CELL BIOCHEMISTRY & FUNCTION

CELL BIOCHEMISTRY AND FUNCTION
Cell Biochem Funct 2015; **33**: 128–133.
Published online 6 March 2015 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/cbf.3096

Breast cyst fluid heparan sulphate is distinctively *N*-sulphated depending on apocrine or flattened type

Ferdinando Mannello[1], Francesca Maccari[4], Daniela Ligi[1], Martina Santi[1], Francesco Gatto[2], Robert J. Linhardt[3], Fabio Galeotti[4] and Nicola Volpi[4]*

[1]*Department of Biomolecular Sciences, Section of Clinical Biochemistry, Unit of Cell Biology, University "Carlo Bo", Urbino 61029, Italy*
[2]*Department of Biological and Chemical Engineering, Chalmers University of Technology, Gothenburg 41296, Sweden*
[3]*Departments of Chemistry and Chemical Biology, Chemical and Biological Engineering and Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, USA*
[4]*Department of Life Sciences, University of Modena and Reggio Emilia, Modena 41125, Italy*

Breast cyst fluid (BCF) contained in gross cists is involved with its many biomolecules in different stages of breast cystic development. Type I apocrine and type II flattened cysts are classified based on biochemical, morphological and hormonal differences, and their different patterns of growth factors and active biocompounds may require different regulation. In a previous paper, hyaluronic acid in a very low content and chondroitin sulphate/dermatan sulphate were identified and characterized in BCF. In this new study, various apocrine and flattened BCFs were analyzed for HS concentration and disaccharide pattern. Apocrine HS was found specifically constituted of *N*-acetyl groups contrary to flattened HS richer in *N*-sulphate disaccharides with an overall *N*-acetylated/*N*-sulphated ratio significantly increased in apocrine compared with flattened (13.5 vs 3.7). Related to this different structural features, the charge density significantly decreased (~−30%) in apocrine versus flattened BCFs. Finally, no significant differences were observed for HS amount (~0.9–1.3 $\mu g\,ml^{-1}$) between the two BCF types even if a greater content was determined for flattened samples. The specifically *N*-sulphated sequences in flattened BCF HS can exert biologic capacity by regulating growth factors activity. On the other hand, we cannot exclude a peculiar regulation of the activity of biomolecules in apocrine BCF by HS richer in *N*-acetylated disaccharides. In fact, the different patterns of growth factors and active biocompounds in the two types of cysts may require different regulation by specific sequences in the HS backbone possessing specific structural characteristics and distinctive chemical groups. Copyright © 2015 John Wiley & Sons, Ltd.

KEY WORDS—breast cancer; breast cyst fluids; heparan sulphate; glycosaminoglycans; human gross breast cyst disease

LIST OF ABBREVIATIONS—BC, breast cancer; BCF, breast cyst fluid; GAG, glycosaminoglycan; GCBD, gross cystic breast disease; HS, heparan sulphate

INTRODUCTION

Gross cystic breast disease (GCBD) is the most frequent lesion of the breast, with an incidence of ~7% in the Western world.[1] GCBD is considered an advanced form of fibrocystic lesion in which active molecules present in breast cyst fluid (BCF) play a role in different stages of cystic development. Different developmental stages may occur in cyst evolution, and these biologically active compounds may influence GCBD evolution and breast cancer (BC) risk.[2] The lining of gross cysts (GCs) is a simple layer of apocrine or flattened epithelium that may disappear during cyst evolution leaving a bare connective tissue surface.[1] In the early apocrine stage, both myoepithelial and apocrine hypertrophic/hyperplastic epithelial cells undergo lysis releasing proteolytically activated bioactive compounds into BCFs (type I secretive cysts).[3–5] Before the fibrotic disappearance, the final stage is characterized by flattened cells (type II transudative cysts)[3–7] lining GCs and containing a BCF composition similar to plasma. As a consequence, two main GC types may develop, type I secretive and type II transudative cysts.[1,8,9] Type I cysts, lined by apocrine metabolically active epithelial cells, produce and secrete many compounds such as growth factors, specific proteins, proteinases, steroids and peptide hormones. These compounds accumulate within the cyst compartment, promoting cell proliferation and inducing apocrine cells to atypia, hyperplasia or preneoplastic alterations related to BC, as suggested by epidemiological and clinical studies.[1,7–10] On the contrary, type II transudative cysts are mainly characterized by a composition similar to that of plasma in which biomolecules are derived from plasma drainage and lined by flattened

*Correspondence to: Nicola Volpi, Department of Life Sciences, University of Modena and Reggio Emilia, Via Campi 213/D, Modena 41125, Italy. E-mail: volpi@unimo.it

Received 12 December 2014
Revised 30 January 2015
Accepted 2 February 2015

epithelium without hypertrophic metabolism and no epidemiological evidence of BC predisposition.

