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Aberrant glycosylation of α3 integrins as diagnostic markers in epithelial ovarian cancer

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ABSTRACT

Background: Glycans are strongly involved in stability and function of integrins (ITG) and tetraspanin protein CD63 and their respective interaction partners as they are dysregulated in the tumorigenic processes. Glycosylation changes is a universal phenomenon of cancer cells. In this study, glycosylation changes in epithelial ovarian cancer (EOC) are explored using tetraspanin and integrin molecules.
Methods: ITG and CD63 were immobilized from 10 EOC and 5 benign ovarian cyst fluid on microtiter wells and traced with 3 glycan binding proteins (STn, WGA, UEA) conjugated on europium nanoparticles. Total protein measurements (ITG & CD63 immunoassays) were also performed. The most promising glycovariant candidates identified were then clinically evaluated on the whole cohort of 77 ovarian cyst fluids. Additional testing was performed in ascites fluid samples of liver cirrhosis (n = 2) and EOC (n = 4).
Results: Sialylated Tn antibody based glycovariants of ITGα3 ($ITG\alpha3^{STn}$) and CD63 ($CD63^{STn}$) performed better than corresponding protein epitope-based immunoassays, $ITG\alpha3^{IA}$ and $CD63^{IA}$ respectively. Combined ITGα3 based assays ($ITG\alpha3^{IA}$ + $ITG\alpha3^{STn}$) detected 49 out of 55 malignant & borderline cases without detecting any of the 22 benign and healthy cysts.
Conclusion: Our findings indicate the potential diagnostic application of $ITG\alpha3^{STn}$ along with total $ITG\alpha3^{IA}$, which could help reduce the unnecessary surgeries. The results encourage studying further the potential use of these novel assays to detect EOC at earlier clinical stages.

1. Introduction

CA125 levels in blood and *trans*-vaginal imaging are currently routinely used in diagnosis of epithelial ovarian cancer (EOC) [1,2]. However, not all patients express elevated levels of CA125 in the early stages, and with imaging, it is often difficult to distinguish tumor formation from functional cysts in pre-menopausal ovaries [3]. Hence, new approaches have constantly been explored for early detection of EOC.

In the search for new biomarkers, serum has been the most common source, but proteomic profiles of other body fluids have also been evaluated [4–7]. Ovarian cyst fluid (CF) is a source rich in proteins secreted directly by ovarian tumor cells, and hence can offer direct targets for new diagnostic techniques in early-stage EOC [8]. Few studies so far have reported diagnostic and prognostic markers in ovarian CF [9–12]. Ascitic fluid, another potential diagnostic target, is composed of constituents such as lipids, proteins, extracellular vesicles

Abbreviations: AUC, Area under the curve; BSA, Bovine Serum Albumin; CA125, Cancer Antigen 125; CA15-3, Cancer Antigen 15-3; CF, Cyst Fluid; CI, Confidence Interval; EOC, Epithelial Ovarian Cancer; Eu-NP, Europium (III)-Chelate-dyed nanoparticles; EV, Extracellular vesicles; GV, Glycovariant; IA, Immunoassay; ITGs, Integrins; LC, Liver Cirrhosis; mAb, Monoclonal Antibody; n, Number; NP, Nanoparticles; ROC, Receiver Operating Characteristic; RT, Room Temperature; S/B, Signal/Background; SN, Sensitivity; SP, Specificity; STn, Sialyl-Tn antigen; TRF, Time-resolved fluorescence; UEA, Ulex Europaeus Agglutinin; WGA, Wheat Germ agglutinin.

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(EV) and integrins (ITGs) [13–15], which can be used as diagnostic targets.

