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Collagen degradation products measured in serum can separate ovarian and breast cancer patients from healthy controls: A preliminary study

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Abstract.
BACKGROUND: During cancer the otherwise tightly controlled homeostasis of the extracellular matrix (ECM) is disturbed. The protein composition changes, the ECM stiffens and increased levels of proteases are secreted. The combination of these processes result in release of specific protein fragments (e.g. collagens) to the circulation, which when measured may reflect disease pathogenesis.
OBJECTIVE: To investigate if biomarkers of protease-degraded collagen could differentiate ovarian and breast cancer patients from healthy controls when measured in serum.
METHODS: The levels of markers reflecting MMP-degradation of type I (C1M), type III (C3M) and type IV (C4M, C4M12) collagen were assessed in serum from ovarian cancer patients ($n = 10$), breast cancer patients ($n = 14$) and healthy controls ($n = 49$) using validated ELISAs. The markers were compared using one way ANOVA and AUC was calculated.
RESULTS: All markers were significantly elevated in serum from ovarian cancer patients ($p < 0.0001$) and breast cancer patients ($p < 0.04$–0.0001) compared to healthy controls. Furthermore, diagnostically the markers were able to differentiate ovarian (AUROC 90%–93%) and breast cancer patients (AUROC 76%–93%) from healthy controls, with C1M being the strongest differentiator of disease vs. controls.
CONCLUSION: Four serum biomarkers reflecting altered MMP-mediated collagen turnover were able to differentiate ovarian and breast cancer patients from healthy controls.

Keywords: Cancer, extracellular matrix, collagen, biomarkers, breast cancer, ovarian cancer, matrix metalloproteinase, degradation, remodeling, tumor microenvironment

1. Background

Women's health is significantly influenced by the occurrence of cancers, such as breast and ovarian cancers. Breast cancer is the leading cause of cancer death among women in the US and EU [1,2], and ovarian cancer, although less frequent, is often discovered in the late clinical stages where the cancer has already spread and no curative interventions are possible [3]. Taken together, the medical needs for breast and ovarian cancer are early diagnosis, prognosis and prediction of a therapeutic response. One of the best ways to achieve this is to use serum biomarkers [4].

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Table 1
Patient characteristics

Group	No. of patients	Stage				Gender, % females	Age, years
		I	II	III	IV		Mean ± SD (range)
Ovarian cancer	10	1	2	7	–	100%	54 ± 11 (37–74)
Breast cancer	14	–	12	2	–	100%	57 ± 10 (41–79)
Healthy controls	49					100%	63 ± 14 (31–84)

Extracellular matrix (ECM) turnover could be targets for novel and improved serum biomarkers. Normal tissue homeostasis of the ECM is tightly controlled; here, old and damaged proteins of the ECM are degraded and replaced with new proteins. During cancer however this process is disturbed [5,6]; the protein composition changes, the ECM stiffens and increased levels of proteases are secreted. This leads to shift in the homeostasis of the ECM of which especially the interstitial fibrillar type I and III collagen, as well as the basement membrane protein type IV collagen play important roles [7,8].

As a consequence of the altered matrix remodeling an increased production of turnover products, i.e. protein fragments, are released into the circulation [5]. These protein fragments can, as they carry specific pathology dependent neo-epitopes, provide a unique tissue fingerprint of the combination of the involved proteases and the composition of the collagen. The aim of this study was to investigate whether protein fingerprints of specific MMP-generated collagen fragments may differentiate breast and ovarian cancer patients from healthy controls when measured in serum.

2. Methods

2.1. Patient serum samples

Serum was collected, after informed consent and approval by appropriate Institutional Review Board, from 14 patients with breast cancer and 10 patients with ovarian cancer prior to resection, and 49 controls with no symptomatic or chronic disease (Table 1). Patient samples were obtained from the commercial vendor Asterand (Detroit, MI) and the healthy controls were a pool of samples from Asterand and two other study populations (BA0058-05-003) and [9]. All patients signed an informed consent and the study confirms with The Code of Ethics of the World Medical Association (Declaration of Helsinki). According to Danish law, it is not required to get ethical approval when measuring biochemical markers in previously collected samples; hence, there was no additional ethical approval for this particular study. Samples were collected, processed and stored in a similar fashion until analyzed, and all analyses were performed blindly.

2.2. ELISA measurements and procedure

Using well-characterized and validated competitive ELISAs, levels of MMP 2, 9 and 13-degraded collagen type I (C1M) [10], MMP-9 degraded collagen type III (C3M) [11], and MMP-9 and 12 degraded collagen type IV (C4M, C4M12) [12,13] were assessed in the serum samples.

