Exploratory analysis of CA125-MGL and –STn glycoforms in the differential diagnostics of pelvic masses

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Running title: CA125 glycoforms in EOC diagnostics
Keywords: CA125, epithelial ovarian cancer, differential diagnostics, glycosylation, pelvic mass
Abbreviations: CA125=Cancer antigen 125, IA=immunoassay, MGL= macrophage galactose-type lectin, STn= Sialyl-Thomsen-nouveau, EOC=epithelial ovarian cancer

Impact statement

Measurement of glycovariant forms of CA125 assists in a more accurate diagnosis and therefore helps to avoid unnecessary surgery of patients with elevated conventional CA125 due to benign conditions. The glycovariant assays were particularly useful in the differential diagnostics of pelvic masses with marginally elevated conventional CA125.

Aberrant glycosylation is known to be associated with malignant transformation in essentially all types of human cancers. In this study, the detection of two cancer associated CA125 glycovariants is based on a lectin and a glycan specific antibody coated on nanoparticles, providing high analytical sensitivity.

This is the first demonstration of nanoparticle assisted glycovariant measurement on a large clinical cohort with malignant and benign ovarian tumors.

Abstract

1. Background

The cancer antigen 125 (CA125) immunoassay (IA) does not distinguish epithelial ovarian cancer (EOC) from benign disease with the sensitivity needed in clinical practice. In recent studies, glycoforms of CA125 have shown potential as biomarkers in EOC. Here, we assessed the diagnostic abilities of two recently developed CA125 glycoform assays for patients with a pelvic mass. Detailed analysis was further conducted for postmenopausal patients with marginally elevated conventionally measured CA125 levels, as this subgroup presents a diagnostic challenge in the clinical setting.

2. Methods

Our study population contained 549 patients diagnosed with EOC, benign ovarian tumors and endometriosis. Of these, 288 patients were postmenopausal and 98 of them presented with marginally elevated serum levels of conventionally measured CA125 at diagnosis. Preoperative serum levels of conventionally measured CA125 and its glycoforms (CA125-MGL and CA125-STn) were determined.

3. Results

The CA125-STn assay identified EOC significantly better than the conventional CA125-IA in postmenopausal patients (85% vs 74% sensitivity at a fixed specificity of 90%, $p = 0.0009$). Further, both glycoform assays had superior AUCs compared to the conventional CA125-IA in postmenopausal patients with marginally elevated CA125. Importantly, the glycoform assays reduced the false positive rate of the conventional CA125-IA.

4. Conclusions

The results indicate that the CA125 glycoform assays markedly improve the performance of the conventional CA125-IA in the differential diagnosis of pelvic masses. This result is especially valuable when CA125 is marginally elevated.

Background

Ovarian cancer is the leading cause of death among patients with gynecological malignancies in developed countries (1). Although the relative 5-year survival rate has slightly improved during the last decades, it still falters at 47% (2). Patients often do not experience symptoms until the disease reaches an advanced stage (3), and delayed diagnosis is a major factor in the poor survival of patients. Current biomarkers applied in clinical practice detect advanced disease with adequate sensitivity and specificity; however, detection of early disease remains a highly challenging task (4–6).

Cancer antigen 125 (CA125) is a well-validated biomarker in the diagnostics of ovarian cancer. Yet, CA125 has several critical limitations: it detects only approximately 50% of stage I ovarian cancers (5) and it is also elevated in a variety of benign conditions and non-ovarian malignancies (6). Regardless of its limitations, CA125 is considered the single best biomarker in the diagnostics of epithelial ovarian cancer (EOC) (7). The circulating CA125 antigen defined by immunoassays is derived from the mucin-like type I membrane glycoprotein called MUC16 (8,9). It has one intracellular domain and two extracellular domains: the intracellular carboxy terminus, the tandem repeat domain and the amino terminal domain (10). Of particular interest for this study is the extracellular amino terminal domain, which consists of densely linked O- and N-glycosylation sites (10). Glycosylation is a process heavily influenced by the oncogenic transformation of the cell (9). Previous studies have indicated that the N-glycosylation of CA125 is altered in ovarian cancer (11,12). Therefore, the assessment of cancer specific glycoforms may offer opportunities for improving the performance of CA125 as a tailored biomarker.