The electrolytic composition of BCF samples has revealed a wide variation of Na^+, K^+ and Cl^- levels ranging from a profile characteristic of extracellular fluids to one characteristic of the intracellular compartment.[1,7–11] This variation is attributed mainly to changes in Na/K ATPase activity, a key enzyme in the development of GCBD.[1] Type I cysts show a high level of K^+ and a low level of Na^+, with a Na^+/K^+ ratio generally <3. On the contrary, type II cysts have low K^+ and high Na^+ concentrations for a Na^+/K^+ ratio >3.[1,10] Different risk profiles of BC have been found in GC subtypes (see[1] for review) with type I apocrine GC frequently associated with an increased risk.[11] However, several controversial results have been reported.[8]

BCFs also contain many biologically active compounds secreted or derived by the lysis of the apocrine epithelial cells lining the cysts. In an effort to identify GC at higher BC risk depending on the two cyst types, several tumour markers have been evaluated in BCFs.[9,10] In particular, growth factors have been analyzed to elucidate a possible relationship between GCBD, cyst types and BC.[1,9–11] Peculiar differences were found between apocrine and flattened cysts, the former being particularly rich in epidermal growth factor (EGF) while the latter being abundant in transforming growth factor (TGF)-beta, basic fibroblast growth factor (b-FGF) and insulin-like growth factor-binding protein-3.[1,9–11]

Along with other active molecules, glycosaminoglycans (GAGs), unbranched structurally complex polysaccharides composed of repeating units of alternating uronic acids and amino sugars,[12] and their macromolecular associations proteoglycans (PGs) play major roles in multiple cancer-related processes.[13] In fact, GAGs bind to a large variety of ligands,[14] thus regulating growth factor signalling, cellular behaviour, inflammation, angiogenesis and the proteolytic environment. Deregulated expression of GAGs, as well as of enzymes involved in their biosynthesis and degradation.[15] contribute to the different steps of tumour progression and correlate with clinical prognosis in several malignant neoplasms.[13]

In a previous study,[16] we determined the presence of GAGs in BCF of GCs subdivided into apocrine and flattened in a cohort of GCBD-affected women. We were able to identify chondroitin sulphate (CS)/dermatan sulphate (DS), heparan sulphate (HS) and a very low content of hyaluronic acid. Furthermore, the CS/DS species were quantified and structurally characterized for molecular mass, disaccharide composition and overall charge density. Over the past, no structural evidence was available for HS. However, over the past decade, HS has been shown to bind and regulate the activities of many proteins generally by specific interaction to particular sequences within polysaccharide chains, thereby underscoring the importance of its determination in GCBD.[17] Because of its capacity to non-covalently interact with many proteins, HS has important emerging roles in oncogenesis, and its (and heparin) derivatives represent potential therapeutic strategies for human cancers.[18,19] This is more important by considering that BCF contain many polypeptides, enzymes and growth factors such as b-FGF, EGF, insulin-like growth factors 1 and 2, platelet-derived growth factor (PDGF) and various TGFs (see[1] for review). In this study, we report for the first time the disaccharide pattern and structural characterization of HS purified from BCF of apocrine and flattened GCs with the aim of finding a correlation between HS structure and BCF type and composition.

MATERIALS AND METHODS

BCF collection

As described previously,[16] BCF samples were collected by fine needle aspiration from 25 women GCBD-affected after informed consent. The study was performed according to the ethical standards of the Helsinki Declaration of 1975 (as revised in 1983), and the approval of the Ethics Committee of the University Carlo Bo of Urbino (protocol 18/CE) was obtained. The patients were recruited after attending the Senology Center of the Hospital of Urbino and subdivided according to the menopausal stage (aged 29–52 years). The size of the GCs varied from 1 to 6.5 cm in diameter, and the volume of BCFs ranged between 1.5 and 40 ml. Occurrence of cancer was always excluded by clinical, echographic, mammographic and cytological examinations.

Women that had taken any hormonal medication for at least 6 months prior to the study, or reporting pregnancy, lactation or clinical symptoms concerning the breast within 3 years prior this study, as well as those who had been medically treated for breast diseases within 6 months prior to the study, were excluded from the recruitment.