In brief, a 96-well pre-coated streptavidin plate was coated with biotinylated peptide dissolved in optimized assay buffer. The plate was incubated for 30 minutes at 20°C and subsequently washed five times in washing buffer (20 mM Tris, 50 mM NaCl, pH 7.2). 20 μl of selection peptide or sample was added to the appropriate wells, followed by 100 μl of a HRP-conjugated target-specific monoclonal antibody. The plate was incubated for 2 hours at 20°C or over night at 4°C and washed five times with washing buffer. Finally 100 μl of TMB (Kem-En-Tec) were added and the plates were incubated for 15 minutes at 20°C in darkness. All of the incubation steps were performed while shaking at 300 rpm. The TMB reaction was stopped by adding 100 μl of stopping solution (1% HCL) and measured at 450 nm with 650 nm as reference. A calibration curve was plotted using a 4-parametric mathematical fit model.

2.3. Statistical analysis

The levels of the individual biomarkers in serum from the controls and patients were Log10 transformed and compared using one way analysis of variance (ANOVA). The p-values were adjusted to account for multiple comparisons using Dunnetts's method. To evaluate the markers ability to separate ovarian and breast cancer patients from healthy controls receiver operating characteristics (ROC) was created and area under the ROC curved (AUC) was calculated for each biomarker after adjusting for age. The age adjustment

