analytical chemistry

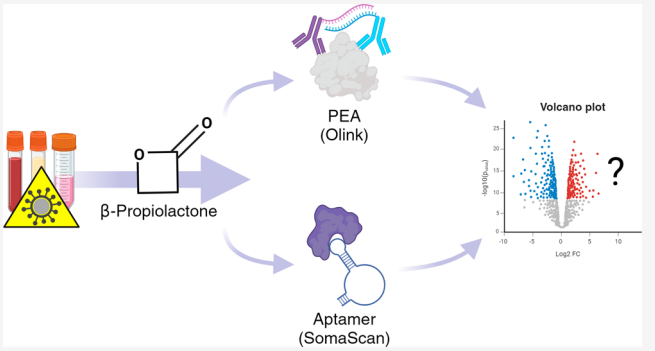
pubs.acs.org/ac
Article

Sample-Treatment with the Virucidal β-Propiolactone Does Not Preclude Analysis by Large Panel Affinity Proteomics, Including the Discovery of Biomarker Candidates

Vanessa M. Beutgen, Aditya M. Bhagwat, Anna Mary Steitz, Silke Reinartz, Rolf Müller, and Johannes Graumann*

Cite This: Anal. Chem. 2024, 96, 9332−9342
Read Online

ABSTRACT: Virus inactivation is a prerequisite for safe handling of high-risk infectious samples. β-Propiolactone (BPL) is an established reagent with proven virucidal efficacy. BPL primarily reacts with DNA, RNA, and amino acids. The latter may modify antigenic protein epitopes interfering with binding properties of affinity reagents such as antibodies and aptamers used in affinity proteomic screens. We investigated (i) the impact of BPL treatment on the analysis of protein levels in plasma samples using the aptamer-based affinity proteomic platform SomaScan and (ii) effects on protein detection in conditioned medium samples using the proximity extension assay-based Olink Target platform. In the former setup, BPL-treated and native plasma samples from patients with ovarian cancer (n = 12) and benign diseases (n = 12) were analyzed using the SomaScan platform. In the latter, conditioned media samples collected from cultured T cells with (n = 3) or without (n = 3) anti-CD3 antibody stimulation were analyzed using the Olink Target platform. BPL-related changes in protein detection were evaluated comparing native and BPL-treated states, simulating virus inactivation, and impact on measurable group differences was assessed. While approximately one-third of SomaScan measurements were significantly changed by the BPL treatment, a majority of antigen/aptamer interactions remained unaffected. Interaction effects of BPL treatment and disease state, potentially altering detectability of group differences, were observable for less than one percent of targets (0.6%). BPL effects on protein detection with Olink Target were also limited, affecting 3.6% of detected proteins with no observable interaction effects. Thus, effects of BPL treatment only moderately interfere with affinity proteomic detectability of differential protein expression between different experimental groups. Overall, the results prove high-throughput affinity proteomics well suited for the analysis of high-risk samples inactivated using BPL.



INTRODUCTION

The investigation of viral diseases with the capacity to cause global pandemics or severe local outbreaks is a major challenge for medical research. Newly emerging OMICs technologies are well suited to gain deeper understanding of the associated pathophysiology and may open new treatment avenues. Blood and its derivatives provide highly informative samples that are easily accessed and subjected to comprehensive analysis. Research on high-risk infectious samples, however, poses a significant hazard to scientific personnel, and samples from patients with particularly hazardous virus disease may only be handled in BSL-4 laboratories. Safe sample processing outside of high biosafety laboratories requires prior effective inactivation of virus activity by virucidal agents such as β-propiolactone (BPL). BPL-treated samples are considered noninfectious, and efficacy was reported for a broad range of viruses.[1] Seeking an agent with virucidal efficacy paralleled by minimal effects on other blood components, Gerald LoGrippo in 1960 selected BPL from a collection of 23 virucides. BPL is unstable in aqueous solutions, as it gradually hydrolyses. Its half-life is highly temperature dependent with 3−4 h at room temperature, yielding the degradation products β-propionic acid and hydracrylic acid derivates.[1,2]

Large-scale affinity proteomic platforms are increasingly used in clinical research, as they imply beneficial characteristics in particular for the analysis of blood samples, as compared to more classical mass spectrometry-based proteomic methodology. Such analyses may provide new insight into pathophysi-