The different types of breast GCs were characterized and classified as type I secretive apocrine or type II transudative flattened based on their Na^+/K^+ ratio, according to previous classification.[1,3–5,16] $Na^+$and K^+ concentrations in BCFs were measured by flame photometry (Perkin Elmer), with and without an internal standard at 589 and 776 nm, respectively.[20] Log-normalized Na/K ratio values were employed throughout the analysis to best separate the populations of breast cysts.[1,20]

Heparan sulphate determination

BCFs were centrifuged at 19 000 g for 30 min at 4 °C and the supernatants stored at −30 °C until assay. HS determination was carried out by means of high-performance liquid chromatography (HPLC) with on-line fluorescence detector and electrospray ionization mass spectrometry (ESI-MS) as described in.[21,22] After extraction and purification, HS disaccharide mapping was performed after enzymatic treatment by using a cocktail of heparinases I, II and III.[20,21] Released unsaturated disaccharides by the action of lyases were fluorotagged with 2-aminoacridone (AMAC) and separated by HPLC-fluorescence detector-ESI-MS.

Statistical analysis

All the assays were carried out in triplicate and the data expressed as mean ± standard error of the mean. The highest

density interval (HDI) for the mean difference in each measurement on HS between flattened and apocrine samples was calculated using Bayesian estimation under the following assumptions: measurements are sampled from a *t*-distribution of unknown and to be estimated normality (i.e. degrees of freedom); high uncertainty on the prior distributions; the marginal distribution is well approximated by a Markov chain Monte Carlo sampling with no thinning and chain length equal to 100 000. The estimation was performed using *BEST*[23] (the previous assumptions are reflected by the default parameters). A difference in a measurement between flattened and apocrine is deemed significant if the HDI for the difference does not include zero.

RESULTS

A common strategy for the detailed structural analysis of HS involves exhaustive enzymatic digestion with heparinases able to reduce these biomolecules to its disaccharide building blocks that are then separated and quantified by analytical techniques.[21,22] HS extracts purified from BCF samples were treated with heparinases, heparin lyases, that catalyse its β-eliminative cleavage, yielding eight main disaccharides having a 4,5-unsaturated uronic acid residue at their nonreducing ends.[21,22] The generated unsaturated disaccharides by the enzymatic action were fluorotagged with AMAC to significantly increase detection sensitivity and to improve their resolution in HPLC (Figure 1). HS is generally found to be constituted of a high percentage of non-sulphated disaccharide IVA with an overall charge density of ~0.30–0.60 typical for this kind of biopolymers.[21] Minor yet not negligible percentages of other disaccharides, monosulphated, disulphated and trisulphated in various positions, are generally present inside the HS backbone. These minor constituents may play an important role for HS biological properties and represent specific markers of diseases.[24]

Fifteen samples of type I secretive apocrine BCF and ten samples of type II transudative flattened BCF were analyzed for HS concentration and disaccharide pattern (Table 1). No significant differences were observed for HS amount between apocrine and flattened BCF present in a content of ~0.9–1.3 $\mu g\,ml^{-1}$ (Table 1) even if we report a trend towards a greater abundance for flattened samples. The determination of the HS disaccharide pattern in the BCF samples allowed a quantitative evaluation and compositional analysis of the main non-sulphated and variously sulphated disaccharides. The percentage of non-sulphated *N*-acetylated disaccharide IVA was found significantly increased in apocrine compared with flattened BCF (+21.3%; Figure 2). On the contrary, the specifically *N*-sulphated disaccharides, species IVS and IIIS, were observed to decrease by ~67% and ~25%, respectively (Figure 2). The ratio between non-sulphated *N*-acetylated IVA and *N*-sulphated IVS disaccharides explains 79% of the variation in the HS disaccharide pattern between type I secretive apocrine and type II transudative flattened BCF samples, as revealed by principal component analysis (Figure 1 in the supporting information). Indeed, this ratio was significantly increased in apocrine compared with flattened BCFs (13.5-fold vs 3.7-fold; Figure 3). These results confirm that HS in apocrine BCFs is mainly constituted of non-sulphated *N*-acetyl groups as opposed to flattened BCFs, which are rich in *N*-sulphated disaccharides. Finally, the charge density significantly decreases by about 30% in type I secretive apocrine vs type II transudative flattened BCFs (Figure 2), mainly because of the observed differences in *N*-acetylated/*N*-sulphated disaccharides.