Methods previously utilized in glycoprofiling involve hydrophilic interaction chromatography (HILIC), high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and mass spectrometry (MS) (13). Other potentially advantageous approaches include the use of monoclonal antibodies and lectins (13). Lectins are proteins that have the capability to recognize specific carbohydrate moieties of glycoconjugates (14) which make them attractive candidates for the development of more precise measurement methods of biomarkers. In recent studies, the sensitivity and specificity of conventionally measured cancer biomarkers have been significantly improved with lectin-based glycoprofiling in liver (15,16), breast (17,18) and prostate cancer (19). We recently reported that nanoparticle aided CA125 glycoprofiling is a promising method for improving the accuracy of the conventional CA125-IA in EOC diagnosis (20,21). Recombinant human macrophage galactose-type lectin (MGL) and Sialyl-Thomsen-nouveau (STn) antibody showed great promise as

candidates for the detection of cancer associated CA125 glycoforms. Similar, microarray based results have been reported also by others on STn- and ST-glycoforms of CA125 (22).

In the current study, we examined for the first time the potential of two novel MGL- and STn-based assays on two preoperative clinical cohorts to distinguish EOC from benign pelvic masses. We evaluated the glycoform assays separately, combined and with the conventional CA125-IA.

Material and Methods

1. Participants

Participants were prospectively recruited at the Department of Obstetrics and Gynecology at the Turku University Hospital in Finland from 2009 to 2017 and at the Oslo University Hospital in Norway from 2012 to 2015. Patients with abnormal pelvic processes and/or elevated serum CA125 were included. Further, only patients with histologically confirmed disease were included in the study. The study population consisted of 617 patients, of which 264 patients were diagnosed with EOC and 317 patients with benign pelvic diseases (Table 1). In addition, healthy controls (n = 36) admitted to the hospital for tubal sterilization were included in the current study. Transvaginal ultrasound and/or a CT-scan were carried out for patients with elevated serum CA125 levels to estimate the risk of EOC. Tissue samples were obtained during diagnostic surgery and the histopathological diagnosis and disease stage were affirmed by a pathologist specialized in gynecological pathology. For patients diagnosed with EOC the disease stage was determined in accordance with the guideline of The International Federation of Gynecologists and Obstetricians (FIGO 2014). This study has been approved by the Ethics Committee in the hospital district of Southwest Finland and the Regional Ethics Committee in South East Norway.

2. The conventional CA125 immunoassay

A blood sample was drawn at the time of diagnosis: preoperatively and prior to treatments. The sample analyses were performed in a similar manner in both cohorts. The serum samples were collected using vacuum tubes with gel and clot activator. Samples were incubated for 30-60 minutes at room temperature and centrifuged for 15 minutes. The samples were stored in -70 - -80 °C. The serum CA125 values (U/ml) for the Turku EOC cohort and benign controls were determined using a clinically well-established ECLIA method on the Cobas e 601 instrument or a Modular E170 automatic analyzer (Roche Diagnostic GmbH, Mannheim, Germany). The Oslo EOC cohort and endometriosis controls were measured manually using the CA125 EIA kit (Fujirebio Diagnostics Inc., Malvern, PA, USA) in accordance with the guidelines of the

manufacturer. The two assays have been shown to correlate well (r=0.98, (23)) and have the same cut-off of 35 U/ml.

3. CA125 glycoform measurement with CA125-MGL and CA125-STn immunoassays

In-house time resolved fluorometry (TRF) immunoassays for CA125-MGL and CA125-STn have been described before (20,21). Briefly, biotinylated capture Ov185 monoclonal antibody or Ov185 F(ab')2 (50 ng/30 µl /well) were immobilized to streptavidin-coated low-fluorescence microtiter wells (Kaivogen Oy, Turku, Finland) in the assay buffer for 60 min at room temperature (RT) without shaking. After washing twice, 25 µl of standard (OVCAR-3 cell line purified CA125) or diluted serum sample was added in triplicates and incubated for 60 min at RT with shaking. Samples were diluted 1:5 and 1:10 in buffer solution for CA125-MGL and CA125-STn, respectively. After washing twice for CA125-MGL and four times for CA125-STn, the captured CA125 antigen was incubated with the Eu+3-chelate-doped Fluoro-MaxTM polystyrene nanoparticles (NPs) (Seradyn Inc., Indianapolis, IN) conjugated with human lectin-MGL (1×10^7 /25 µl /well) and STn-mAb STn-NPs-conjugates (5×10^6/25 µl /well) for 90 minutes at RT with shaking. After incubation, the wells were washed six times with wash buffer. The time-resolved fluorescence for Eu+3 was then measured from dry wells using VictorTM 1420 Multilabel counter.