Received: September 13, 2023
Revised: May 7, 2024
Accepted: May 7, 2024
Published: May 29, 2024

https://doi.org/10.1021/acs.analchem.3c04116
Anal. Chem. 2024, 96, 9332−9342

ology and advance biomarker research. Being able to analyze the blood proteome in high-risk infectious diseases in safe virus-inactivated samples may thus provide new impulses for diagnosis and treatment of live-threatening viral disease. So far, studies investigating the effect of BPL on downstream assays have come to diverging conclusions. Ball et al. tested the effects of BPL on immunological analysis of various blood components of common interest in the context of HIV infection.[3] They tested for G, A, and M class immunoglobulins, complement components C3 and C4, haptoglobin, α1-antitrypsin, C-reactive protein, and others using a Beckman Immunochemistry Analysis System. Measured plasma concentrations of these proteins did not change significantly following BPL treatment. They further tested for concentrations of various autoantibodies and did not detect clinically relevant alterations either. Similar conclusions were derived from a second study that investigated the effect of BPL on antibody measurements by ELISA.[4] Here, serum levels of antibodies against tetanus toxin, diphtheria toxin, *Haemophilus influenzae* type b capsular polysaccharide, and pneumococcal polysaccharide in BPL-treated and untreated samples from the same subjects were analyzed. Antibody concentrations in treated and untreated samples showed high correlation, leading to the conclusion that virus inactivation by BPL does not interfere with accuracy of measurements in ELISA, at least not for the concentrations tested. These findings, however, appear not generally reproducible or generalizable to all proteins. Although α1-antitrypsin (AAT) levels were previously reported to be unchanged by BPL treatment, this result was not reproduced in other work[5] investigating the impact of sample pH, incubation time, and temperature on apparent protein abundance. A decrease in measured AAT abundance was observed to correlate with pH, time, and BPL concentration. Samples analyzed were treated with relatively high BPL concentrations and not buffered under all conditions tested. AAT levels were further found to continuously decrease with storage time following BPL incubation.

Taking together the findings of these studies, it may be hypothesized that low levels of BPL in a buffered sample do not influence detectability of a majority of blood proteins when storage time between treatment and measurement remains limited. BPL concentrations commonly used in virus inactivation protocols range from 0.025% to 1%.[6] Optimal concentrations depend on the target pathogen and must be determined experimentally.[7] Lower concentrations may generally be desirable to reduce unwanted side effects, e.g., on proteins. More recently, the effects of BPL on different nucleobase analogues and amino acid residues were investigated systematically, and a plethora of BPL-related protein modifications were reported,[6] inviting speculation on the mechanisms of the observed impact of BPL on affinity/epitope interaction.[8]

With the appearance of next-generation plasma and serum proteomics in biomarker discovery studies, a need arises for the investigation of the impact of BPL treatment on blood-derived and other sample types in large-scale, high-throughput affinity proteomic analysis, as provided by the SomaScan and the proximity extension assay (PEA)-based Olink platform. To validate the use of such technologies on BPL-treated samples, two main questions must be answered:[1] Does the treatment affect the measurements obtained by the platform, and[2] if so, to what extent does it impact the detection of differential protein expression between study groups? We set out to answer both questions and evaluated BPL-treatment compatibility with such platforms by analyzing the effect of low-concentration, bicarbonate-buffered BPL treatment on 50% diluted plasma samples of patients with benign gynecological diseases (n = 12) or ovarian cancer (n = 12) with respect to protein detectability in the aptamer-based proteomic platform SomaScan. We further analyzed effects on protein detectability in conditioned medium (CM) samples from cultured ascite-derived T cells with (n = 3) or without (n = 3) anti-CD3 antibody stimulation in the PEA-based Olink Target platform. The presented results demonstrate the feasibility of affinity proteomic analyses for differential protein expression analysis in samples treated with the virucidal BPL, including samples derived from subjects with infectious disease.

MATERIALS AND METHODS

Plasma Samples. Heparin-plasma samples were collected from patients with high-grade ovarian carcinoma (n = 12) and patients with noncancerous (benign) tumors (n = 12). Samples were collected according to the guidelines of the Declaration of Helsinki with the informed consent of the patients and approval by the ethics committee of Marburg University (205/10). All patients agreed in writing to the publication of pseudonymized data derived from clinical material. Plasma was diluted 1:2 with PBS prior to storage at −80 °C.

Conditioned Medium Samples. Conditioned medium (CM) samples (n = 3 biological replicates) from a recently published study were reanalyzed to identify BPL-related effects on protein detection and quantification using the Olink Target platform.[9] Briefly, T cells from ascites of three patients were cultured with or without antibody-mediated stimulation of CD3 activation and supernatants were collected after 2 days. For details regarding culturing conditions, refer to ref 9.

β-Propiolactone (BPL) Treatment. Prior to analysis using the SomaScan or Olink Target platforms, plasma and CM samples were buffered by adding sodium hydrogen carbonate ($NaHCO_3$) to a final concentration of 45 mM and one sample per subject was either mock or BPL treated. To that end, BPL stock (98% BPL solution, 13.6 mol/L) solution was freshly diluted with dH_2O to yield a 50% working solution, which was added to the samples resulting in a final concentration of 0.05% BPL. BPL and mock (dH_2O) treated samples were incubated for 72 h at 4 °C.