CD63 is a cell surface membrane protein and is a part of the tetraspanin family [16]. CD63 is the first tetraspanin to be characterized [17] and is reported to have cell surface glycosylations [18]. Many studies have reported its role in cancer metastasis [19,20]. ITGs are a diverse family of glycoproteins and, in cancer, overexpression of ITGs leads to migration and invasion by cancer cells in the extracellular matrix [21]. ITGs have been shown to have a fundamental role in cancer progression and metastasis [22]. ITGs profile of EV across ovarian cancer cells in a study showed significant expressions of alpha integrins α2, α3, α6, and αv, as well as beta integrins β1 and β4 [23]. In cancer, a wide range of glycosylation changes have been observed [24,25]. Alteration of ITG glycosylation is frequently detected in tumors and has been previously reported [26–28]. ITG *N*-glycosylations, both sialylation and core fucosylation, play a role in modulating ITG functions during cancer [29]. Truncated *O*-glycan structures T, Tn and sialyl-Tn (STn) are expressed early in tumorigenesis and are correlated with poor survival in carcinoma patients. *O*-sialylation, specifically STn antigen, in integrins has been well characterized [24,30].

Various glycan binding proteins (like lectins) and antibodies can be used as tools to detect altered glycosylations in cancer [31]. We have previously reported that STn on conjugation with highly fluorescent europium nanoparticles (Eu-NP), enhances the cancer specificity of conventional CA125 and CA15-3 in EOC detection [32,33]. Also, aberrantly fucosylated ITGα3 using UEA-lectin Eu-NP have been reported in urine in bladder cancer patients [34]. Significant differences were also found between control and cases in breast cancer serum using tetraspanin CD63 with anti-CD63 Eu-NP assays [35]. However, there is still a need for novel biomarkers for better detection at early stages.

In the present study, we explore fucosylation, GlcNAc and *O*-sialylation changes in EOC using reporter molecules targeting CD63 tetraspanin and ITGs. We report the detection of STn glycoconjugates of CD63 and ITGs in ovarian CF and ascites samples using high performance Eu-NP assays.

2. Materials and Methods

2.1. Study design

This study included a cohort of 77 ovarian CF samples, out of which 15 were used for initial testing with 3 different binder proteins and the shortlisted candidates were then tested on the whole cohort. The CF cohort has been described earlier by Wang et al, 2016 [36]. These include non-neoplastic cysts (n = 10), benign tumors (n = 12), borderline tumors (n = 24) and invasive cancers (malignant n = 31). The interesting candidates were then further tested in ascitic fluid samples (n = 6), where 2 were liver cirrhosis as benign condition and 4 EOC samples.

2.2. Materials

The anti-tetraspanin and anti-integrin antibodies binding to the tetraspanin CD63 and integrins ITGαv, ITGα3, ITGβ8 were purchased from BD science (Vantaa, Finland). The yellow streptavidin coated 96 well microtiter plates, wash buffer and the assay buffer were obtained from Kaivogen Oy (Turku, Finland). For time resolved fluorescence (TRF) measurement, Hidex Sense from Hidex Oy (Turku, Finland) was used. Europium (III)-chelate-doped Fluoro-Max^{TM} polystyrene nanoparticles (95 nm in diameter) (Eu-NP) were acquired from Seradyn Inc. (Indianapolis, IN, USA). The glycan binding plant lectins UEA and WGA were purchased from Vector laboratories (Burlingame, CA, USA) and STn1242 monoclonal antibody (mAb) was provided by Fujirebio Diagnostics AB (Göteborg, Sweden).

2.3. Methods

The tetraspanin and ITGs selected for glycovariant screening in 15 ovarian CF samples includes CD63, ITGα3, ITGαv and ITGβ8, which were immobilized using respective biotinylated capture antibodies. These were screened with 2 lectins (UEA, WGA) and 1 antibody (STn) with different specificities, coated on Eu-NP and used as tracers. The tracers STn, UEA and WGA have binding specificities to *O*-sialyl Tn antigen, α-linked fucose and N-Acetylglucosamine respectively. CD63 and ITGα3 IA were also included where same antibody was used as biotinylated capture and as Eu-NP coated tracer.