The sample analyses were performed by a blinded investigator; that is, the diagnosis of the patient was not known to the sample analyzer.

4. Statistical analyses

Initially, a sample size calculation was conducted to ensure sufficient statistical power. With our sample size, a sufficient power of 80% was reached (24). The sample size calculation was performed for both CA125-MGL and CA125-STn using the G*Power software (25). In order to adjust for the potential effects of age in the patient groups, linear regression was utilized to correct for age related trends in total CA125 and the two glycovariants (see Supplemental data for further details). For biomarker values, normality was evaluated applying the Shapiro-Wilk test and it was also assessed visually. The average biomarker values and standard deviations (SD) for conventionally measured CA125, CA125-MGL and CA125-STn were calculated in different diagnostic groups, separately in both the Norwegian and the Finnish cohorts. To correct for the non-Gaussian skewness of the data, a logarithmic transformation was performed. To explore the differences between diagnostic groups, confidence intervals were visually compared and the p-

value was calculated utilizing the one-way ANOVA test. Antecedently, Levene's test was applied to assess the equality of variances. Multiple comparisons were performed with the Tukey's honest significant difference (HSD) test or the Games-Howell test, according to the homogeneity of variances. Cut off values (2.0 U/ml for CA125-MGL and 10.0 U/ml for CA125-STn) identified in earlier studies were used to determine the false positive rate of the glycoform assays using a separate cohort (20,21). The established cut off value of 35 U/ml was used for the conventional CA125-IA. Receiver operating characteristic (ROC) curves were set to evaluate the diagnostic abilities of these biomarkers and the areas under curves (AUCs) were used to benchmark performance with p-values computed using the DeLong method. Biomarker competence was further evaluated by calculating the sensitivity for CA125, CA125-MGL and CA125-STn at a fixed specificity of 90%. Sensitivities were compared with the Chi-squared test. $P < 0.05$ was considered significant in all analyses. Statistical analyses were carried out in R (Version 3.3.3.) (26) and IBM SPSS software (IBM Corp. Released 2016. IBM SPSS Statistics for Macintosh, Version 24.0. Armonk, NY: IBM Corp.).

Results

1. CA125-MGL and –STn are potential novel biomarkers in the differential diagnostics of pelvic masses

We first evaluated the concentrations of CA125-MGL, CA125-STn and the conventional CA125-immunoassay (IA) separately in the Norwegian and Finnish cohorts (Supplemental data: Table 1 and 2). As the average serum biomarker levels were similar in the two cohorts, they were combined to increase the power of the study (Table 1). In this cohort, all three markers detected high-grade and low-grade serous, endometrioid and clear cell ovarian carcinomas from the benign conditions. For the patients with mucinous carcinoma, none of the assays showed significant elevation, although over 3-fold higher mean concentrations were observed for total CA125 and CA125-MGL.

We evaluated the performance of the glycovariant assays in the differential diagnostics of pelvic masses by calculating the number of patients with a benign disease and false positive biomarker values. With the CA125-MGL and CA125-STn assays, biomarker levels exceeding the cut off value in benign controls were observed in only 27.8% and 17.0% of patients (Table 2). This illustrates the improved specificity of the glycovariant assays, as 36.6% of the benign controls exceeded the cut off value of the conventional CA125-IA (Table 2). Even though the serum concentration of CA125-MGL was low in benign diseases (overall mean 3.9 U/ml, Table 1), we

detected a statistically significant elevation of the mean CA125-MGL in the sera of patients with benign ovarian neoplasms ($p = 0.011$) and endometriosis ($p < 0.001$) when compared with healthy controls (Table 1). Similarly, the mean CA125-STn level was elevated in women with endometriosis ($p = 0.017$). However, the clinical significance of the small but statistically significant elevation of the glycovariant markers in different benign conditions remains to be addressed in future studies. There were no differences between the serum CA125-STn levels in healthy controls and patients with benign neoplasms ($p = 0.14$) or non-neoplastic diseases ($p = 0.58$). Overall, the increase in serum CA125 glycoforms in benign diseases was modest while the conventionally measured CA125 was notably elevated in all of the benign subgroups compared to healthy controls: neoplasms ($p < 0.001$), non-neoplastic diseases ($p = 0.047$) and endometriosis ($p < 0.001$).