SomaScan Assay. All plasma samples were analyzed on the SomaScan v4.1 platform by SomaLogic Inc. (Boulder, CO, USA). SomaScan is an aptamer-based affinity proteomics platform that analyzes approximately 7,000 proteins in small sample volumes simultaneously.[10] The platform provides semiquantitative protein readouts reported as *Relative Fluorescence Units* (RFU). Features receiving a quality flag after data normalization by SomaLogic were included in downstream analyses probing possible effects of BPL treatment.

Olink Target. The CM samples were analyzed on the Olink Target Inflammation panel at the Fraunhofer-Institut für Translationale Medizin and Pharmakologie ITMP (Frankfurt a.M, Germany). Olink Target uses the proximity extension assay (PEA) technology, a dual-antibody-based assay format, recognizing different epitopes on the target protein, thus increasing specificity.[11] Each antibody-pair is covalently linked to a complementary single-stranded DNA probe, which hybridizes with its counterpart upon reaching close proximity. The resulting sample and assay specific DNA barcodes are further amplified, followed by quantification using qPCR.

https://doi.org/10.1021/acs.analchem.3c04116
Anal. Chem. 2024, 96, 9332−9342

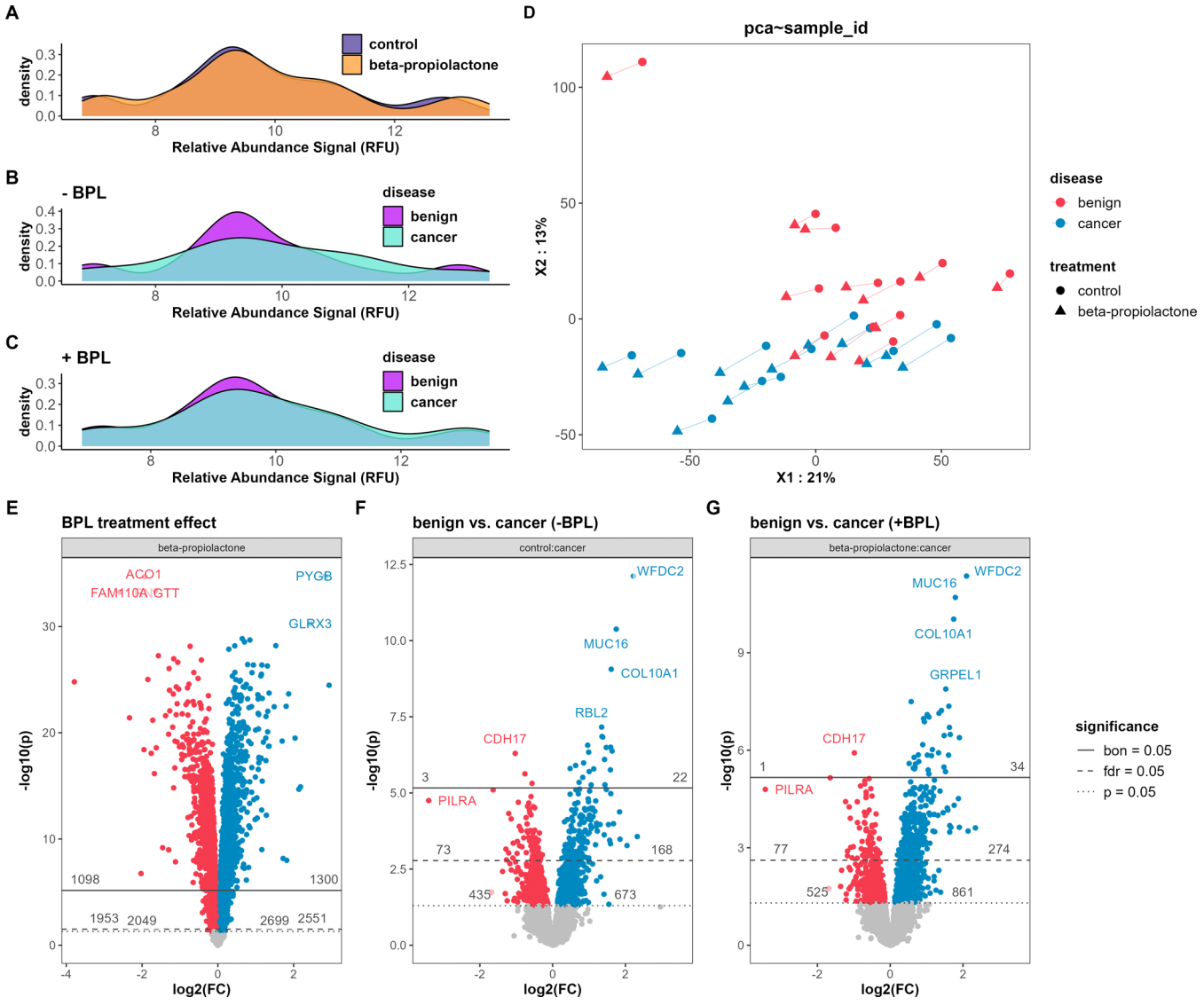


Figure 1. (A–C) Feature density plots showing the distribution of measured protein RFUs. (A) Comparison of RFU distribution in BPL-treated (orange) and untreated (blue) samples. (B, C) Comparison of RFU distribution in cancer (turquoise) and benign (purple) samples, without (B) and with (C) BPL treatment. (D) Principal Component analysis. PCA with coloring based on disease state (benign = red, cancer = blue); shape indicates treatment (circle = untreated, triangle = BPL-treated); samples from the same subjects are connected with a line. (E–G) Volcano plots of proteins with differential plasma abundance. (E) Volcano plot showing differences in SomaScan measurements between BPL treated and untreated samples, assessed by limma. Plotted