Anti-CD63 and anti-integrin antibodies were biotinylated with 40-fold molar excess of biotin isothiocyanate for 4 h at room temperature (RT) as described previously [31]. The purification of the biotinylated antibodies was done using NAP^{TM}-5 gel-filtration columns (GE Healthcare, Schenectady, NY, USA) with the use of 50 mmol/L Tris-HCl (pH 7.75), containing 150 mmol/L NaCl and 0.5 g/L NaN_3. The biotin labelled antibodies were stored in 1 g/L BSA at 4 °C. The application of Eu-NP as bio-conjugated reporter has been described previously [37]. The amino groups of CD63 and integrin binding mAb and glycan binding proteins (lectins) were covalently bound to the activated carboxyl group of the Eu-NPs. The antibody/lectin coated Eu-NP were stored using the buffer 10 mM Tris-HCl, pH 7.8, supplemented with 0.1% BSA and 0.01% sodium azide at + 4 °C. The particles were thoroughly vortexed before every use to disperse the aggregates.

2.3.1. In-house TRF based assays

Both the CF and ascitic fluid measurements were performed similarly as follows. The biotinylated CD63 and ITG antibodies were immobilized 40 ng/25 μl/well on streptavidin coated microtiter plate in assay buffer for 1 h at RT. The plate was washed twice with wash buffer and the cyst or ascitic fluid was added 25 μl/well in triplicates with 1:20 dilution in assay buffer and incubated for 1 h at RT with slow shaking. The plate was again washed twice and the lectin/antibody coated Eu-NPs were added 1e7/25 μl/well and incubated for 1 h at RT with slow shaking. The final wash was done 6 times and the TRF measurement was taken in Hidex Sense (λ_{ex} = 340 nm and λ_{em} = 615 nm).

2.3.2. Statistical analysis

The heatmap of CF screening was plotted using RStudio [38] software with ggplot2 [39] R packages. The box plot analysis for the CF and the bar graphs for the ascitic fluids was performed with Origin, version 2016 for windows. The p-values were calculated using the 2-sample *t*-test in Origin, where p-value below 0.05 was considered significant. Receiver operating characteristics (ROC) were determined and compared, and the area under the curve (AUC) values at 95% CI were calculated using SPSS version 28 statistical software package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Screening of EOC specific CD63 and integrin glycovariants in ovarian cyst fluid

The antibodies that capture the respective ITGs and CD63 were used along with a small panel of different sugar binding lectins/ antibodies coated on Eu-NPs as tracers. The results from cyst fluids (n = 15) which includes benign (n = 5) and EOC (n = 10) are shown in Fig. 1.

Among the ITG and CD63 glycovariants that were screened, the sialyl-Tn (STn) glycosylation seems to be specifically elevated in EOC cyst fluid. The S/B ratio of all 5 benign cyst fluid samples were almost negligible i.e. close to 1 with the STn GVs of ITGs and CD63. Also, on comparing the CD63^{IA} and ITGα3^{IA} to the CD63^{STn} and ITGα3^{STn} GV, the GV combinations showed significant discrimination ($p < 0.01$).