2. The glycoform assays improve the detection of EOC in postmenopausal patients with marginally elevated CA125 levels

The receiver operating curves (ROC) were computed for the whole cohort, the postmenopausal subgroup and within this subgroup for samples with marginally elevated conventional CA125 values, which is the most difficult group for differential diagnosis (Figure 1). ROC curves were also computed for the premenopausal subgroup; however, the subgroup was excluded from further analyses as the number of patients with EOC was low resulting in a lack of statistical power. For the whole cohort, the combination of CA125-MGL and –STn performed with highest sensitivity at fixed 90% specificity (61.6%, $p=0.014$ vs. 54.3% for the CA125-IA), although there was no statistically significant improvement in the CA125-MGL+STn AUC compared to that of the CA125-IA ($p = 0.107$) (Figure 1).

We focused on the postmenopausal subgroup as the majority of patients diagnosed with EOC are included in this category. The combined glycoform assays exceeded the diagnostic ability of the conventional CA125-IA in this subgroup ($p = 0.021$, AUC comparison, Figure 1). The CA125-MGL assay alone did not notably improve the sensitivity of the conventional assay, while the sensitivity of the CA125-STn assay was superior in comparison to the conventional assay. Subgroup specific focus was emphasized in the analysis of postmenopausal patients with marginally elevated conventional CA125 values to examine the diagnostic potential of the glycoform assays. As expected, the performance of the conventional assay was suboptimal in this subgroup (AUC 0.734; 25.4% sensitivity at 90% specificity, Figure 1). Both of the glycoform

assays had a significantly better AUC compared to the conventional assay (p = 0.042 and p = 0.0023 for CA125-MGL and –STn, respectively). However, combining the glycoform assays did not bring considerable improvement to the diagnostic abilities of the CA125-STn assay in these patients.

Discussion

In this study, the main novel finding was the improved differential diagnostics of pelvic masses by use of detection of CA125 glycoforms. The glycoform assays reduced the number of false positive test results of the conventional CA125-IA. Further, the glycoforms performed especially well in the postmenopausal subgroup of patients with marginally elevated levels of conventionally measured CA125. This is the first study in which the potential of CA125 glycoforms has been examined in an extensive cohort.

The improved distinction of EOC from benign diseases is of critical importance. In our cohort, the majority of EOCs were diagnosed at an advanced stage and consequently the conventional CA125-IA performed adequately in this population. Instead, when we focused on the patients with marginally elevated CA125, the advantage of glycoform assays became more apparent. The low specificity of the conventional CA125-IA might lead to additional testing and emotional anguish in patients (27,28). It is of note that the further diagnostic tests typically involve invasive procedures, e.g. a diagnostic laparoscopy, which is suboptimal use of resources and expose the patients to unnecessary surgical procedures. Furthermore, about 20% of advanced stage disease, in addition to 50% of early stage disease, does not show elevated CA125 (5). Thus, there is an urgent clinical need for more precise biomarkers in the differential diagnostics of pelvic masses.

Currently, pelvic masses are evaluated preoperatively with multimarker panels such as the Risk of Ovarian Malignancy Algorithm (ROMA), Risk of Malignancy Index (RMI) and the Copenhagen Index (CPH-I) (29,30). These panels build upon serum levels of CA125, HE4, menopausal status (ROMA, RMI), age (CPH-I) and ultrasound parameters (RMI). The diagnostic abilities of these algorithms have been reported to be comparable, with a sensitivity of 67% – 94% and a specificity of 75% – 92% (4,29–31). Based on the presented study, our CA125 glycoform assays may bring additive value to current diagnostic metrics. The robust and inexpensive assays can be easily utilized in the clinical laboratories with standard equipment. The ideal combination of CA125 glycoforms and other serum biomarkers in EOC differential diagnostics remains an interesting research question and warrants further studies.

Several factors can influence the modification of glycosylation in oncogenic cells. These include epigenetic, genetic, metabolic, inflammatory and environmental mechanisms (32). In ovarian cancer, the MUC16 glycoprotein interacts with galactose-binding lectins that are overexpressed and secreted into the tumor matrix. These galactose-binding lectins might conduct tumor progression by facilitating cell adhesion and by promoting the binding of tumor cells to laminin and fibronectin (33). Thus, the aberrant glycoforms detected from the sera of EOC patients might be a part of a larger 'metastatic code' of the disease (9). In addition of being promising biomarkers in the diagnostics of malignancy, the measurement of aberrant glycoforms could also be a feasible approach to select patients who would most likely benefit from glycan-based therapies in the future (9,34).