are 1098 proteins with lower measurements (red) and 1300 proteins with higher measurements (blue) after BPL treatment (bon. adj. $p < 0.05$). (F) Volcano plot showing differentially abundant proteins in cancer measured without BPL treatment. Three proteins are downregulated (red), while 22 proteins are upregulated (blue). (G) Differentially abundant proteins in benign vs cancer measured after BPL sample treatment. One protein is significantly downregulated (red) and 34 proteins are upregulated (blue) in cancer (bon. adj. $p < 0.05$).

Relative protein abundance is reported as *Normalized Protein eXpression* (NPX) values.

Amino Acid Composition. Calculation of relative amino acid frequencies was carried out for the top 100 proteins with significantly (Bonferroni corrected $p < 0.05$) altered values on the SomaScan platform, resulting from BPL treatment. Human proteome information for SomaScan v4.1 targets was downloaded in FASTA format from UniProt (13.10.2022). Swissprot-reviewed entries and canonical sequences (no isoforms) were considered exclusively, resulting in 6,388 relevant protein sequences. The retrieved sequence data was used to calculate reference values for expected amino acid frequencies normalized to 1 and compared to the observed frequencies of the top 100 proteins significantly affected by BPL.

Survival Associations. Overall survival data were retrieved from the PRECOG database[12] at https://precog.stanford.edu.

Statistical Analysis. SomaScan and Olink data was analyzed in R (v4.2.3) and RStudio (v2023.03.1) using the "autonomics" package version 1.11 (Bhagwat A, Cotton R, Hayat S, Graumann J (2024). autonomics: Unified statistical Modeling of Omics Data. DOI: 10.18129/B9.bioc.autonomics). During data import, the "autonomics" package initially performs a $\log_2$ transformation of the RFU values. All RFU presented are $\log_2$ transformed.

RESULTS

SomaScan Assay. Overall, only minor reductions in signal intensity were found following BPL treatment, indicating that

