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Targeted Mass Spectrometry of Longitudinal Patient Sera Reveals LTBP1 as a Potential Surveillance Biomarker for High-Grade Serous Ovarian Carcinoma

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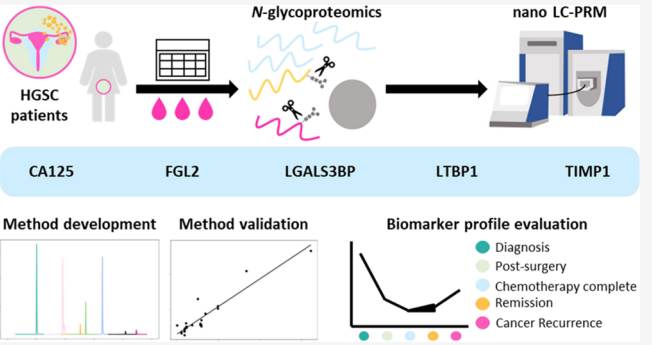
ABSTRACT: High-grade serous ovarian carcinoma (HGSC) is the most prevalent subtype of epithelial ovarian cancer. The combination of a high rate of recurrence and novel therapies in HGSC necessitates an accurate assessment of the disease. Currently, HGSC response to treatment and recurrence are monitored via immunoassay of serum levels of the glycoprotein CA125. CA125 levels predictably rise at HGSC recurrence; however, it is likely that the disease is progressing even before it is detectable through CA125. This may explain why treating solely based on CA125 increase has not been associated with improved outcomes. Thus, additional biomarkers that monitor HGSC progression and cancer recurrence are needed. For this purpose, we developed a scheduled parallel reaction monitoring mass spectrometry (PRM-MS) assay for the quantification of four previously identified HGSC-derived glycopeptides (from proteins FGL2, LGALS3BP, LTBP1, and TIMP1). We applied the assay to quantify their longitudinal expression profiles in 212 serum samples taken from 34 HGSC patients during disease progression. Analyses revealed that LTBP1 best-mirrored tumor load, dropping as a result of cancer treatment in 31 out of 34 patients and rising at HGSC recurrence in 28 patients. Additionally, LTBP1 rose earlier during remission than CA125 in 11 out of 25 platinum-sensitive patients with an average lead time of 116.4 days, making LTBP1 a promising candidate for monitoring of HGSC recurrence.



KEYWORDS: *targeted proteomics, N-glycosylation, serum biomarker, surveillance biomarker, high-grade serous ovarian carcinoma, ovarian cancer, biomarker validation, LTBP1, CA125, PRM*

1. INTRODUCTION

High-grade serous ovarian carcinoma (HGSC), the most prevalent subtype of Epithelial Ovarian Cancer (EOC), represents the fifth most common cause of cancer death for women in Canada, the US, and Europe.[1] The disease is characterized by low survival rates, especially after metastasis: only 30% of EOC patients survive for 5 years[1,2] and less than 20% for 10 years.[3] A significant contributing factor to HGSC's elevated mortality is its high recurrence rate of 70−95%.[4] As novel treatments become available it is vital to monitor HGSC patients closely during remission for the early detection of cancer recurrence.[5]

Currently, HGSC recurrence as well as its response to treatment (debulking surgery and adjuvant platinum-based chemotherapy) is monitored via serum levels of CA125. CA125 is a large, heavily glycosylated transmembrane protein that is encoded by the MUC16 gene. CA125's extracellular domain is shed by EOC cells[6] and thus CA125 can be detected in body fluids like serum, peritoneal fluid,[7] or ascites fluid.[8] CA125 plays various roles in ovarian cancer, including tumorigenesis,[6] metastasis,[9] and immune evasion.[10]

As a surveillance biomarker, CA125 largely improved the diagnosis of ovarian cancer recurrence compared to traditional physical examination and imaging techniques, which only were able to detect large tumors.[11] Decreasing and increasing CA125 levels predict disease regression and progression in 90% of cases.[6] However, over 60% of patients within the accepted CA125 levels during remission of <35 U/mL still carry small

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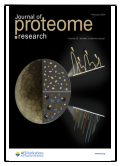

residual tumors.[12] Additionally, CA125 seems to detect cancer recurrence too late as earlier treatment of EOC based on rising CA125 levels instead of symptomatic relapse does not improve overall survival.[13] Thus, more sensitive biomarkers that monitor HGSC recurrence are needed.

Serum is often used for monitoring biomarkers as it is minimally invasive and thus easily attainable. However, the proteomic analysis of biomarkers in serum is challenging since the 22 most abundant proteins that make up 99% of the serum protein mass[14] hinder the detection of low-abundance tissue leakage and signaling proteins, which are often the most promising biomarker candidates. Thus, enrichment methods that target biologically interesting subsets of the serum proteome are needed. In this study, we applied a hydrazide-based approach to enrich *N*-glycosylated proteins from the serum proteome. *N*-glycosylated proteins are often expressed on the cell surface and/or secreted;[15] therefore, the *N*-glycoproteome is a relevant subproteome for the discovery of tumor shedding/leakage biomarkers. Furthermore, glycosylation increases the water solubility of proteins and makes them more resistant to enzymatic degradation,[16] making glycosylated proteins prime candidates for serum biomarkers.

In a previous study, our group utilized a *N*-glycoproteomics approach to identify tumor-derived proteins in sera of HGSC patient-derived xenografts.[17] Parallel Reaction Monitoring (PRM)-MS assays were developed to analyze the levels of biomarker candidates during disease progression in two consecutive patient cohorts (20 patients and 96 samples).[17] Proteins that mirrored tumor load (decrease after surgery and chemotherapy and increase at cancer recurrence) were selected as HGSC biomarker candidates.

In the current study, we attempted to further validate four candidates (FGL2, LGALS3BP, LTBP1, TIMP1) that were proposed by Sinha et al.[17] and compared their performance to CA125 in 212 longitudinal serum samples from an independent cohort of 34 HGSC patients with a special focus on remission. For this purpose, we developed, optimized, and validated a liquid chromatography-PRM-MS assay that enabled the quantification of the candidates in serum at low concentrations. We analyzed whether the candidates mirrored tumor load during disease progression, especially whether they increased prior to diagnosis of disease recurrence via traditional methods (CA125 ELISA and/or radiology).

