculiar subgroup of patients with gross cystic disease of the breast.

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