https://doi.org/10.1021/acs.analchem.3c04116
Anal. Chem. 2024, 96, 9332−9342

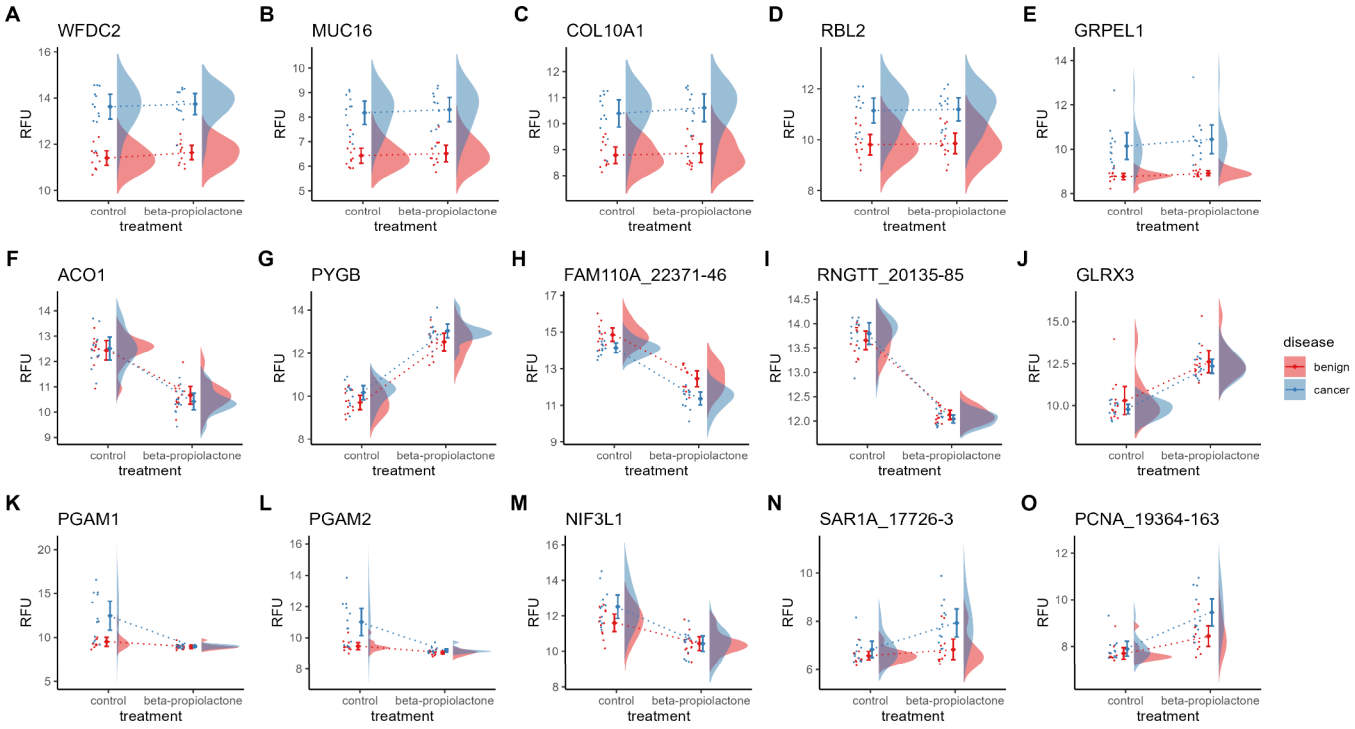


Figure 2. (A–E) Raincloud plots for protein features with most significant differences between benign and cancer samples. (F–J) Raincloud plots of proteins showing the highest diversion of RFUs upon BPL treatment. (K–O) Raincloud plots of proteins showing the highest interaction effects between cancer and BPL treatment.