2. EXPERIMENTAL SECTION

2.1. Enrichment of *N*-Glycosylated Peptides

N-glycosylated peptides were enriched from patient serum samples as described previously with minor modifications.[17] Briefly, 25 μL of serum was added to 75 μL of 2,2,2-trifluoroethanol (TFE):PBS (2:1, v/v) and spiked with 2 pmol of yeast invertase (SUC2, Sigma-Aldrich; UniProt accession-P00724) as a processing control. Proteins were then reduced using 5 mM dithiothreitol (DTT) at 60 °C for 30 min, and free cysteines were carbamidomethylated via incubation with 25 mM iodoacetamide for 30 min at room temperature in the dark. The alkylation reaction was quenched by adding 5 mM DTT before the samples were diluted five times (v/v) with freshly prepared 100 mM ammonium bicarbonate buffer (ABC) (pH 8.0). Afterward, the samples were digested overnight with Trypsin/Lys-C Mix (Promega) at 37 °C (enzyme/total protein 1:100). Trifluoroacetic acid (TFA) was added to a final concentration of 0.5% (v/v) to stop the digestion. Lastly, the samples were centrifuged for 2 min at 12,000× RCF at room temperature before the supernatants were desalted using C18 MacroSpin columns (The Nest Group). After washing the columns with 300 μL acetonitrile (ACN)/0.1% TFA, they were equilibrated two times with 250 μL of 0.1% TFA in water before the peptides were added, washed with 300 μL 0.1% TFA in water, and subsequently eluted in two steps with 200 μL 80% ACN/0.1% TFA. After lyophilization in a vacuum concentrator, peptides were resuspended in 200 μL 100 mM sodium acetate/150 mM sodium chloride (pH 5.5) for *N*-glycopeptide enrichment. *N*-glycopeptides were oxidized by incubating with 10 mM sodium metaperiodate for 30 min in the dark, and the reaction was quenched with 20 mM fresh sodium thiosulfate before coupling the oxidized glycans to hydrazide-coupled paramagnetic beads (glycopeptide:bead 1:1, MagSi-S Hydrazide 3.0, Amsbio) via overnight incubation at room temperature under constant shaking. The next day, the beads were washed 3× each with 1.5 M sodium chloride, water, methanol, 80% ACN, and 100 mM ABC (pH 8.0). Subsequently, covalently bound *N*-glycopeptides were eluted from the beads via enzymatic deglycosylation at asparagine using 5 U PNGaseF (Promega) at 37 °C overnight. The supernatant was collected and combined with two 400 μL washes of the beads with 80% ACN/0.1% TFA before vacuum concentration and subsequent desalting via C18 solid-phase extraction. For desalting, conventional 200 μL pipet tips were packed with five layers of C18 material (Attract SPE Disks, Affinisep). After priming with 20 μL of methanol followed by 20 μL of 0.1% TFA in ACN, the pipet tips were equilibrated with two steps of 20 μL of 0.1% TFA in LC−MS-grade water. Samples dissolved in 200 μL of 0.1% TFA were bound in two steps and washed twice with 20 μL of 0.1% TFA each. Lastly, the peptides were eluted with two steps of 40 μL of 80% ACN/0.1% TFA. The eluted samples were dried by vacuum centrifugation and then reconstituted in 25 μL of 0.1% formic acid in LC−MS-grade water. The peptide concentrations of all samples were determined with a NanoDrop spectrophotometer (Thermo Scientific).

2.2. Development of Scheduled PRM Assays

A QExactive HF mass spectrometer coupled to an Easy-Spray nanoelectrospray ionization source connected to an EASY-nLC 1000 nanoflow UPLC system (Thermo Scientific) was used for the analysis of deglycosylated peptides from human serum. For method development, light and heavy variants of all 7 peptides with heavy Arg (+10) or Lys (+8) and carbamidomethylated cysteines were purchased (AQUA peptides, Thermo Scientific) and spiked into matrix (deglycosylated peptides enriched from commercial human serum; Sigma-Aldrich, H4522) at 5 fmol/μg peptide. Chromatography gradients were optimized by injecting 2 μg of peptide onto a two-column setup consisting of a 2 cm Acclaim PepMap 100 column (id 75 μm, 3 μm, 100 Å) as the precolumn and a 50 cm EASY-Spray PepMap RSLC C18 column (id 75 μm, 2 μm, 100 Å) heated to 45 °C as the analytical column at a flow rate of 250 nL/min. LC gradients of various lengths and gradient compositions (Supplementary Figure S1) were first tested in unscheduled PRM mode and the three best-performing gradients were run afterward in scheduled PRM mode to analyze the peak intensities and the number of points per peak. Normalized Collision Energies (NCEs) of 15, 18, 21, 24, 27, 30, and 33 were tested, and the NCE with the highest fragment ion response was chosen per peptide for the final PRM assay. Ion injection times (IIT) of 300 and 350 ms

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Table 1. Target Inclusion List Depicting Analyte Specific PRM Parameters

protein	peptide sequence	m/z light	m/z heavy	retention time window [min]	NCE
SUC2	AEPILDISNAGPWSR	813.4177	818.4219	38.4−43.4	24
SUC2	FATDTTLTK	499.2637	503.2706	22.3−27.3	21
CA125	DTSVGPLYSGC[+57]R	656.3035	661.3077	27.4−32.4	21
FGL2	VADLTFVVNSLDGK	739.3985	743.4056	40.2−45.2	24
LGALS3BP	ALGFEDATQALGR	674.8464	679.8505	34.2−39.2	21
LTBP1	YVQDQVAAPFQLSDHTGR	678.0009	681.337	30.2−35.2	24
TIMP1	FVGTPEVDQTTLYQR	877.4414	882.4455	31.3−36.3	24

were tested, and the IIT yielding higher ion intensities was chosen for the final PRM method.

2.3. Optimized Parallel Reaction Monitoring (PRM) Assay

The LC−MS/MS setup was as described in the section "Development of Scheduled PRM Assays". The flow rate was 250 nL/min, and the gradient started with 100% mobile phase A (0.1% (v/v) FA). Mobile phase B (0.1% (v/v) FA in ACN) was increased to 8% in 5 min, then to 27% over 30 min. Afterward, mobile phase B was raised to 95% in 2 min and the system was rinsed with 95% B for 9 min followed by 3 washes going from 2 to 95% B in 8 min intervals, to reduce carry-over of FGL2 (Supplementary Figure S2). PRM-MS analyses were run at a resolution of 120,000 at 200 m/z (fwhm), with an automatic gain control (AGC) target of 2×10^5, 350 ms maximum fill time, and an isolation window of 1.4 m/z. The inclusion list containing retention time windows, m/z, and optimal collision energies is depicted in Table 1.