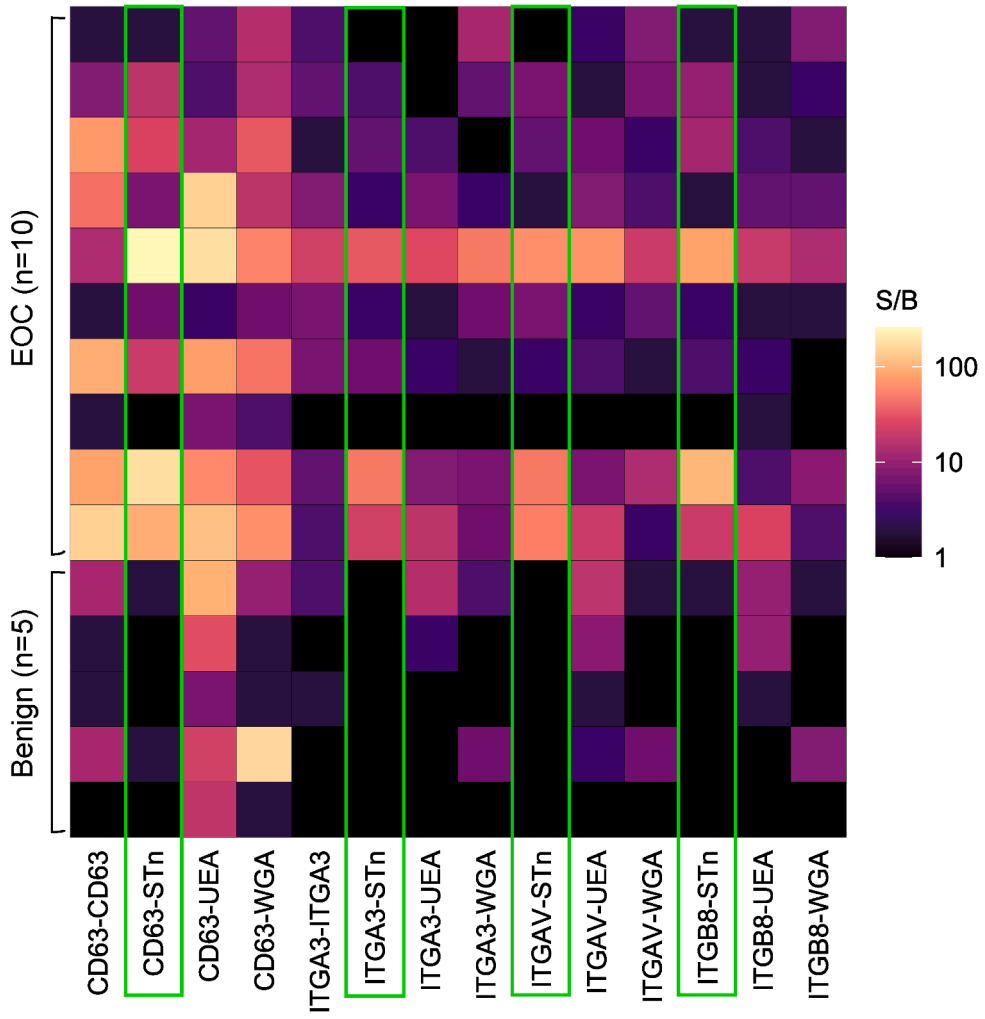

Fig. 1. Glycoprofiling of CD63 and integrins. The heat map represents the signal to background (S/B) ratio of CD63 and different integrin based glycovariant assays with cyst fluid samples (n = 15) which includes benign (n = 5) and EOC (n = 10).

3.2. CD63 and ITGα3 glycovariants in cyst fluid

After the initial screening, the best combinations were tested on a cohort of 77 cyst fluid samples, which included 15 from screening and 62 new samples. These included $CD63^{STn}$ and $ITG\alpha3^{STn}$ GVs that were compared with their protein epitope-based immunoassays, $CD63^{IA}$ and $ITG\alpha3^{IA}$ respectively (Fig. 2).

$CD63^{IA}$ discriminates borderline significantly from non-malignant cases ($p < 0.0001$), whereas there is no significant discrimination of non-malignant with malignant samples ($p = 0.23$). The GV of CD63 however, shows significant discrimination ($p < 0.005$) of non-malignant with both borderline as well as malignant cases. The median S/B ratio of malignant in $CD63^{STn}$ assay is also 5-fold higher than the $CD63^{IA}$. The most significant discrimination of borderline with non-malignant cases is seen with $ITGA3^{IA}$ ($p < 0.00005$). $ITGA3^{IA}$ also significantly discriminates the malignant samples ($p < 0.01$). The GV of ITGA3 ($ITGA3^{STn}$), significantly discriminates non-malignant with both borderline and malignant cases ($p < 0.01$). Most of the non-malignant patients show S/B ratio close to the background, with the least deviation seen with GVs. Further analysis was done to study the detection rate and if any of the GV or IA on combinations complemented each other to improve the assay performance.

In case of CD63 based assays, the AUC of $CD63^{IA}$ was 0.637, which increased to 0.945 with $CD63^{STn}$ GV. At 100% specificity (SP), the detection with CD63 increased from 20% with its IA to 74.5% with its GV. The AUC of $ITG\alpha3^{IA}$ and $ITG\alpha3^{STn}$ was 0.912 (100% SP, 70.9% SN) and 0.940 (100% SP, 83.6% SN) respectively. The only additive advantages were seen on combination of both the ITGα3 assays which showed the highest AUC at 0.976 (100% SP, 89.1% SN). (Fig. 3).