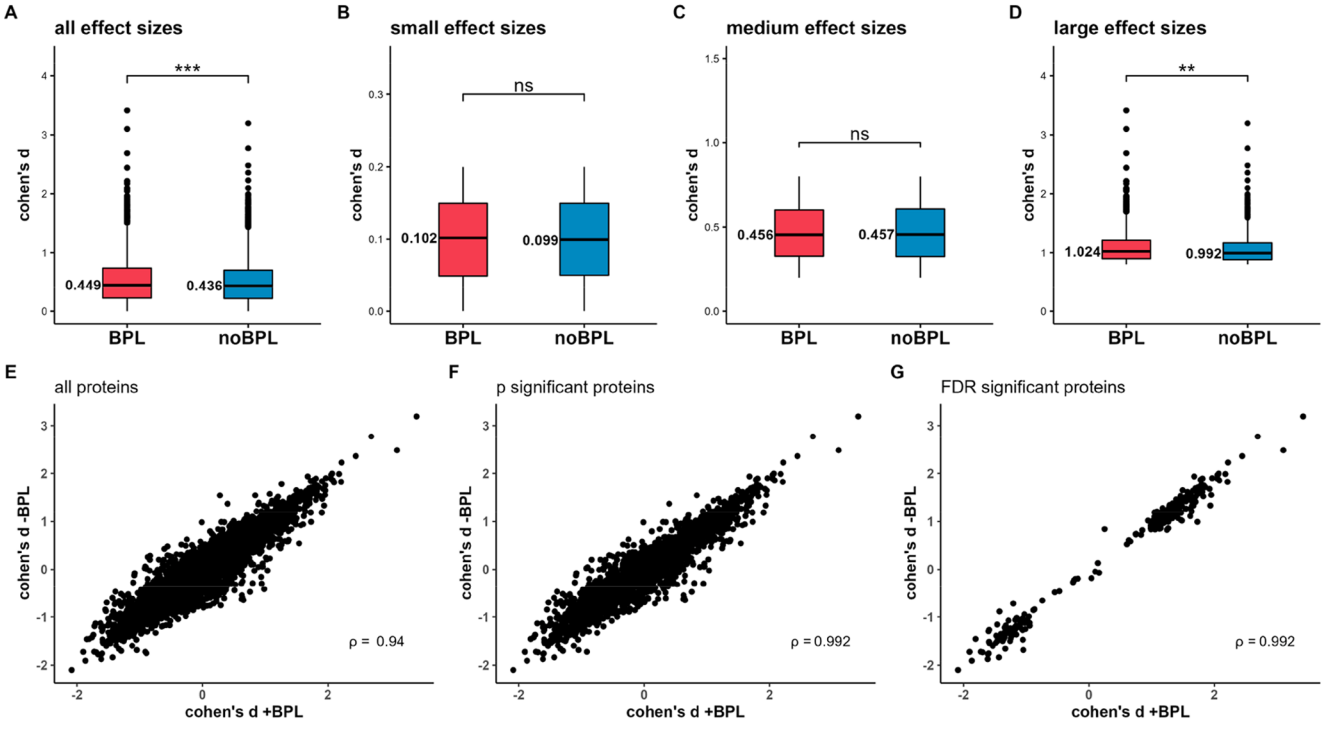


Figure 3. Analysis of changes in Cohen's *d* effect sizes between SomaScan protein targets upon BPL treatment. (A) Cohen's *d* over all protein features. Comparison of effect sizes between benign and ovarian cancer samples for features showing small effect sizes ($d < 0.2$) (B), medium effect sizes ($d > 0.2$ and $d < 0.8$) (C), and large effect sizes ($d > 0.8$) (D). (E–G) Correlation of Cohen's *d* effect sizes between cancer and benign sample protein level means with and without BPL treatment. Pearson correlation of all (E, $\rho = 0.94$), *p* significant (F, $\rho = 0.98$), and FDR significant (G, $\rho = 0.98$) proteins.

global protein detectability using SomaScan remains unaffected (Figure 1A). The effect was lower than what was observed for protein abundance differences between cancer and benign samples in each condition (Figure 1B,C). This was also

https://doi.org/10.1021/acs.analchem.3c04116
Anal. Chem. 2024, 96, 9332–9342

reflected in principal component analysis, where an unidirectional shift was observed upon BPL treatment (Figure 1D). However, separation between benign and cancer samples was preserved, as protein abundances shifted to the same extent for both groups and relative levels appeared to remain unaltered.

Differential expression analysis using Bayesian moderated *t* testing as implemented by limma,[13] supported these assumptions (Figure 1E−G). This analysis revealed that 2398 of ∼7000 (34%) targets showed significantly (Bonferroni corrected $p < 0.05$) different measurements after BPL treatment independent of disease state (Figure 1E). Of these, 1098 proteins were measured with reduced abundance, while 1300 were measured at higher abundance, when comparing BPL treatment to mock controls. The proteins most affected by BPL treatment were ACO1 (SomaScan SeqId: 20054-289, PYGB (SomaScan SeqId: 24414-3), FAM110A (SomaScan SeqId: 22371-46), RNGTT (SomaScan SeqId: 20135-85), and GLRX3 (SomaScan SeqId: 16596-25). Figure 1F,G shows the differential expression of proteins in samples from subjects with benign gynecological diseases versus patients with ovarian cancer without (Figure 1F) and with BPL (Figure 1G) treatment, respectively. As apparent from the volcano plots, the same three proteins (WFDC2, MUC16, and COL10A1) emerge to strongly differentiate ovarian cancer from benign plasma samples irrespective of BPL treatment.

From the RFU distribution of the most BPL treatment-affected proteins in differential expression analysis, it is apparent that, despite significant altered measurements, samples from patients with benign gynecological diseases and samples from ovarian cancer patients are affected to a comparable extent and the relative ratio between benign and cancer samples was maintained, preserving detectability of group differences. This is exemplified in Figure 2A−E for the five proteins with most significantly changed abundance between ovarian cancer and benign samples in our data set, as detected by differential expression analysis. Figure 2F−J shows the proteins for which the assays were most affected by BPL treatment. ACO1, FAM110A, and RNGTT were measured with significantly lower abundance following BPL treatment, while PYGB and GLRX3 were measured with higher abundance. The ratios between protein levels in cancer versus benign samples, however, did not change significantly. On the other hand, a small number of protein targets presented with significantly changed ratios, exemplified by the five strongest affected targets in Figure 2K−O.

To understand how BPL treatment impacts the effect size between measured protein abundances in benign versus cancer samples, both with and without BPL treatment globally, we calculated Cohen's *d*[14] for all protein features. Figure 3A represents the corresponding graphical analysis for all effect sizes. The median effect size between protein abundances in benign vs cancer samples in BPL treated samples was $d = 0.449$ and $d = 0.436$ in untreated control comparisons. The resulting effect size difference $\Delta d = 0.013$ appeared significant ($p < 0.05$). Breaking this down to small ($d < 0.2$), medium ($0.2 < d < 0.8$), and large ($d > 0.8$) effect sizes, it became apparent that primarily large effect sizes were impacted by the BPL-mediated increase (Figure 3B−D). Globally, the benign vs cancer effect sizes for every assayed protein, both with and without BPL treatment, showed an excellent correlation ($\rho > 0.9$) (Figure 3E−G).