2.4. PRM Assay Validation

The linear range, limit of detection (LOD), and limit of quantification (LOQ) were all determined in the matrix, by spiking varying amounts of heavy AQUA peptides into deglycosylated peptides enriched from commercial human serum (Sigma-Aldrich, H4522). Some analytes could not be quantified in the matrix; therefore, a fixed amount of light AQUA peptide (100 fmol for FGL2, 10 fmol for all other analytes) was added to ensure that a ratio of heavy to light peptide could be measured. For determining the linear range, heavy peptide spike-ins of 2.5, 5, 10, 25, 50, 100, 250, 500, and 1,000 fmol (FGL2) or 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 fmol (all other analytes) were tested. Some measurements at lower concentrations had signal-to-noise ratios (S/N) of less than 3, so after experimental determination of the LOD and LOQ (see the following paragraph), measurements below the LOD and LOQ were excluded from the linear range curves.

The limit of detection was estimated using the ratio of heavy to light peptide via the following formula:

$$\mathrm{LOD} = \mathrm{mean(blank)} + 1.645 \times \mathrm{SD(blank)} + 1.645 \times \mathrm{SD(lowconcentratedsample)} (n = 10)^{18}$$

As a blank, pooled patient serum spiked with 10 fmol of light AQUA peptides was used. Heavy AQUA peptides were added to the blank at 1 fmol (CA125, SUC2-FATDTTLTK), 5 fmol (LTBP1, SUC2-AEPILDISNAGPWSR, TIMP1), or 10 fmol (FGL2, LGALS3BP) to create the low concentration sample (tested low concentrations were estimates from the linear range experiments). After determining a preliminary LOD via this method, pooled patient serum was spiked with heavy AQUA peptides at concentrations of 1×, 1.5×, 2×, 2.5×, 3×, 5×, and 10× the preliminary LOD ($n \geq 5$) to determine the true, experimental LOD and the LOQ. The LOD was defined as the concentration at which the signal-to-noise (S/N) was above 3, and the LOQ as the concentration at which the S/N was above 3 and the coefficient of variation (CV) was below 20%.[18] To determine the signal-to-noise ratio of the three quantifier transitions, we compared the maximum signal intensity of each transition within the peak time window to the maximum signal intensity of any of the monitored transitions within 3× the peak time window in Skyline (Supplementary Figure S3).

2.5. PRM Quantification of Patient Cohort

Serum samples (212) from 34 patients (n = 5−8/patient) were obtained through the University Health Network GYN blood biobank (Supplementary Table S1) via an ethics-approved protocol (#11-0022). All cases were diagnosed with advanced-stage disease, received debulking surgery, were subsequently treated with cytoreductive chemotherapy, and were clinically diagnosed with cancer recurrence based on clinical assessment with a CA125 ELISA and/or radiology. A 100% CA125 increase within 3 months was considered indicative of recurrence in patients with CA125 serum levels of 10−35 Units/mL during remission, while a 25% increase signaled recurrence in patients with CA125 serum levels of ≥35 Units/mL during remission.

Patients (12/34) received neo-adjuvant chemotherapy prior to surgery. Patients (9/34) relapsed within 6 months and were classified as platinum-resistant, and 25/34 relapsed in over 6 months and were classified as platinum-sensitive. For platinum-sensitive patients, we received 1−3 samples taken during disease remission.

CA125 serum levels determined with a clinically approved ELISA were available for 205/212 samples. For 42/205 samples, the serum sample used for ELISA was taken on a different day than the serum sample used for this study, with a median difference of 21.5 days (exact time differences noted in Supplementary Table S1).

Twenty-five microliters of serum were processed randomly based on the patient, as described earlier. After enrichment of deglycosylated peptides, 2 μg were spiked with 10 fmol (CA125, FGL2, LTBP1, SUC2-FATDTTLTK, TIMP1) or 100 fmol (LGALS3BP, SUC2-AEPILDISNAGPWSR) of heavy AQUA peptides, injected into the LC−MS/MS system and analyzed with our optimized PRM assay.

For 44 samples, more than 25 μL of serum was available so that processing replicates/stability tests could be performed as follows: one serum sample was thawed, a 25 μL aliquot was taken and processed, the remaining 25 μL was frozen again and stored at −80 °C for 3−4 months before the sample was thawed again and reprocessed. Additionally, 17 samples were loaded onto the column twice to test the measurement reproducibility.

2.6. Processing of PRM Data

All chromatograms were manually inspected using Skyline to ensure correct identification of the target peptides via comparing their retention times and transition ion ratios to their respective

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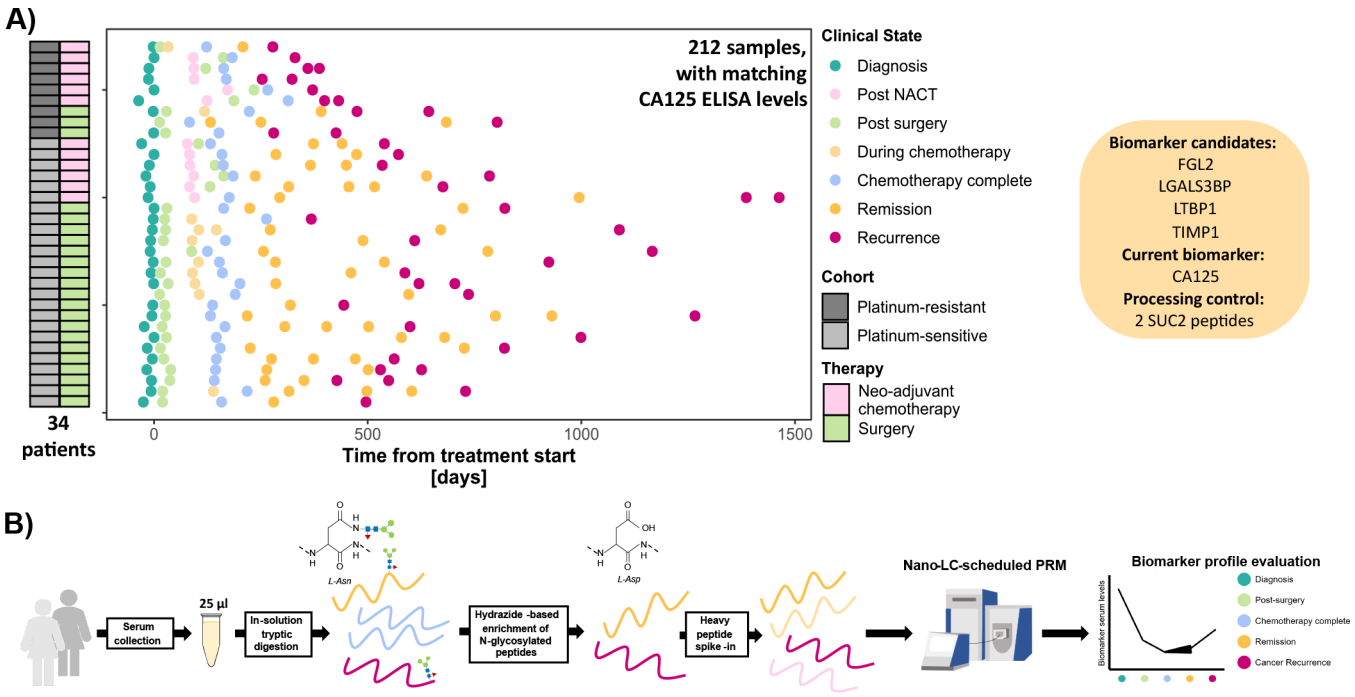