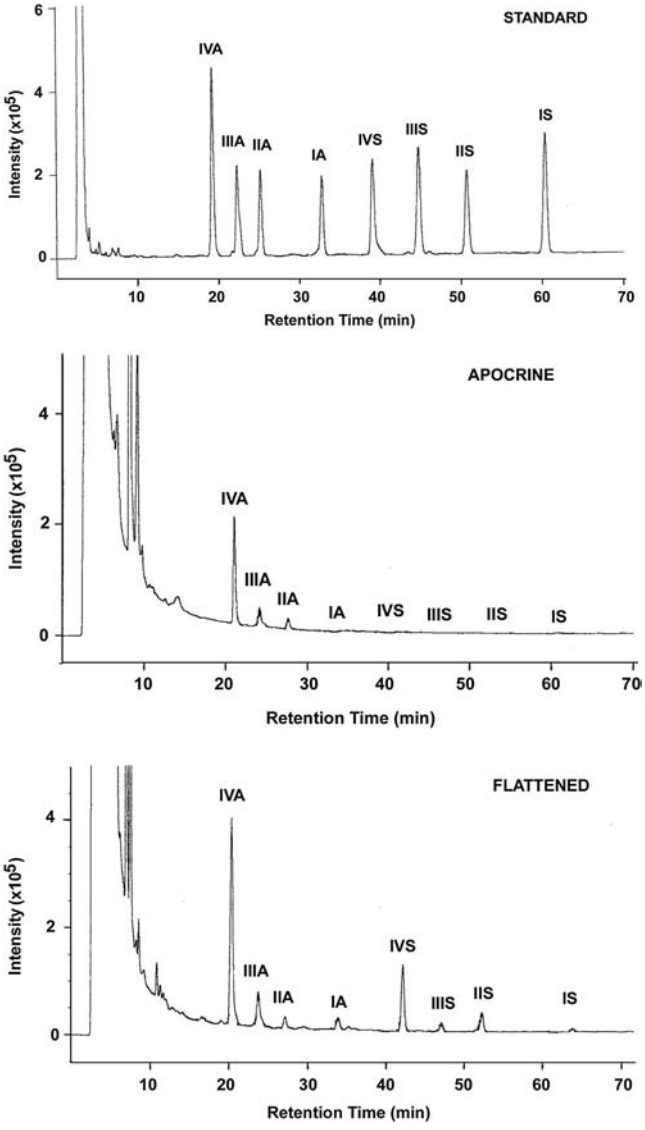
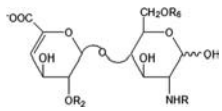


Figure 1. High-performance liquid chromatography separation and fluorimetric detection of the HS unsaturated disaccharides after derivatization with 2-aminoacridone. A separation of eight standard disaccharides, a sample representative of type I apocrine and one for type II flattened BCF is illustrated. IVA, ΔUA-GlcNAc. IIIA, ΔUA2S-GlcNAc. IIA, ΔUA-GlcNAc,6S. IA, ΔUA2S-GlcNAc,6S. IVS, ΔUA-GlcNS. IIIS, ΔUA2S-GlcNS. IIS, ΔUA-GlcNS,6S. IS, ΔUA2S-GlcNS,6S. ΔUA: unsaturated uronic acid. GlcN: glucosamine. Ac: acetyl group. S: sulphate group

Table 1. Total content and disaccharide composition (in %) of HS from 15 type I secretive apocrine and ten type II transudative flattened



	Type I secretive apocrine	Type II transudative flattened
μg HS (ml BCF)$^{-1}$	0.89 ± 0.14	1.32 ± 0.37
IVA (ΔUA-GlcNAc)	74.1 ± 1.7	61.1 ± 2.3
IIIA (ΔUA2S-GlcNAc)	3.4 ± 0.3	4.9 ± 1.5
IIA (ΔUA-GlcNAc,6S)	12.0 ± 1.0	11.4 ± 0.9
IA (ΔUA2S-GlcNAc,6S)	0.1 ± 0.1	0.1 ± 0.1
IVS (ΔUA-GlcNS)	5.5 ± 0.5	16.7 ± 1.6
IIIS (ΔUA2S-GlcNS)	1.5 ± 0.6	2.0 ± 0.3
IIS (ΔUA-GlcNS,6S)	2.7 ± 0.3	3.0 ± 0.4
IS (ΔUA2S-GlcNS,6S)	0.6 ± 0.1	0.8 ± 0.1
Charge density	0.32 ± 0.02	0.46 ± 0.02

Data are illustrated as means ± standard error.
The scheme illustrates the HS unsaturated disaccharides produced by the action of heparin lyases. R may be an acetyl or sulphate group; R_2 and R_6 may be a sulphate group or H.
Charge density (the sulphate-to-carboxyl ratio) was calculated considering the presence and the percentage of carboxyl and sulphate groups for each disaccharide.
ΔUA = unsaturated uronic acid; GlcN = glucosamine; Ac = acetyl group; S = sulphate group.