3.3. CD63 and ITGα3 glycovariants in ascitic fluid

The best CD63 and ITGα3 based assays from the cyst fluid were selected and tested in EOC ascitic fluid (n = 4) using liver cirrhosis (LC) ascitic fluid (n = 2) as benign control. In the $ITG\alpha3^{STn}$ GV assay, the counts of 3 out of 4 EOC were higher than the 2 LC ascitic fluid, whereas only 1 EOC was higher than LC in $ITG\alpha3^{IA}$ and $CD63^{STn}$. (Fig. 4).

4. Discussion

Emerging data suggest that cancer-derived extracellular vesicles are strongly glycosylated, being rich in specific glycoconjugates like tetraspanin family proteins and integrins (ITGs). This puts a focus on glycoisoform of expressed glycoconjugates to be cancer relevant [34,40]. Our study was driven by the underlying principle that glycosylation changes occur in cancerous cell surface, released glycoproteins or integrin associated glycoconjugates and absent in non-cancerous conditions [29]. However, the reliable recognition of the cancer specific glycoisoforms requires a specific, robust, and preferably cost-efficient diagnostic platform for wide-spread use in the clinics. We have

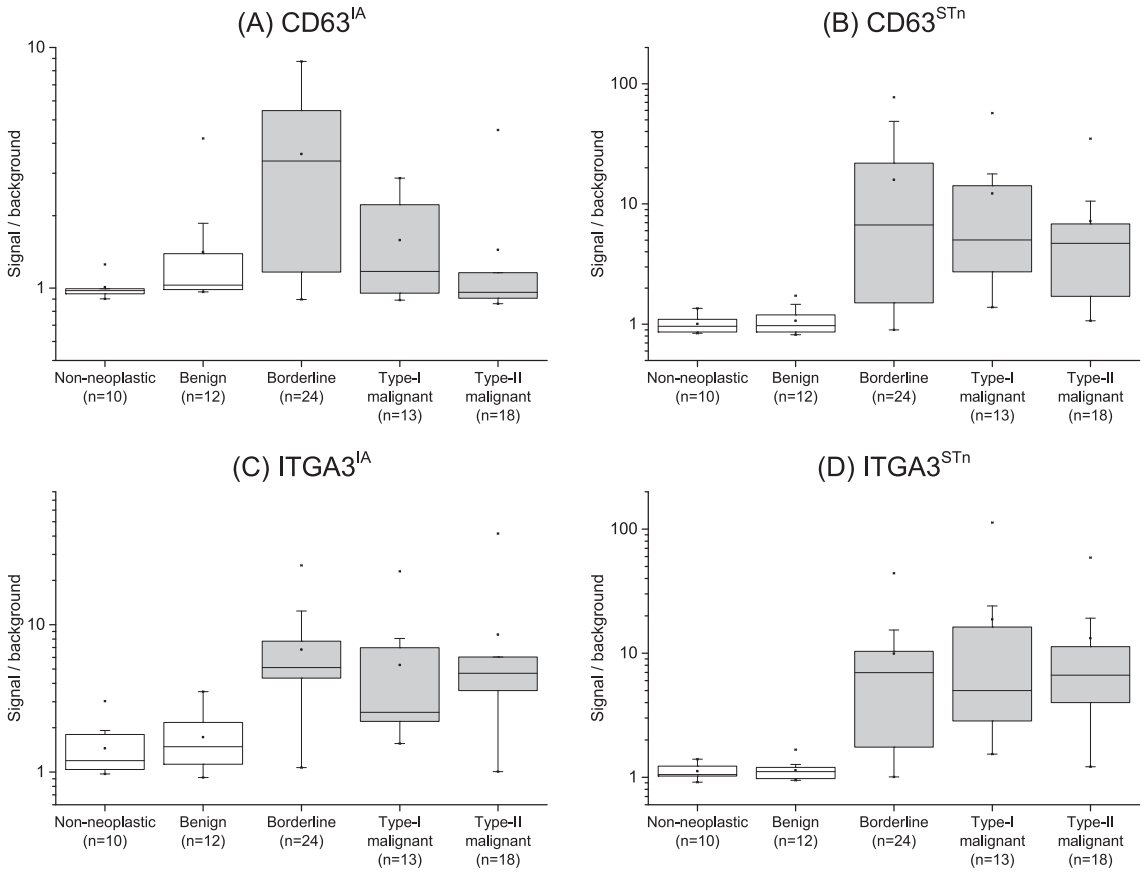

Fig. 2. Glycoconjugates of CD63 and ITGα3 in the ovarian cyst fluid. Box plots of non-neoplastic (n = 10), benign (n = 12), borderline (n = 24), type-I malignant (n = 13) and type-II malignant (n = 18) in (A, B) CD63^{IA} and its GV CD63^{STn} and (C, D) ITGα3^{IA} and its GV ITGα3^{STn}.