Figure 1. Experimental design. (A) Cohort overview. (B) *N*-glycoproteomics workflow applied in this study.

heavy labeled SIS peptide. The areas under the curve (AUC) of the three most intense fragment ions per precursor were extracted using Skyline.[19] Then, the ratios of endogenous (light) peptide to heavy AQUA peptide (=light/heavy ratio) were imported into the *R* statistical environment, and further processed, evaluated, and visualized. The light/heavy ratio of each analyte was standardized to the light/heavy ratio of the processing control SUC2 (added as intact protein at the beginning of sample processing). Injection replicates were combined after this standardization while processing replicates were combined after additional standardization to the concentration at diagnosis (per analyte, per patient). In the case of single replicates < LOD or LOQ, the replicate with the better quantification was chosen and the other replicate was discarded. For the analysis of longitudinal profiles and slopes between time points, all analyte concentrations were standardized to their level at diagnosis per patient. The slope was additionally divided by the time difference between the two contrasted time points to account for differences in intervals between serum sampling. For the evaluation of response to treatment, missing time points (e.g., at diagnosis) were replaced by the sample closest to the missing time point (e.g., postsurgery or post-NACT). The correlation between CA125 quantification via ELISA and our PRM method was determined for all samples > LOD for which the date of PRM sample collection matched the date of the ELISA. An overview of all obtained quantification results can be found in Supplementary Table S2.

3. RESULTS

3.1. Method Optimization and Validation

The goal of this study was to verify the behavior of four previously discovered HGSC biomarker candidates (FGL2, LGALS3BP, LTBP1, TIMP1)[17] in sera sampled from HGSC patients during disease progression (Figure 1A). To enable quantification of the biomarker candidates at low concentrations during cancer remission, we systematically developed a targeted proteomics assay (Figure 1B), starting with liquid chromatography (LC) optimization.

For the optimized scheduled PRM assay, we chose the 55 min gradient as it yielded the highest peak areas for 3 out of 4 novel biomarker candidates (FGL2, LTBP1, TIMP1) while resulting in more than 12 data points per peak for all analytes (Figure 2A). Next, normalized collision energies (NCE) between 15 and 33% were tested, and the NCE resulting in the highest peak area was selected for the optimized method (Figure 2B). An increase in the ion injection time from 300 to 350 ms resulted in higher peak areas for all analytes except CA125 (Figure 2C), with a median increase of 4-fold. However, this increase in signal intensity came at a cost of points per peak (median 19 vs 17). Since we expected the concentrations of our analytes to be near the limit of detection during disease remission, we chose the 350 ms ion injection time to increase the likelihood of detection in all our cohort samples.

In the final PRM assay, all analytes were baseline-separated (Figure 2D) and the three most intense transitions were extracted via Skyline for quantification (Figure 2E). Since some transitions of LTBP1 and FGL2 showed interfering signals in serum at very low concentrations (Supplementary Figure S3), other transitions were chosen for the quantification of these analytes. The optimized PRM method could quantify all analytes with limits of detection (LOD) and quantification (LOQ) as low as 0.12/0.2 fmol (Figure 3A).

3.2. Performance of the Optimized PRM Method in Cohort Samples

The developed PRM-MS assay was able to detect all new biomarker candidates in 187 of 212 samples (Figure 3B), showing the utility of a targeted *N*-glycoproteomic approach for the quantification of HGSC biomarkers in human serum. Samples with biomarker concentrations below the LOD were mostly collected during remission of HGSC.

CA125, in contrast, could only be detected in 35 samples via PRM, usually at diagnosis and/or recurrence of the disease.

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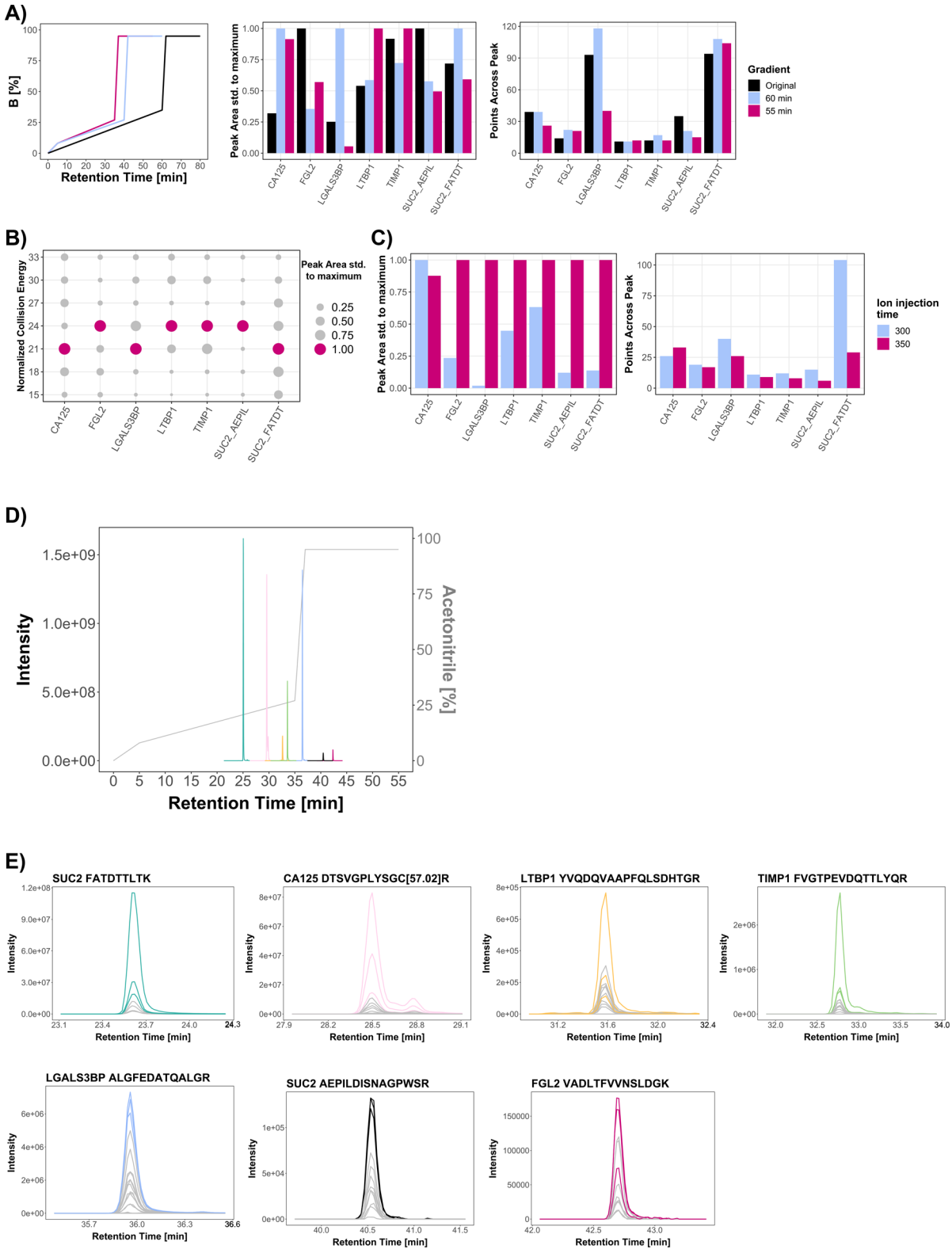