DISCUSSION

HS and heparin have been shown to bind and regulate the activities of many proteins, such as enzymes, growth factors, extracellular matrix (ECM) proteins and the cell surface proteins of pathogens.[18,19,25] In turn, these interactions control many biological processes such as cell signalling, morphogenesis *in vivo*, wound healing, cancer cell proliferation and metastasis.[17,26,27] Many cytokines and growth factors contain specific sequences rich in lysine/arginine residues able to interact with HS mediated by specific ionic binding.[28] The HS macromolecules present in HSPGs bind both growth factor ligands and receptors to form a ternary complex that enhances signalling and can promote carcinogenesis.[28] On the contrary, a high local content of cell surface HSPGs can decrease growth factor signalling complexes. Finally, HSPGs located at the surface of cells can also be released to enhance or suppress cell signalling and influence cancer cell biology.[28]

Several studies have focused on the relationship between HS structure and activity, and specific recognition required by defined sequences within the HS chain.[17,18,28,29] Compared with type I apocrine BCFs, we observed a more sulphated HS in type II flattened BCF and in particular a significantly higher percentage of *N*-sulphate groups. Conversely, we observed a decrease in non-sulphated *N*-acetylated residues in the HS present in type I apocrine fluid. A more sulphated HS in flattened BCF with

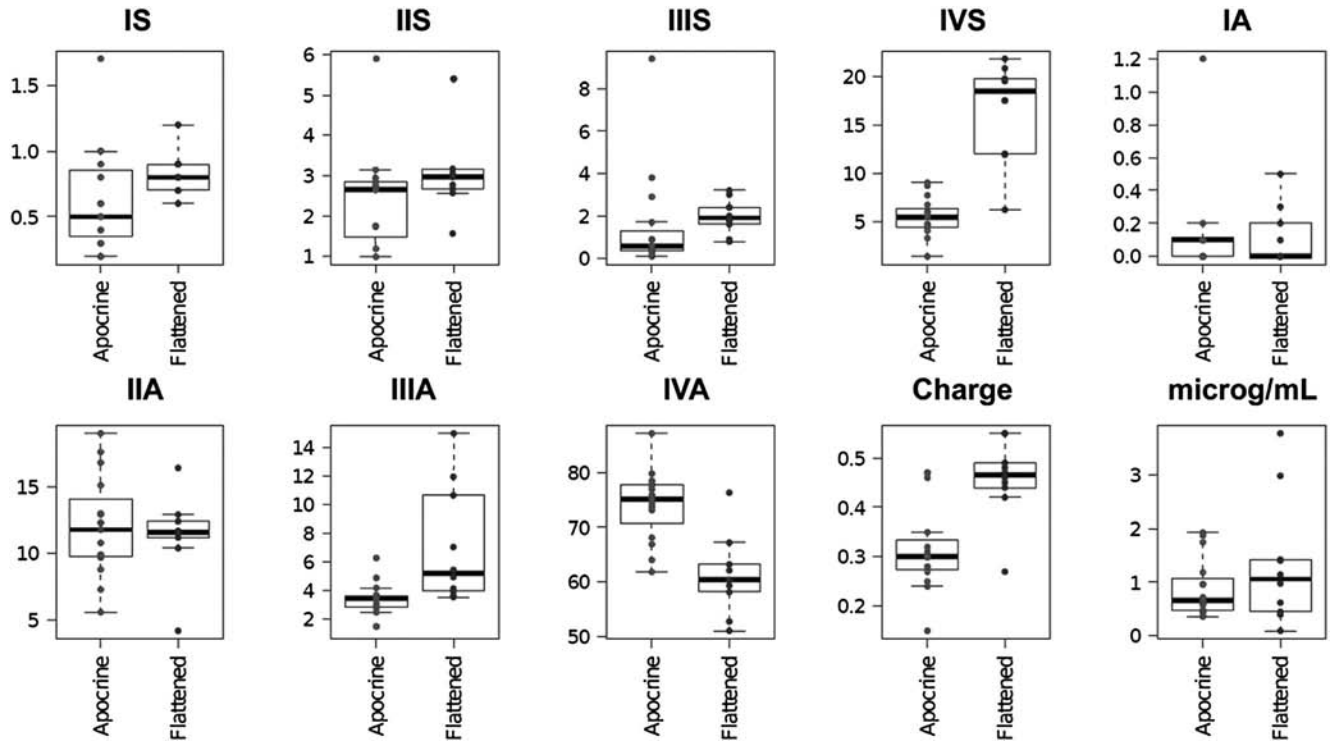


Figure 2. Boxplots of percentage for the eight different HS disaccharides (I-IVS and I-IVA) and total HS charge density and concentration (in μg ml^{-1}) in type I apocrine versus type II flattened BCF samples