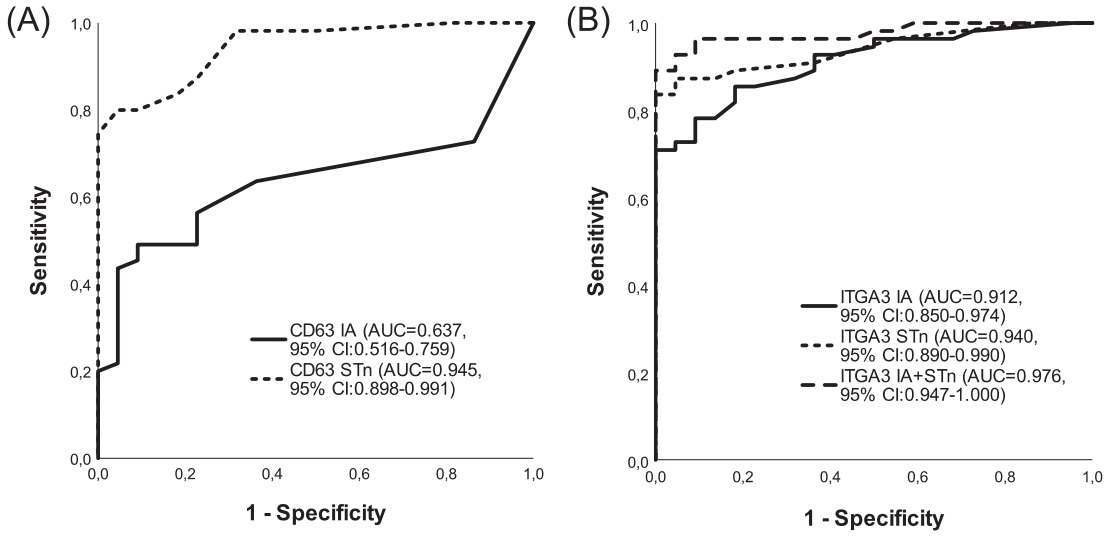

Fig. 3. ROC plots of CD63 and ITGα3 based assays in ovarian cyst fluid. Cyst fluid samples (n = 77) include non-malignant (n = 22) and borderline + malignant (n = 55). (A) ROC plot displaying CD63^{IA} (solid line) and CD63^{STn} glycovariant (dotted line). (B) ROC plot displaying ITGα3^{IA} (solid line), ITGα3^{STn} (dotted line) and combination of ITGα3^{IA} and ITGα3^{STn} glycovariant (long dotted line). The AUC and 95% confidence interval (CI) are displayed numerically in the brackets.

previously utilized lectin or anti-glycan antibody conjugated europium nanoparticles (Eu-NP) successfully to explore the glycosylation of conventional tumor biomarkers; CA125, CA15-3 as well as urinary integrins for ovarian, breast and bladder cancer respectively [31–35,41]. In the present study, we used one anti-tetraspanin (CD63) mAb and 3 different anti-ITGs (α3, αv and β8) as capture, and two lectins (WGA and UEA) and an anti-STn-mAb as tracer molecule. We used 5 benign and 10 EOC associated cyst fluids for screening of best assay combinations. We discovered that the Eu-NPs assisted STn-mAb in combination with CD63 (CD63^{STn}) and ITG-α3, -αv (ITGα3^{STn}, ITGαv^{STn}) could discriminate EOC associated cyst fluid from benign patients. To measure total tetraspanin and ITG amounts we included CD63 and ITGα3 immunoassays (IA)