Figure 2. Method optimization. Experiments were performed with heavy internal standard peptides spiked into matrix (deglycosylated peptides enriched from commercial human serum). (A) Liquid chromatography optimization. (B) Normalized collision energy optimization. (C) Ion injection time optimization. (D) Chromatogram of a mix of light and heavy internal standard peptides (10 fmol each) in buffer A measured with the optimized nano LC-scheduled PRM method. (E) Zoom-in on each analyte from Figure 2D, transitions used for quantification are highlighted.

However, we were still able to compare the performance of the novel biomarker candidates to the established HGSC surveillance marker, since all samples had matching CA125 ELISA levels available. The PRM assay determined CA125 concentrations in high correlation with the clinically approved ELISA ($r = 0.94$, Figure 3C), even though it was less sensitive for CA125. One possible explanation for this difference in sensitivities is that immunoassays detect analytes of interest

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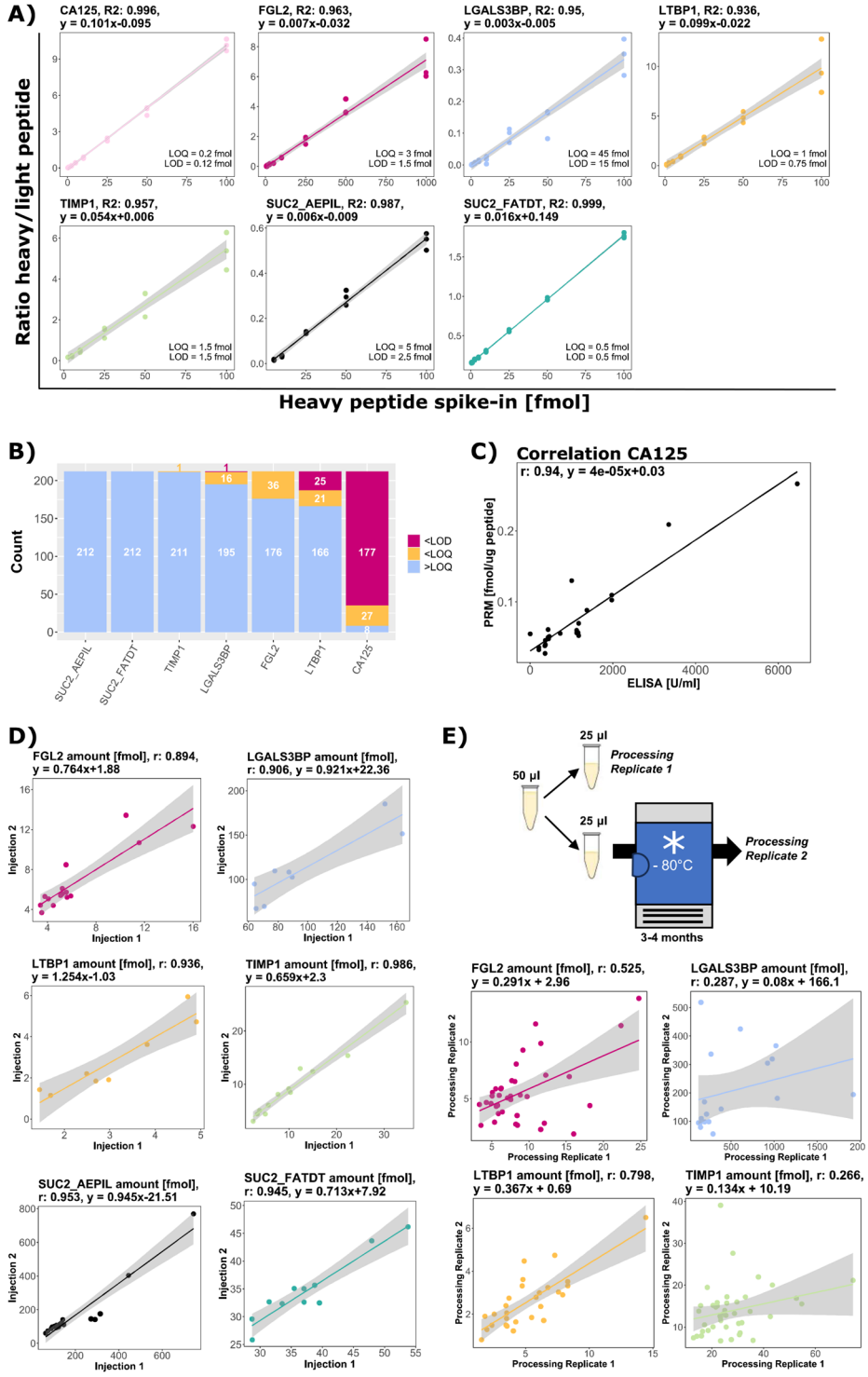

Figure 3. Method validation. (A) Linear ranges, limits of detection (LOD) and quantification (LOQ), determined via spiking heavy internal standard peptides into deglycosylated peptides enriched from commercial human serum. LOD and LOQ are in fmol/2 μg deglycosylated serum peptide and were experimentally verified by spiking pooled patient serum with heavy internal standard peptides at 1× LOD−10× LOD. (B) Detection of the four novel biomarker candidates (TIMP1, LGALS3BP, FGL2, LTBP1), the established biomarker CA125, and two processing control peptides derived from yeast invertase (SUC2) in our 212 cohort samples. (C) Correlation between CA125 serum levels measured via the developed PRM assay and a clinically approved ELISA. Only patient samples > LOD in both assays were considered. (D) Correlation between injection replicates of 17 patient samples, values < LOQ were removed. (E) Correlation between processing replicates of 44 patient samples, values < LOQ were removed.

via immunoselection, which circumvents the masking of the readout signal by high abundant proteins.