The strength of our study is the large cohort of preoperatively evaluated and histologically confirmed cases with a pelvic mass. Unfortunately, there are also some limitations including the age-bias of the cohort used, which we now addressed by correcting for potential age-related trends the data using linear regression. It is of importance to study the CA125 glycovariants in a large age-matched cohort and to tailor such panels to incorporate further patient characteristics. The low portion of EOC patients with marginally elevated CA125 can be seen as a limitation of the study. In addition, the diagnostic abilities of the biomarkers are difficult to evaluate in rare EOC histologies such as clear cell and mucinous cancers as the statistical power is low. Fully exploring each glycovariants reactivity profile separately or in comparison to conventional CA125 calls for additional studies. None of the assays succeeded in clear discrimination of mucinous ovarian cancer from benign disease. A promising method to improve the detection of mucinous carcinomas would be to combine the glycoform assays with serum REG4 measurement, which has shown potential as a histotype-specific biomarker of mucinous ovarian carcinoma (35). Importantly, the glycoform assays performed well in patients with high grade serous ovarian cancer, which is the most important histological type of EOC.

In conclusion, the results suggest that the glycoform assays improve the performance of the conventional CA125-IA particularly in patients with marginally elevated serum CA125 levels. The glycoform assays discriminated EOC from benign diseases more efficiently than the conventional CA125-IA, and the two glycoform assays represents a promising tool in the differential diagnostics of pelvic masses. For these tasks, we suggest using the two novel glycoform assays to explore their exact complementarity with different and more extensive patient cohorts. Further studies are required on the prognostic and predictive value of the glycoform assays

Acknowledgments

The authors wish to thank Lassi Huhtala (University of Turku), Michael Gabriel (University of Turku) and Rolf A. Klaasen (Oslo University Hospital) for technical assistance.

References:

1. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D BF. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin [Internet]. 2018;68:7–30. Available from: http://doi.wiley.com/10.3322/caac.21442
3. Rossing MA, Wicklund KG, Cushing-Haugen KL, Weiss NS. Predictive value of symptoms for early detection of ovarian cancer. J Natl Cancer Inst. 2010;102:222–9.
4. Van Gorp T, Cadron I, Despierre E, Daemen A, Leunen K, Amant F, et al. HE4 and CA125 as a diagnostic test in ovarian cancer: Prospective validation of the Risk of Ovarian Malignancy Algorithm. Br J Cancer [Internet]. Nature Publishing Group; 2011;104:863–70. Available from: http://dx.doi.org/10.1038/sj.bjc.6606092
5. Zurawski VR, Knapp RC, Einhorn N, Kenemans P, Mortel R, Ohmi K, et al. an Initial Analysis of Preoperative Serum Ca-125 Levels in Patients With Early Stage Ovarian-Carcinoma. Gynecol Oncol. 1988;30:7–14.
6. Canney PA, Moore M, Wilkinson PM et al. Ovarian cancer antigen CA125: a prospective clinical assessment of its role as a tumour marker. Br J Cancer. 1984;50:765–9.
7. Terry KL, Schock H, Fortner RT, Hüsing A, Fichorova RN, Yamamoto HS, et al. A prospective evaluation of early detection biomarkers for ovarian cancer in the European EPIC cohort. Clin Cancer Res. 2016;22:4664–75.
8. Sean R. Stowell, Tongzhong Ju and RDC. Protein Glycosylation in Cancer. Annu Rev Pathol Mech Dis. 2015;10:473–510.
9. Fuster MM, Esko JD. The sweet and sour of cancer: Glycans as novel therapeutic targets. Nat Rev Cancer. 2005;5:526–42.
10. McLemore MR, Aouizerat B. Introducing the MUC16 gene: implications for prevention and early detection in epithelial ovarian cancer. Biol Res Nurs. 2005;6:262–7.
11. Saldova R, Struwe WB, Wynne K, Elia G, Duffy MJ, Rudd PM. Exploring the glycosylation of serum CA125. Int J Mol Sci. 2013;14:15636–54.
12. Biskup K, Braicu EI, Sehouli J, Fotopoulou C, Tauber R, Berger M, et al. Serum glycome profiling: A biomarker for diagnosis of ovarian cancer. J Proteome Res. 2013;12:4056–63.
13. Kirwan A, Utratna M, O'Dwyer ME, Joshi L, Kilcoyne M. Glycosylation-Based Serum Biomarkers for Cancer Diagnostics and Prognostics. Biomed Res Int. Hindawi Publishing Corporation; 2015;2015.
14. Hashim OH, Jayapalan JJ, Lee C-S. Lectins: an effective tool for screening of potential cancer biomarkers. PeerJ [Internet]. 2017;5:e3784. Available from: https://peerj.com/articles/3784
15. Korekane H, Hasegawa T, Matsumoto A, Kinoshita N, Miyoshi E, Taniguchi N. Development of an antibody-lectin enzyme immunoassay for fucosylated α-fetoprotein. Biochim Biophys Acta - Gen Subj [Internet]. Elsevier B.V.; 2012;1820:1405–11. Available from: http://dx.doi.org/10.1016/j.bbagen.2011.12.015
16. Matsuura S, Kawai T, Ilirai H. A collaborative study for the evaluation of lectin-reactive alpha-fetoproteins in early detection of hepatocellular carcinoma. Cancer res. 1993;5419–24.