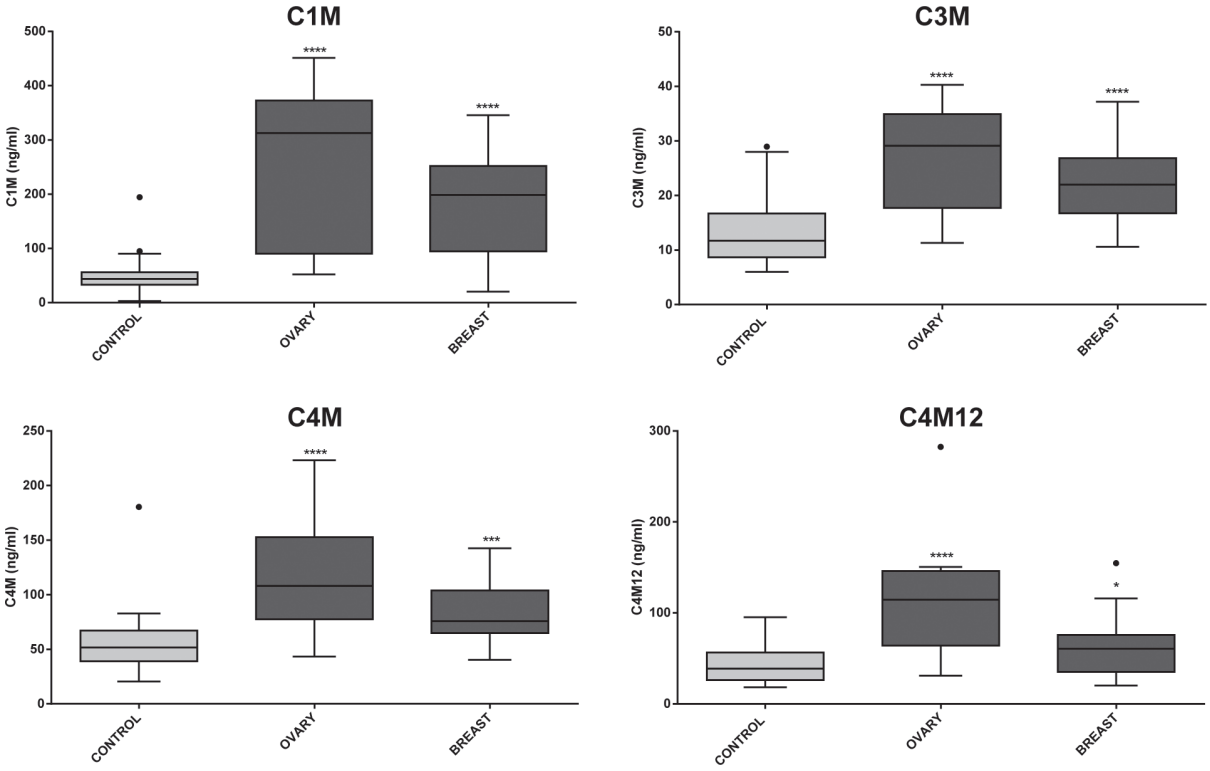


Fig. 1. Serum levels (ng/ml) of C1M, C3M, C4M, C4M12 in patients with ovarian cancer ($n = 10$), breast cancer ($n = 14$) and healthy controls ($n = 49$). The boxes represent the 25th, 50th and 75th percentiles. The whiskers represent the lowest and highest value, except outliers (•), which are higher than 1.5 times the 75th percentile or lower than 1.5 times the 25th percentile. Groups were compared using an ANOVA test on Log10 transformed data. The p-values are adjusted to account for multiple comparisons. Asterisks indicate the following: $^{*}p < 0.05$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

was performed using a multivariable logistic regression model.

The statistical analyses were performed using MedCalc Statistical Software v.12 (MedCalc Software, Ostend, Belgium) and GraphPad Prism v.6 (GraphPad Software, La Jolla, USA). The results were considered statistically significant when the p-value was < 0.05.

3. Results

3.1. MMP-mediated degradation of collagen

Levels of MMP-generated fragments of collagen type I, III and IV were significantly elevated in serum from ovarian and breast cancer patients as compared to controls (Fig. 1). In detail, the level of MMP-degraded collagen type I (C1M) was 6-fold higher in ovarian patients as compared to controls and 4-fold higher in breast cancer patients. The levels of MMP-degraded collagen type III (C3M), and MMP-degraded collagen type IV (C4M and C4M12) were 2-fold higher in ovarian cancer patients as compared to controls and 1.5 fold higher in breast cancer patients. For all markers there were no significant differences between the different tumor stages (Fig. 2). Together, these findings indicate that altered collagen-remodeling is ongoing in both ovarian and breast cancer.

3.2. Diagnostic power of biomarkers to discriminate between healthy controls and patients with breast and ovarian cancer

The area under the ROC curve (AUC) was calculated as a measure of the diagnostic power of the biomarkers when adjusted for age (Fig. 3). The diagnostic power of C1M, C3M, C4M and C4M12 was significant with an AUC $\geqslant$ 90% and $\geqslant$ 76% for ovarian and breast cancer, respectively indicating that the biomarkers are able to differentiate diseased from healthy. C1M proved to be the strongest differentiator of cancer vs. controls with AUC of 93% for both ovarian and breast cancer.

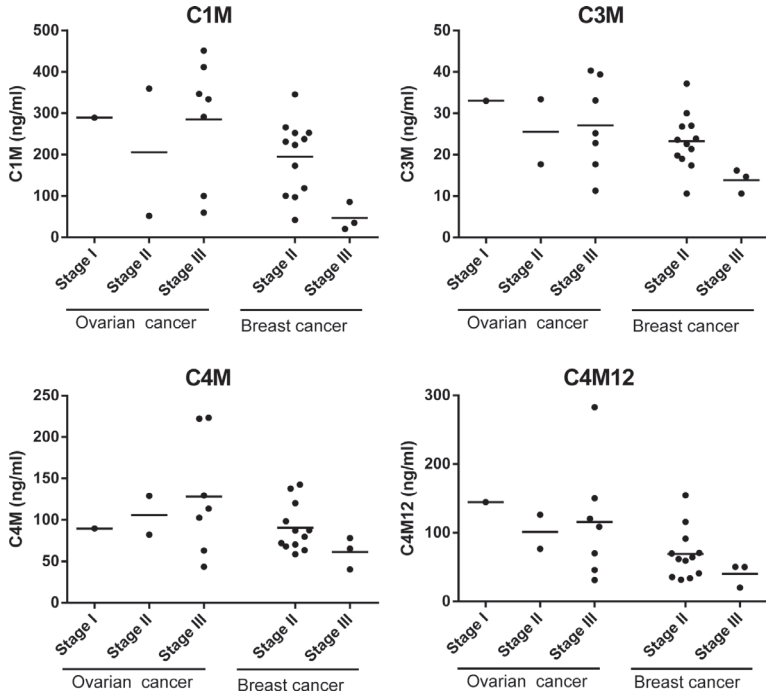
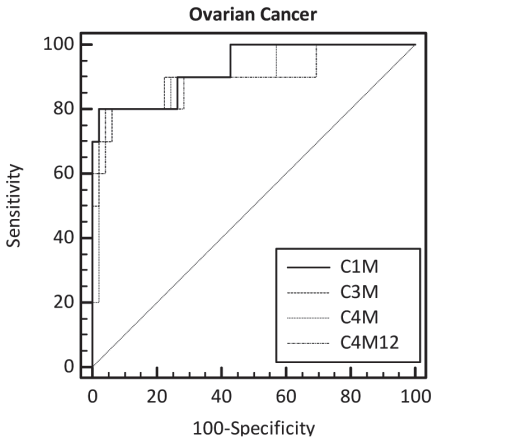
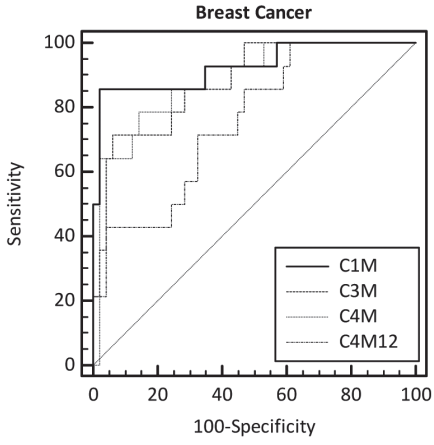