Injection replicates of 17 patient samples revealed a high reproducibility of the PRM assay (r = 0.894−0.986, Figure 3D); 44 processing replicates showed that most analytes do not tolerate multiple freeze−thaw cycles; only LTBP1 was remarkably stable (r = 0.798, Figure 3E).

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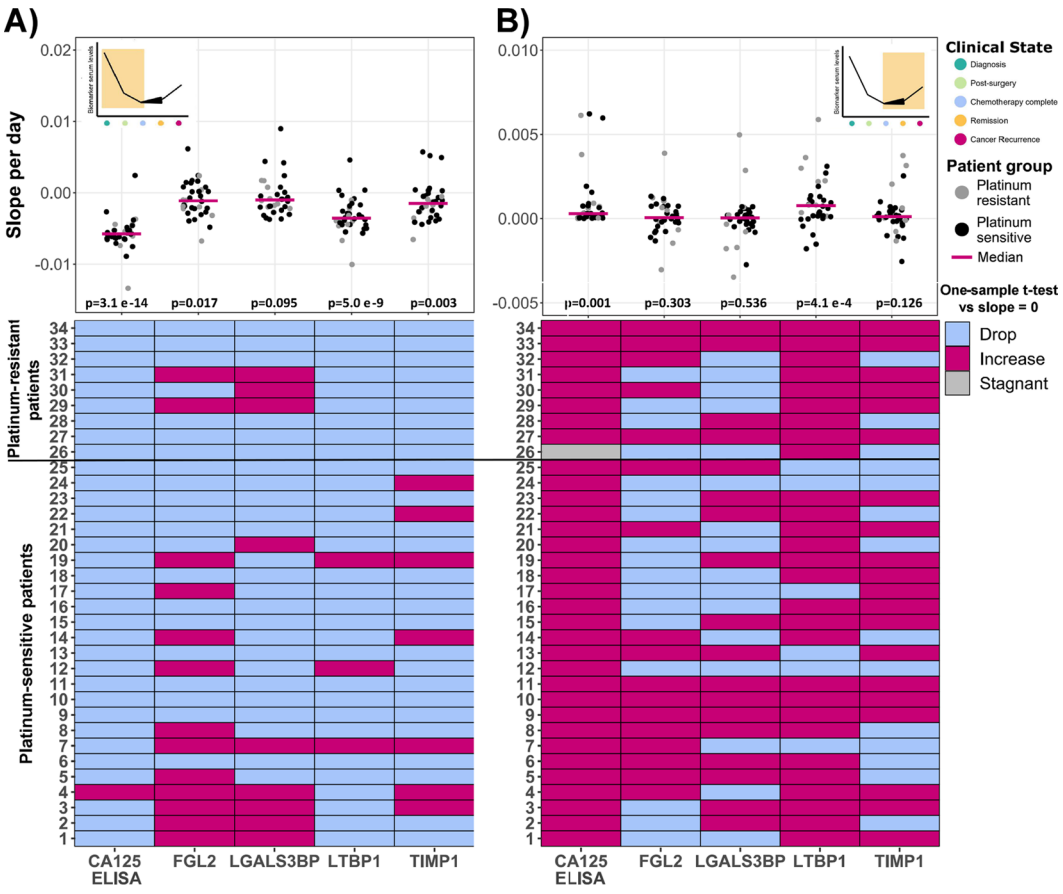

Figure 4. Response of biomarker candidates to cancer treatment and recurrence. (A) Changes in biomarker serum levels between cancer diagnosis and the end of chemotherapy. (B) Changes in biomarker serum levels between the end of chemotherapy and cancer recurrence.

3.3. Response of Biomarker Candidates to Cancer Treatment and Recurrence

Next, we assessed whether the four biomarker candidates mirrored the patients' tumor load. For this purpose, we evaluated whether their levels dropped as a result of cancer treatment and rose with disease recurrence. In our cohort, HGSC recurrence was determined via radiology (detection of new lesions on imaging) and/or CA125 ELISA: a 100% CA125 increase within 3 months was considered indicative of recurrence in patients with CA125 serum levels of 10−35 Units/mL during remission, while a 25% increase signaled recurrence in patients with CA125 serum levels of ≥35 Units/mL during remission.

For analysis of biomarker profiles, we standardized the slope (increase/decrease) of each biomarker candidate to the time interval in which the two evaluated samples were taken since our cohort was comprised of patients with vastly different disease timelines: 9 platinum-resistant patients, who experienced HGSC recurrence within six months after the end of chemotherapy, and 25 platinum-sensitive patients, who developed recurrent disease after more than six months.

All new biomarker candidates (FGL2, LGALS3BP, LTBP1, TIMP1) dropped between HGSC diagnosis and the end of chemotherapy (Figure 4A) and rose at cancer recurrence (Figure 4B), substantiating them as markers of HGSC. LTBP1 performed best among the novel candidates, with 31 out of 34 patients having lower LTBP1 serum levels after chemotherapy and 28 patients showing an increase in cancer recurrence. Additionally, LTBP1 showed a steeper increase between the end of chemotherapy and HGSC recurrence than the FDA-approved biomarker CA125 (Figure 4B), although CA125 showed less interpatient variability with 33/34 patients having increased CA125 levels at recurrence. However, in 8 patients (patients 1, 4, 5, 10, 13, 16, 26, and 30), CA125 did not increase above the required 100%/25% threshold even when HGSC recurrence was diagnosed via other methods (Supplementary Table S1). LTBP1 levels rose in 7 of those 8 patients between the end of chemotherapy and cancer recurrence, with a median increase of 208% (range 58−456%). This indicates that combining CA125 and LTBP1 to monitor HGSC recurrence might be beneficial. However, more patients need to be studied in the future to determine LTBP1's threshold for the detection of HGSC recurrence.