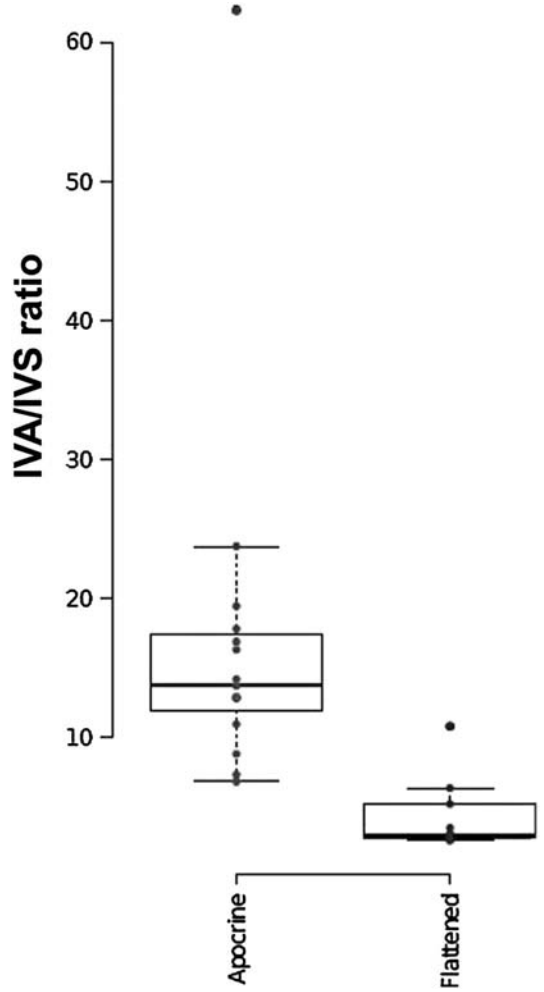


Figure 3. The ratio between non-sulphated *N*-acetylated disaccharide (IVA) and *N*-sulphated disaccharide (IVS) is significantly higher in type I apocrine BCF compared with type II flattened BCF

specifically *N*-sulphated disaccharide/oligosaccharide sequences can exert biologic activity by binding and regulating growth factors activity. On the other hand, we cannot exclude that a yet unexplored regulation of the activity of biomolecules present in apocrine BCF may require HS richer in *N*-acetylated disaccharides/sequences. In fact, major differences have been found for the two main subgroups of gross breast cysts as demonstrated by a wide characterization of different biochemical, morphological and hormonal features.[1] Type I secretive cysts are lined by apocrine metabolically active epithelial cells able to produce a variety of biomolecules, which accumulate within the cyst compartment. Type II transudative cysts are mainly characterized by a biochemical composition similar to that found in plasma. The different patterns of growth factors and active biocompounds in the two types of cysts may therefore require different regulation by specific sequences in the HS backbone possessing specific structural characteristics and distinctive chemical groups. In this regard, type I secretive cysts are rich in EGF contrary to type II transudative showing a high content of b-FGF, TGF and interleukin-4, interleukin-6 and interleukin-8.[1,9] It is worthy of mention that active HS sequences able to bind to b-FGF with high affinity are mainly composed of *N*-sulphated disaccharides essential for the interaction[30,31] and that HS-*N*-deacetylase/*N*-sulfotransferase (NDST) able to catalyse both the HS *N*-deacetylation and *N*-sulfation reactions modulates the activity of b-FGF by mediating its initial binding to HS.[32] Additionally, effective binding of FGF2 to its receptor requires the presence of HS with *N*-sulphated domains localized at the cell surface and ECM.[33] On the other hand, *N*-sulphated HS displays a higher affinity than other GAGs for interleukin-4,[34] and binding of HS to interleukin-8 correlates with the necessary occurrence of *N*-sulphated disaccharides.[35] Finally, reduction of *N*-sulfation has been observed to attenuate PDGF binding *in vitro*, leading to impaired PDGF signalling.[36]

Specific *N*-sulfation and total sulfation state of plasma HS were significantly found increased in patients having indirect lung injury,[24] showing that the sulfation pattern of circulating HS varies according to inciting pulmonary insult. In the two different types of cysts, HS may be produced more *N*-acetylated (type I apocrine) or *N*-sulphated (type II flattened) by the differential tissue-specific action of NDST enzymes able to produce and release in BCF HS polysaccharides having different structural microheterogeneity.