As sample- and assay-specific effects are lost during calculation of Cohen's *d*, we were also interested in individual interaction effects and to that end investigated the interaction coefficients of a two-factor (treatment*disease) limma-driven linear model. Figure 4 shows a contrastogram providing an

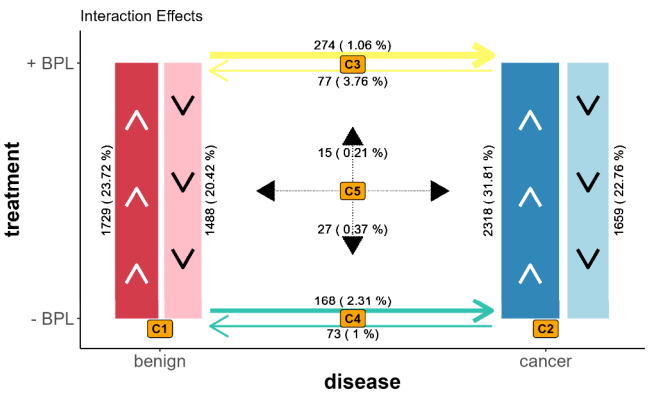


Figure 4. Contrastogram. Graph shows numbers of significantly altered protein levels in different contrasts of two-factor limma-based analysis (FDR < 0.05). Line thickness is proportional to number of proteins identified as differentially expressed. Arrow direction indicates the condition with higher expression. Red (C1: ∼disease/treatment; benign: BPL). Number of altered protein level measurements upon BPL treatment in benign samples only. Levels of 1729 proteins were measured to increase and 1488, decrease. Blue (C2: ∼disease/treatment; cancer: BPL). Numbers of altered protein level measurements upon BPL treatment in cancer samples only. Levels of 2318 proteins were measured to increase and 1659, decrease. Yellow (C3: ∼treatment/disease; BPL: cancer). Numbers of altered protein levels between benign and cancer samples in BPL-treated samples. Here, 274 proteins were measured with higher levels and 77, with lower levels. Green (C4: ∼treatment/disease; no BPL: cancer). Numbers of altered protein levels between benign and cancer samples in untreated samples. Here, 168 proteins were measured with higher levels and 73 proteins, with lower levels. Black (C5: ∼treatment*disease; BPL: cancer). Interaction effects of BPL treatment and disease state on measured protein levels. Fifteen proteins show increased levels while 27 proteins show lower levels.

overview of significantly changed protein levels (FDR < 0.05) in the contrasts investigated. In Figure 4, C1 and C2 show the differences in measured protein abundance within either benign (C1) or cancer (C2) samples, representing the overall effect of BPL on the assay. A large percentage of proteins show altered measurements (44.14% and 54.57%), indicating that BPL changes the measurements of protein expression substantially. The impact on detection of differential protein expression between benign and cancer samples in BPL-treated and -untreated samples is indicated by C3 and C4, respectively. As a general trend, the number of altered proteins between cancer and control plasma samples increased upon BPL treatment. In the untreated samples, abundance of 73 proteins appeared decreased and of 168 proteins increased in cancer samples (Figure 4, C4, green arrows), while the numbers increased in BPL-treated samples to 77 and 274 (Figure 4, C3, yellow arrows), respectively. A total number of 42 (0.6%) assays showed significant interaction effects (C5; 27 positive, 15 negative) (Figure Supporting Information File 2). The observed interaction effects ranged from −2.897 to 1.15 with a mean of 0.015.

We investigated possible reasons for the enhanced and decreased signal levels observed upon BPL treatment. To that