3.4. Performance of CA125 (ELISA) and LTBP1 (PRM) during Disease Progression

Since LTBP1 best reflected the patients' tumor load, we further analyzed its performance during disease progression and compared it with CA125. Both LTBP1 and CA125 mirrored tumor load during disease progression in platinum-sensitive (Figure 5A) and platinum-resistant patients (Figure 5B), with lower levels after cytoreductive surgery and further reduction postchemotherapy.

Some patients showed an increase in CA125 or LTBP1 after surgery, most likely as a direct result of the invasive procedure. Inflammation of the inner pelvic or abdominal cavities is associated with elevated CA125 levels[6] and LTBP1 is involved in wound healing via regulation of the cytokine TGFβ.[20] Samples with an increase in CA125 and/or LTBP1 compared to

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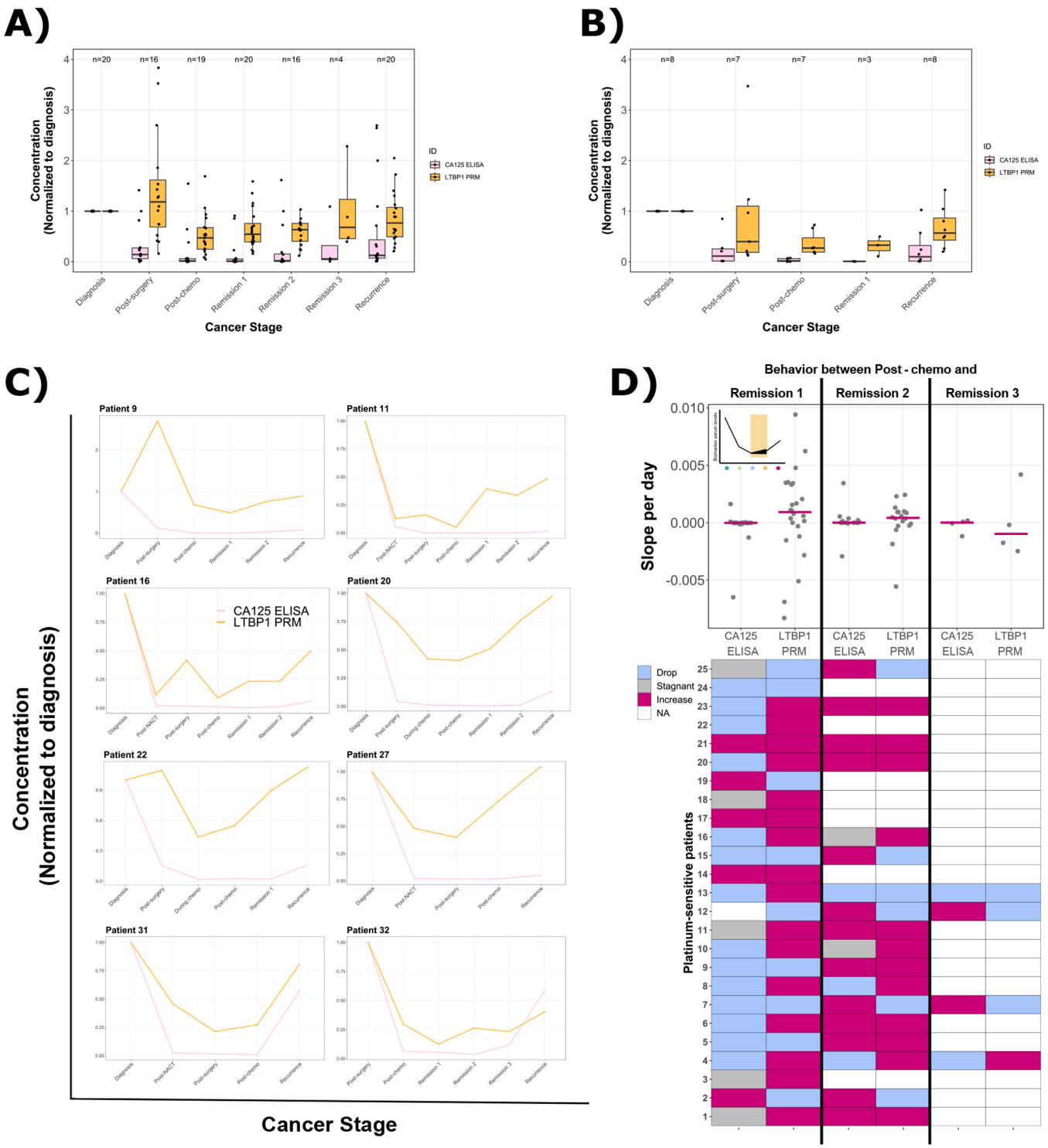

Figure 5. Performance of CA125 (ELISA) and LTBP1 (PRM) during disease progression. (A) Longitudinal profiles of CA125 and LTBP1 in platinum-sensitive patients (n = 20). Only patients for which both CA125 ELISA and LTBP1 PRM levels at diagnosis were available are depicted. (B) Longitudinal profiles of CA125 and LTBP1 in platinum-resistant patients (n = 8). One patient without a diagnosis sample was excluded. (C) Longitudinal profiles of CA125 and LTBP1 in individual patients. Patients 9−22 were platinum-sensitive, and patients 27−32 were platinum-resistant. (D) Changes in CA125 and LTBP1 serum levels of platinum-sensitive patients during disease remission, leading up to cancer recurrence.

diagnosis were on average collected closer to the surgery date than samples with a postsurgery biomarker drop (23.2 vs 27.0 days).

In general, CA125 levels dropped more drastically than LTBP1 as a result of cancer treatment (mainly surgery) but then stayed relatively stagnant until disease recurrence, where a minor increase in serum concentration was observed (Figure 5A/B). LTBP1, in contrast, dropped less substantially after treatment but showed a more pronounced increase between the end of chemotherapy and HGSC recurrence. Additionally, LTBP1 levels rose already during disease remission, as could be seen in the longitudinal profiles of multiple patients (Figure 5C). In a

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J. Proteome Res. 2024, 23, 749−759

few platinum-resistant patients, LTBP1 increased even before the end of chemotherapy which hints that LTBP1 might reflect the beginning of chemotherapy resistance in some patients.

A closer examination of the markers' performance during remission (Figure 5D) revealed that LTBP1 rose in 16/25 patients (median slope all patients = 7.81×10^{-4} Units/day) directly after the end of chemotherapy completion, while CA125 levels mostly dropped or stagnated (median slope = -1.48×10^{-5} Units/day). This early rise of LTBP1 was also observed in five out of the six platinum-sensitive patients that did not show a CA125 increase above the established thresholds at recurrence, indicating that LTBP1 might enable early detection of recurrence even in patients that do not respond with pronounced CA125 serum level increases. Additionally, LTBP1 rose earlier than CA125 in 11 out of 25 patients, with an average lead time of 116.4 days in all 25 patients (Table 2).