The different distributions of several molecules in the related types of GCs suggest that differential mechanisms are involved in the transport of some biocompounds into the cystic compartment, perhaps conditioning the evolution or regression of a gross breast cyst. In this respect, HS with its different structure and distinctive *N*-sulfation may act as a specific modulator of the active BCF components. Although we were unable to directly demonstrate the effect of different HS on BCF biomolecules (and growth factors) because of the limited HS content available for *in vitro* assays after its extraction and purification, our results support a differential role of HS in regulating important processes able to induce the onset and/or progression of benign lesions to malignant transformation or spontaneous regression.[1] In fact, gross cystic disease and BC are hormonally induced diseases,[37] and growth factor profiles of BCF may indicate the presence in breast tissue of a hormonal and proliferative environment permissive to subsequent cancer growth.[38] In this respect, HS with its distinctive sulfation profile may play a key role in regulating growth factors activity. Anyway, further studies are required to demonstrate the diagnostic and prognostic importance of BCF HS in a relationship with GCBD and BC evolution.

CONFLICTS OF INTEREST

We declare that we have no conflicts of interest.

ACKNOWLEDGEMENTS

We thank Dr Sebastiani M. and Dr Manna P. (Centre of Senology, AUSL 1 and 2, Pesaro and Urbino, Italy) for

the recruitment of patients, samples and clinical data collection.

This research was supported by Research Grant Award 2011 (to F. Mannello) from Dr. Susan Love Research Foundation (Santa Monica, CA, USA).

AUTHOR CONTRIBUTIONS

F. M. and N. V. designed and developed the experimental design. N. V., F. M. and F. G. performed the experimental procedures and analyses. N. V. performed the data analysis and wrote the manuscript. R. J. L. supported the analytical procedures. F. G. performed the statistical analysis.

All authors reviewed and approved the study.