17. Wi GR, Moon BI, Kim HJ, Lim W, Lee A, Lee JW, et al. A lectin-based approach to detecting carcinogenesis in breast tissue. Oncol Lett. 2016;11:3889–95.
18. Ideo H, Hinoda Y, Sakai K, Hoshi I, Yamamoto S, Oka M, et al. Expression of mucin 1 possessing a 3`-sulfated core1 in recurrent and metastatic breast cancer. Int J Cancer. 2015;137:1652–60.
19. Belicky S, Černocká H, Bertok T, Holazova A, Réblová K, Paleček E, et al. Label-free chronopotentiometric glycoprofiling of prostate specific antigen using sialic acid recognizing lectins. Bioelectrochemistry. 2017;117:89–94.
20. Gidwani K, Huhtinen K, Kekki H, Vliet S Van, Hynninen J. A Nanoparticle-Lectin Immunoassay Improves Discrimination of Serum CA125 from Malignant and Benign Sources. Clin Chem. 2016;1400.
21. Gidwani K et al. Europium Nanoparticle-Based STn monoclonal antibody recognizes Epithelial Ovarian Cancer-associated CA125 and Improves discrimination of serum CA125 from malignant and benign sources. Submitted. 2018;
22. Chen K, Gentry-Maharaj A, Burnell M, Steentoft C, Marcos-Silva L, Mandel U, et al. Microarray glycoprofiling of CA125 improves differential diagnosis of ovarian cancer. J Proteome Res. 2013;12:1408–18.
23. Eleftherios P. Diamandis, Herbert A. Fritsche, Hans Lilja, Daniel W. Chan MKS. Tumor markers. Physiology, pathobiology, technology, and clinical applications. 1st ed. 2003.
24. Barrett J, Jenkins V, Farewell V, Menon U, Jacobs I, Kilkerr J, et al. Psychological morbidity associated with ovarian cancer screening: Results from more than 23 000 women in the randomised trial of ovarian cancer screening (UKCTOCS). BJOG An Int J Obstet Gynaecol. 2014;121:1071–9.
25. Buys SS. Effect of Screening on Ovarian Cancer Mortality. Jama [Internet]. 2011;305:2295. Available from: http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.2011.766
26. Moore R, -Raughley MJ, Brown A, Robison K, Miller C, Allard J. Comparison of a Novel Multiple Marker Assay Versus the Risk of Malignancy Index for the Prediction of Epithelial Ovarian Cancer in Patients with a Pelvic Mass. Am J Obs Gynecol. 2013;203:1–14.
27. Minar L, Felsinger M, Cermakova Z, Zlamal F, Bienertova-Vasku J. Comparison of the Copenhagen Index versus ROMA for the preoperative assessment of women with ovarian tumors. Int J Gynecol Obstet. 2018;140:241–6.
28. Anton C, Carvalho F, Oliveira E, Maciel G, Baracat E, Carvalho J. A comparison of CA125, HE4, risk ovarian malignancy algorithm (ROMA), and risk malignancy index (RMI) for the classification of ovarian masses. Clinics [Internet]. 2012;67:437–41. Available from: http://clinics.org.br/article.php?id=751
29. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. Nat Rev Cancer [Internet]. Nature Research; 2015 [cited 2016 Nov 22];15:540–55. Available from: http://www.nature.com/doifinder/10.1038/nrc3982
30. Liu F, Rabinovich GA. Galectins as modulators of tumour progression. Nat Rev Cancer. 2005;5.
31. Hudak JE, Bertozzi CR. Glycotherapy: New Advances Inspire a Reemergence of Glycans in Medicine. Chem Biol. 2015;21:16–37.
32. Lehtinen L, Vesterkvist P, Roering P, Korpela T, Hattara L, Kaipio K, et al. REG4 is highly expressed in mucinous ovarian cancer: A potential novel serum biomarker. PLoS One. 2016;11.