Table 2. Time Interval between the End of Treatment and the First Biomarker Increases before Recurrence in Platinum-Sensitive Patients

	time of first increase after treatment end [days]		
patient	LTBP1	CA125	lead time LTBP1 vs CA125 [days]
1	118	118	0
2	201	102	−99
3	71	409	338
4	98	385	287
5	210	231	21 (due to differences in sample draw dates)
6	120	357	237
7	325	227	−98
8	52	451	399
9	571	571	0
10	433	534	101
11	204	288	84
12	238	238	0
13	799	1155	356
14	182	182	0
15	413	315	−98
16	241	241	0
17	406	444	38 (due to differences in sample draw dates)
18	84	419	335
19	427	301	−126
20	131	386	255
21	131	131	0
22	78	443	365
23	126	525	399
24	never rises	105	NA
25	560	560	0
average	**259.1**	**375.5**	**116.4**

However, the intervals in which serum was sampled during remission were quite large in some patients (Supplementary Table S1) which might have artificially increased the determined lead time. Nonetheless, LTBP1 rose mostly in the first sample taken during remission (15 of 25 patients), while CA125 increased only in the second sample (13 of 25 patients). Thus, using LTBP1 as a surveillance biomarker might improve the early detection of HGSC recurrence.

4. DISCUSSION

In this study, we aimed to find biomarkers for monitoring HGSC recurrence. For this purpose, we investigated the serum levels of the established HGSC surveillance biomarker CA125 and four novel HGSC biomarker candidates proposed by Sinha et al.[17] in 212 longitudinal serum samples from 34 patients.

All biomarker candidates (FGL2, LGALS3BP, LTBP1, and TIMP1) mirrored HGSC progression, with LTBP1 showing the most pronounced increase at cancer recurrence. LTBP1, Latent Transforming Growth Factor Beta (TGFβ) Binding Protein 1, is a secreted and extracellular matrix (ECM) protein that targets latent TGFβ to the ECM and releases its active form upon enzymatic cleavage.[21] TGFβ is known to inhibit the proliferation of early-stage ovarian carcinoma while conversely promoting proliferation in late-stage ovarian cancer.[22] TGFβ as well as LTBP1 have been shown to be overexpressed in ovarian carcinoma cells.[23] These findings suggest a biological role of LTBP1 in ovarian cancer, which further supports its suitability as a marker for HGSC. LTBP1 has also been implicated in other cancers, including breast,[24] hepatocellular carcinoma,[25] and esophageal squamous cell carcinoma.[26] Thus, its specificity for detecting HGSC versus other malignancies should be investigated before considering it as a potential diagnostic biomarker.

This study, however, aimed to explore surveillance biomarkers for HGSC, and LTBP1 levels mirrored the tumor load in most patients. The most promising finding was that LTBP1 rose earlier during remission than did the approved HGSC surveillance biomarker CA125, with an average lead time of 116.4 days. This indicates that LTBP1 anticipates HGSC recurrence earlier than CA125, making it an ideal biomarker candidate.

A combination of LTBP1 with CA125 could be very useful for HGSC surveillance. Adding biomarkers to CA125 can improve sensitivity and especially selectivity for detecting primary and recurrent ovarian cancer, as has been shown for human epididymis protein 4 (HE4).[27−29] Our study shows that LTBP1 is a promising marker for early detection of recurrent HGSC and thus a good candidate for future studies investigating HGSC biomarker panels.

LTBP1 levels decreased in 6 out of 34 patients after a previous increase during HGSC remission but with a general upward trend toward recurrence (patients 4, 11, 12, 15, 21, 32, Supplementary Figures S4−S5). Additionally, 8 patients showed a decrease in LTBP1 levels at recurrence after a previous increase during remission (patients 1, 4, 8, 10, 14, 17, 18, 23, Supplementary Figure S4). However, all of these patients still had higher levels of recurrence than at nadir. CA125 ELISA levels showed more stable trends during remission, but this mostly meant unchanging, very low levels (median of 10 U/ml). LTBP1, in contrast, appears to be more sensitive, but the current PRM assay demonstrated fluctuations in signal that warrant future studies exploring LTBP1's thresholds for HGSC recurrence. Further development of the LTBP1 PRM assay involving antibody-based enrichment might increase the sensitivity and precision. Additionally, the inclusion of more LTBP1-derived peptides into the PRM method might further improve the assay's performance, although this would require switching from a *N*-glycoproteomics approach to a more generic enrichment approach (i.e., antibody capture). Alternatively, the assay could be completely shifted to an immunoassay like ELISA, however, cross-reactions and nonspecific binding of

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J. Proteome Res. 2024, 23, 749−759

antibodies without subsequent target identification by MS might impact the specificity of quantitation.

In summary, we evaluated the four novel HGSC surveillance biomarker candidates FGL2, LGALS3BP, LTBP1, and TIMP1, that were derived from a previous study of longitudinal serum samples (20 patients and 96 samples).[17] Our study of 212 longitudinal samples from 34 patients revealed that all markers reflected the tumor load of multiple patients with LTBP1 mirroring disease progression in most HGSC patients. Additionally, LTBP1 rose earlier prior to cancer recurrence than the established surveillance biomarker CA125. Thus, LTBP1 is a promising novel biomarker for monitoring HGSC progression and recurrence.

■ ASSOCIATED CONTENT

Data Availability Statement

The raw MS data associated with this manuscript was submitted to the Mass Spectrometry Interactive Virtual Environment and is available under the ID MSV000092904.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.3c00629.

Supplementary Figures S1-S5: Tested liquid chromatography (LC) gradients; change of LC gradient to reduce FGL2 carry-over between runs; interfering signals at low concentrations of LTBP1 and FGL2; longitudinal profiles of platinum-sensitive patients; and longitudinal profiles of platinum-resistant patients (PDF)

Supplementary Table S1: Cohort overview (XLSX)

Supplementary Table S2: PRM quantification results (XLSX)

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Author Contributions

Conceived and designed the experiments: D.W., T.K.; Performed the experiments: D.W., S.K.; Analyzed the data: D.W., V.I.; Interpreted the data: D.W., T.K.; Contributed reagents/materials/analysis tools: T.K., M.B., T.M.; Wrote the paper: D.W., T.K., M.B.

Notes

The authors declare no competing financial interest.

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