REFERENCES

1. Mannello F, Tonti GA, Papa S. Human gross cyst breast disease and cystic fluid: bio-molecular, morphological, and clinical studies. *Breast Cancer Res Treat* 2006; **97**: 115–29.
2. Mannello F, Malatesta M, Gazzanelli G. Breast cancer in women with palpable breast cysts. *Lancet* 1999; **354**: 677–8.
3. Dixon JM, Scott WN, Miller WR. Natural history of cystic disease: importance of cyst type. *Br J Surg* 1985; **72**: 190–2.
4. Beccati D, Grilli N, Schincaglia P, *et al*. Apocrine cells in breast cyst fluid and their relationship to cyst type: a morphometric study. *Eur J Cancer Clin Oncol* 1988; **24**: 597–602.
5. Naldoni C, Bruzzi P, Bucchi S, *et al*. Cohort study of women affected by gross cystic disease: correlation between cationic and hormonal composition of breast cyst fluid and the risk of breast cancer. *Ann NY Acad Sci USA* 1990; **586**: 272–5.
6. Vizoso F, Fueyo A, Allende MT, *et al*. Evaluation of human breast cysts according to their biochemical and hormonal composition, and cytological examination. *Eur J Surg Oncol* 1990; **16**: 209–14.
7. Dixon JM, Miller WR, Scott WN. The morphological basis of human breast cyst populations. *Br J Surg* 1983; **70**: 604–6.
8. Ebbs SR, Bates T. Breast cyst type does not predict the natural history of cystic disease or breast cancer. *Br J Surg* 1988; **75**: 702–4.
9. Zanardi S, Pensa F, Torrisi R, *et al*. Presence and distribution of growth factors in breast cyst fluid. *Ann N Y Acad Sci* 1996; **784**: 542–9.
10. Zanardi S, Valenti G, Torrisi R, *et al.* Identification of breast cyst subpopulations: biochemical and morphological features. *Cancer Detect Prev* 1991; **15**: 273–6.
11. Fleisher M, Bradlow HL, Schwartz MK, *et al*. The anion gap in human breast cyst fluid: a possible high risk indicator for breast cancer. *Clin Chem* 1984; **30**: 940.
12. Jackson RJ, Busch SJ, Cardin AD. Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. *Physiol Rev* 1991; **71**: 481–539.
13. Yip GW, Smollich M, Götte M. Therapeutic value of glycosaminoglycans in cancer. *Mol Cancer Ther* 2006; **5**: 2139–48.
14. Mulloy B, Linhardt RJ. Order out of complexity - protein structures that interact with heparin. *Curr Opin Struct Biol* 2001; **11**: 623–28.
15. Mizumoto S, Ikegawa S, Sugahara K. Human genetic disorders caused by mutations in genes encoding biosynthetic enzymes for sulfated glycosaminoglycans. *J Biol Chem* 2013; **288**: 10953–61.
16. Mannello F, Maccari F, Ligi D, *et al*. Characterization of oversulfated chondroitin sulfate rich in 4,6-O-disulfated disaccharides in breast cyst fluids collected from human breast gross cysts. *Cell Biochem Funct* 2014; **32**: 344–50.
17. Knelson EH, Nee JC, Blobe GC. Heparan sulfate signaling in cancer. *Trends Biochem Sci* 2014; **39**: 277–88.
18. Powell AK, Yates EA, Fernig DG, *et al.* Interactions of heparin/heparan sulfate with proteins: appraisal of structural factors and experimental approaches. *Glycobiology* 2004; **14**: 17R–30R.
19. Turnbull JE, Miller RL, Ahmed Y, *et al.* Glycomics profiling of heparan sulfate structure and activity. *Methods Enzymol* 2010; **480**: 65–85.
20. Malatesta M, Mannello F, Sebastiani M, *et al.* Ultrastructural characterization and biochemical profile of human gross cystic breast disease. *Breast Cancer Res Treat* 1998; **48**: 211–9.
21. Galeotti F, Volpi N. Online reverse phase-high-performance liquid chromatography-fluorescence detection-electrospray ionization-mass spectrometry separation and characterization of heparan sulfate, heparin, and low-molecular weight-heparin disaccharides derivatized with 2-aminoacridone. *Anal Chem* 2011; **83**: 6770–7.
22. Volpi N, Galeotti F, Yang B, *et al.* Analysis of glycosaminoglycan-derived, precolumn, 2-aminoacridone-labeled disaccharides with LC-fluorescence and LC-MS detection. *Nat Protoc* 2014; **9**: 541–58.
23. Kruschke JK. Bayesian estimation supersedes the *t* test. *J Exp Psychol Gen* 2013; **142**: 573–603.
24. Schmidt EP, Li G, Li L, *et al.* The circulating glycosaminoglycan signature of respiratory failure in critically ill adults. *J Biol Chem* 2014; **289**: 8194–202.
25. Bernfield M, Gotte M, Park PW, *et al.* Functions of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem* 1999; **68**: 729–77.
26. Lander AD, Selleck SB. The elusive functions of proteoglycans: *in vivo* veritas. *J Cell Biol* 2000; **148**: 227–32.
27. Kraushaar DC, Dalton S, Wang L. Heparan sulfate: a key regulator of embryonic stem cell fate. *Biol Chem* 2013; **394**: 741–51.
28. Capila I, Linhardt RJ. Heparin-protein interactions. *Angew Chem Int Ed Engl* 2002; **41**: 391–412.
29. Casu B, Naggi A, Torri G. Heparin-derived heparan sulfate mimics to modulate heparan sulfate-protein interaction in inflammation and cancer. *Matrix Biol* 2010; **29**: 442–52.
30. Turnbull JE, Fernig DG, Ke Y, *et al.* Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate. *J Biol Chem* 1992; **267**: 10337–41.
31. Walker A, Turnbull JE, Gallagher JT. Specific heparan sulfate saccharides mediate the activity of basic fibroblast growth factor. *J Biol Chem* 1994; **269**: 931–5.
32. Ishihara M, Guo Y, Wei Z, *et al.* Regulation of biosynthesis of the basic fibroblast growth factor binding domains of heparan sulfate by heparan sulfate-N-deacetylase/N-sulfotransferase expression. *J Biol Chem* 1993; **268**: 20091–5.
33. Naimy H, Buczek-Thomas JA, Nugent MA, *et al*. Highly sulfated nonreducing end-derived heparan sulfate domains bind fibroblast growth factor-2 with high affinity and are enriched in biologically active fractions. *J Biol Chem* 2011; **286**: 19311–9.
34. Lortat-Jacob H, Garrone P, Banchereau J, *et al*. Human interleukin 4 is a glycosaminoglycan-binding protein. *Cytokine* 1997; **9**: 101–5.
35. Spillmann D, Witt D, Lindahl U. Defining the interleukin-8-binding domain of heparan sulfate. *J Biol Chem* 1998; **273**: 15487–93.
36. Abramsson A, Kurup S, Busse M, *et al.*. Defective N-sulfation of heparan sulfate proteoglycans limits PDGF-BB binding and pericyte recruitment in vascular development. *Genes Dev* 2007; **21**: 316–31.
37. Hess JC, Sedghinasab M, Moe RE, *et al*. Growth factor profiles in breast cyst fluid identify women with increased breast cancer risk. *Am J Surg* 1994; **167**: 523–30.
38. Ness JC, Sedghinasab M, Moe RE, *et al*. Identification of multiple proliferative growth factors in breast cyst fluid. *Am J Surg* 1993; **166**: 237–43.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site.