Fig. 2. Serum levels (ng/ml) of C1M, C3M, C4M, C4M12 divided into stage of disease for both ovarian and breast cancer. Mean is illustrated by horizontal line.



	AUC %	CI	Sens	Spec	P-value
C1M	93	83 to 98	80	98	<0.0001
C3M	92	83 to 98	80	94	<0.0001
C4M	91	80 to 97	80	96	<0.0001
C4M12	90	79 to 96	80	88	<0.0001



	AUC %	CI	Sens	Spec	p-value
C1M	93	83 to 98	86	98	<0.0001
C3M	88	78 to 95	71	94	<0.0001
C4M	88	78 to 95	79	86	<0.0001
C4M12	76	63 to 86	100	30	0.0003

Fig. 3. Age adjusted receiver operating characteristic (ROC) of C1M, C3M, C4M and C4M12 for the diagnosis of ovarian and breast cancer.

4. Discussion

The aim of this study was to investigate whether C1M, C3M, C4M and C4M12 could differentiate breast and ovarian cancer patients from healthy controls when measured in serum. Interestingly, all markers were significantly elevated in serum from both breast and ovarian cancer patients compared to the healthy controls. These findings are in accordance with previous reports which found elevated levels of type

IV collagen in serum from breast and ovarian cancer patients [14,15] and elevated levels of collagen type I and III in the tissue of breast and ovarian cancer patients [7,16]. Furthermore, the desmoplatic reaction in cancer share many similarities with general fibrosis where the fragments of collagen type I [10], type III [11], and type IV [13] also have been found elevated in serum and linked to ECM remodeling.

Developing neo-epitope based biomarkers that mirror the ECM remodeling that occur in cancer could be a way to meet the unmet need for novel non-invasive biomarkers. Neo-epitopes based biomarkers have the ability to be specific to different cancer subtypes, as the composition of ECM and MMPs changes not only between tissues but also between cancer subtypes [17–19]. Furthermore, as stromal changes are a well recognized component of pre-invasive lesions, the markers have the potential to be used as an early marker of cancer. In this study, increased levels of the collagen degradation markers were elevated in all stages of ovarian and breast cancer , emphasizing their potential to be applied in the early onset of cancer.

The biomarkers provided excellent diagnostic information (Fig. 2). However, to fully analyze the diagnostic applicability of the collagen degradation markers used here, other types of cancer and diseases should be included in the study. Other limitations include a small sample size and the cross-sectional nature of the study. A larger longitudinal study is therefore needed to understand the full potential of these biomarkers. Furthermore, from this study we cannot conclude if the biomarkers are secreted from the tumor tissue or is part of a systemic effect.

In conclusion, we found that four serum biomarkers measuring altered MMP-mediated collagen turnover were able to differentiate ovarian and breast cancer patients from healthy controls in this small cancer cohort study. As these markers reflect altered disease pathogenesis they may increase the understanding of disease mode of action and, if validated in larger clinical studies, provide an improved and additional clinical tool for stratifying and monitoring patients according to subtype/ disease severity.

Acknowledgement

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Conflicts of interest

Willumsen N., Bager C.L., Leeming D.J., Karsdal M.A., and Bay-Jensen A.C. are employed at Nordic Bioscience A/S which is involved in the discovery and development of biochemical markers. Karsdal M.A. owns stocks at Nordic Bioscience. Smith, V. and Dornan, D. are employed at Gilead Sciences Inc. involved in drug development.

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