Table 1. Patient characteristics. Comparisons of means of benign diseases to healthy controls (Ref. 1) and EOC to benign diseases (Ref. 2).

Histology	N	Age, y mean	CA125 (U/ml) mean ± SD	p - value	CA125-MGL (U/ml) mean ± SD	p - value	CA125-STn (U/ml) mean ± SD	p - value
Healthy	**36**	**37**	**10.3 ± 6.0**	**Ref. 1**	**0.7 ± 0.9**	**Ref. 1**	**1.8 ± 1.7**	**Ref. 1**
Benign	**317**		**46.9 ± 78.0**	**Ref. 2**	**3.9 ± 23.1**	**Ref. 2**	**9.1 ± 27.5**	**Ref. 2**
Neoplasms	126	56	59.2 ± 108.9	0.001*	3.6 ± 12.8	0.011*	6.0 ± 14.8	0.14
Non-neoplastic	8	43	49.9 ± 68.1	0.047*	2.3 ± 2.6	0.96	4.9 ± 5.6	0.58
Endometriosis	183	36	38.3 ± 45.3	< 0.001*	4.2 ± 28.5	< 0.001*	11.4 ± 33.9	< 0.001 *
Ovarian cancer	**232**							
High grade serous	158	65	1321.5 ± 2211.1	< 0.001*	116.9 ± 127.4	< 0.001*	767.5 ± 1036.2	< 0.001*
Low grade serous	24	59	549.3 ± 705.7	< 0.001*	45.9 ± 81.5	< 0.001*	576.2 ± 2001.7	< 0.001*
Clear cell	15	59	562.0 ± 1579.8	0.014*	24.3 ± 51.5	< 0.011*	95.5 ± 193.1	0.035*
Endometroid	23	63	336.4 ± 358.6	< 0.001*	20.1 ± 25.1	< 0.001*	105.6 ± 194.8	0.001*
Mucinous	12	60	110.3 ± 107.7	0.103	50.5 ± 153.1	0.157	17.8 ± 37.6	1.00
Stage (FIGO2014)								
I	50	63	229.3 ± 372.5	< 0.001*	21.8 ± 75.6	< 0.001*	111.8 ± 274.6	0.002*
II	10	61	771.8 ± 940.6	< 0.001*	68.1 ± 55.6	< 0.001*	842.2 ± 1970.5	< 0.001*
III	122	65	1171.2 ± 1793.4	< 0.001*	112.3 ± 133.0	< 0.001*	770.6 ± 1241.5	< 0.001*
IV	43	62	1738.9 ± 3069.7	< 0.001*	122.3 ± 114.1	< 0.001*	707.8 ± 932.4	< 0.001*
Unknown	7							

* serous cystadenoma (21), mucinous cystadenoma (27), fibroma (16), follicular cyst (15), mature teratoma (13), leiomyoma (6), histology not specified (28)

** infection (5), adnexal torsion (3)

Table 2. The number of false positive controls (N and %) with the conventional CA125-IA, CA125-MGL and CA125-STn assays.

Histology	CA125 (>35.0 U/ml)		CA125-MGL (>2.0 U/ml)		CA125-STn (>10.0 U/ml)	
	N	%	N	%	N	%
Benign (all)	116	36.6	88	27.8	54	17.0
Neoplasms	50	39.7	35	27.8	12	9.5
Non-neoplastic	3	37.3	3	37.5	1	12.5
Endometriosis	63	34.4	50	27.3	41	22.4

Figure legends:

Figure 1. ROC curves, AUCs, AUC comparisons and the sensitivities at fixed specificity (90%) for the conventional CA125, CA125-MGL, CA125-STn assays and for the combined glycoform assays (CA125-MGL+STn) in different subgroups.