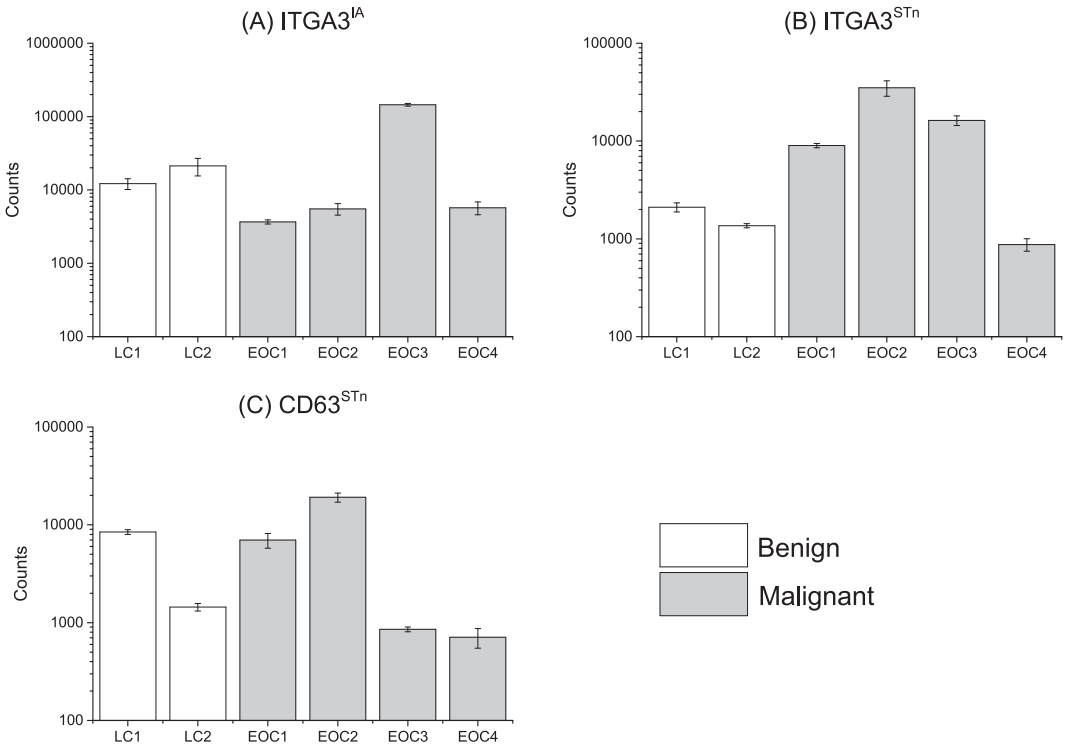

Fig. 4. CD63 and ITGα3 based assays in ascitic fluid. The illustration represents ascitic fluids of liver cirrhosis (LC) as benign control (n = 2) and EOC as malignant (n = 4). Eu-NP based assays were performed and the counts (signals) were plotted on the Y axis.

where we used the same mAb as capture and tracer ($CD63^{IA}$ and $ITG\alpha3^{IA}$).

The clinical evaluation of the best CD63 and ITGα3 based assays was done on 77 ovarian cyst fluids, including 55 malignant + borderline EOC and 22 benign and healthy i.e., non-malignant patients. As expected, we observed better discrimination with our novel $ITG\alpha3^{STn}$ and $CD63^{STn}$ assays compared to their corresponding total protein levels. Surprisingly $ITG\alpha3^{IA}$ also gives significant discrimination and further complements the $ITG\alpha3^{STn}$ GV assay. At 100% specificity, although most (~90%) of the 55 malignant + borderline cysts were detectable with the combination of $ITG\alpha3^{STn}$ and $ITG\alpha3^{IA}$ in their fluidic compartment, 6 did not. Four of these six cysts occurred in borderline tumors and two in type-I cancer, while 100% of the type-II cancers were detectable by the ITGα3 based assays. In the same cyst fluids in a study by Wang et al 2016 [36], at 100% SP, only 87% of the tumor-DNA mutations were detectable (54 malignant + borderline and 18 non-malignant). ctDNA could not be extracted from 4 non-neoplastic and 1 borderline sample. The same cohort has also been tested for MUC1 and MUC16 based GV markers in our recently published study [33]. The two type-I cysts which are not detected with ITG based assays are detected with the STn glycoform of MUC1. Further studies are needed to see the potential additive advantages of ITG assays to mucin based GV assays.

A study by He et al 2018 [42] revealed that ITGβ8 expression was substantially elevated in ovarian cancer tissues compared to that in normal ovarian tissues. In addition, they demonstrated that elevated ITGβ8 expression was independently associated with shorter overall survival and recurrence-free survival in high-grade serous OC. These findings reveal the potential clinical value of ITGβ8 expression as a prognostic indicator in high-grade serous ovarian cancer. Ricardo et al 2015 [43], used proximity ligation assay in tissue lesions to demonstrate the selective expression of Tn and STn glycoforms of MUC16 and MUC1 in serous ovarian cancer. In a previous study [34], we reported aberrantly fucosylated ITGα3 ($ITG\alpha3^{UEA}$) in bladder cancer urine samples while in this study we report the aberrant expression of STn antigen on ITGα3 in ovarian cyst fluid. This might indicate tissue specific glycosylation in cancer, i.e., the same glycoprotein (ITGα3) having different glycan epitope in different cancers. Xia et al 2005 [44], evaluated expression levels of CD63 and ITGα5 using RT-PCR and hybridization in-situ in tissue material and reported their low expression and negative correlation with ovarian cancer. In another study, we reported that differential diagnosis of primary breast cancer may be aided by detecting cancer-associated glycosylation of MUC1 and MUC16 with WGA, and total concentration of CD63 ($CD63^{IA}$) [35]. In this study, $CD63^{IA}$ also shows significant discrimination ($p = 0.008$, AUC = 0.637) between malignant and non-malignant in ovarian cyst fluid but their STn containing glycovariant ($CD63^{STn}$) was superior ($p = 0.0021$, AUC = 0.945). To our knowledge, we have demonstrated for the first time here that altered glycans on ITGα3 in ovarian tumors are also present in their associated cyst fluids and ascitic fluid. Further studies examining the ITGs glycosylation in patient blood are required to confirm whether glycoforms of ITGs can serve as a prognostic/predictive factor and a diagnostic tool for the detection of ovarian cancers.

Our study, though only proof-of-principle, illustrates one route to improve the management of patients with ovarian cysts. One of the limitations of this study spans from the number of samples; this proof of principle assay calls for further clinical and technical evaluation with well-defined patient cohorts in the future.

5. Conclusion

The novel $ITG\alpha3^{STn}$ could be a valuable diagnostic biomarker in EOC. The assay can be used to discriminate the malignant from the non-malignant patients without any pre-analytical processing of the samples. Our data shows that specific dysregulation of integrin glycosylation can be considered as a biomarker in oncology.

CRediT authorship contribution statement

Shruti Jain: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization.

Priyadharshini Parimelazhagan Santhi: Investigation. **Rufus Vinod:** Investigation. **Shamima Afrin Ruma:** Validation. **Kaisa Huhtinen:** Resources. **Kim Pettersson:** Writing – review & editing, Project administration, Funding acquisition. **Karin Sundfeldt:** Resources, Writing – review & editing, Funding acquisition. **Janne Leivo:** Writing – review & editing, Supervision. **Kamlesh Gidwani:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of interest: The authors declare no conflict of interest.

Ethics approval statement: The study was approved by the local ethics committee in Gothenburg and was performed in accordance with the Declaration of Helsinki and all its amendments.

Patient consent statement: Informed consent was obtained from all individual participants included in the study.

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