https://doi.org/10.1021/acs.analchem.3c04116
Anal. Chem. 2024, 96, 9332−9342

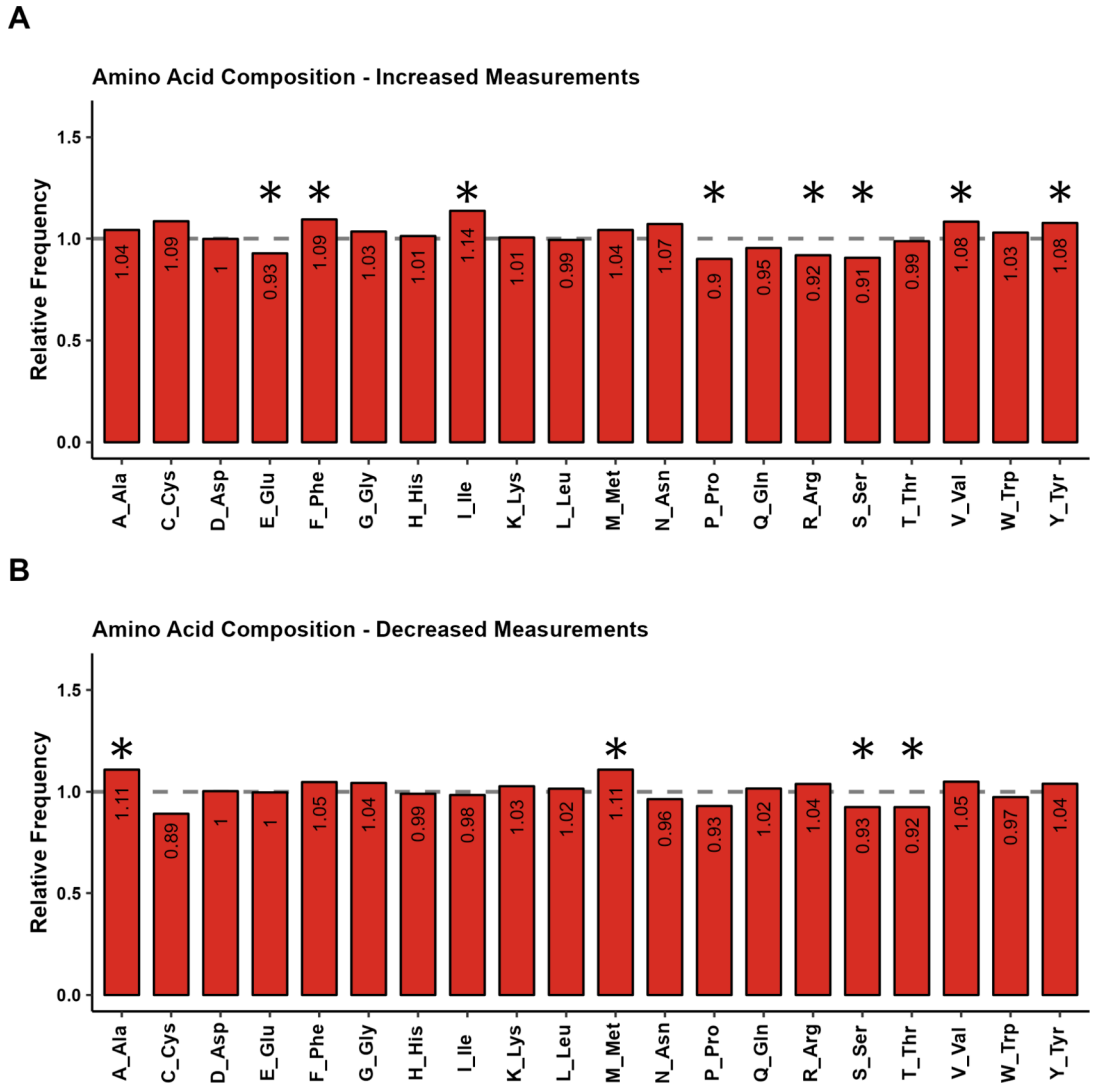


Figure 5. Amino acid compositions of proteins with increased RFU values (A) and decreased RFU values (B) after BPL treatment. Amino acid composition shows relative frequencies of each amino acid in the enhanced or impaired assays. Dashed line representing the expected frequency with regard to all proteins in the SomaScan panel normalized to 1. Asterisk indicates statistical significance (one-sample t test, $p < 0.05$) (Supporting Information File 2).

end, and as BPL is known to be most reactive toward specific amino acid residues (especially cysteine, methionine, and histidine),[6] we analyzed whether the amino acid composition of strongly affected proteins reflected this and calculated the relative abundance of every amino acid in the top 100 significantly altered measurements. The relative amino acid frequencies for proteins with increased measurements ranged from 0.9 to 1.14 with significantly (one-sample t test, $p < 0.05$) increased phenylalanine, isoleucine, valine, and tyrosine and decreased glutamic acid, proline, arginine, and serine frequencies. The highest changes were observed for isoleucine and proline with frequencies >10% deviating from expected values (Figure 5A). The relative amino acid frequencies for proteins with decreased abundance measurements ranged between 0.89 and 1.11, with a significant increase of alanine and methionine frequency of 11% and a significant decrease of serine and threonine frequency (Figures 5B and Supporting Information File 2).

Even though the identification of ovarian cancer biomarkers was not a main goal, the present study also revealed a number of novel potential candidates that were not detectable by our previous analysis based on smaller Olink and SomaScan assay panels,[15] notably COL10A1 (Figure 1F,G). Intriguingly, interrogation of the in PRECOG database[12] revealed that COL10A1 gene expression is strongly associated with a short survival of ovarian cancer (z-score of 5.40, corresponding to a p-value of 4.2×10^{-6}), emphasizing the potential relevance of this finding. Our analysis also identified proteins clinically used as plasma biomarkers for ovarian cancer, i.e., WFDC2 and MUC16 (15−19), further validating the SomaScan technology for biomarker discovery.

Olink Target Inflammation Panel. Samples analyzed here replicate an experiment that was part of a recently published study.[9] In brief, the subject of this analysis was the identification of differential protein secretion of cultured T cells isolated from ascites, upon CD3 stimulation. The aim of the analysis was the identification of actors promoting TRAIL expression in NK cells. Protein levels were determined using the PEA-based Olink Explore 3072 platform. A subset of equivalent samples was used here to analyze BPL-related effects on protein detectability by the PEA-based Olink Target 96 Inflammation Panel. With this panel, 92 target proteins

https://doi.org/10.1021/acs.analchem.3c04116
Anal. Chem. 2024, 96, 9332−9342

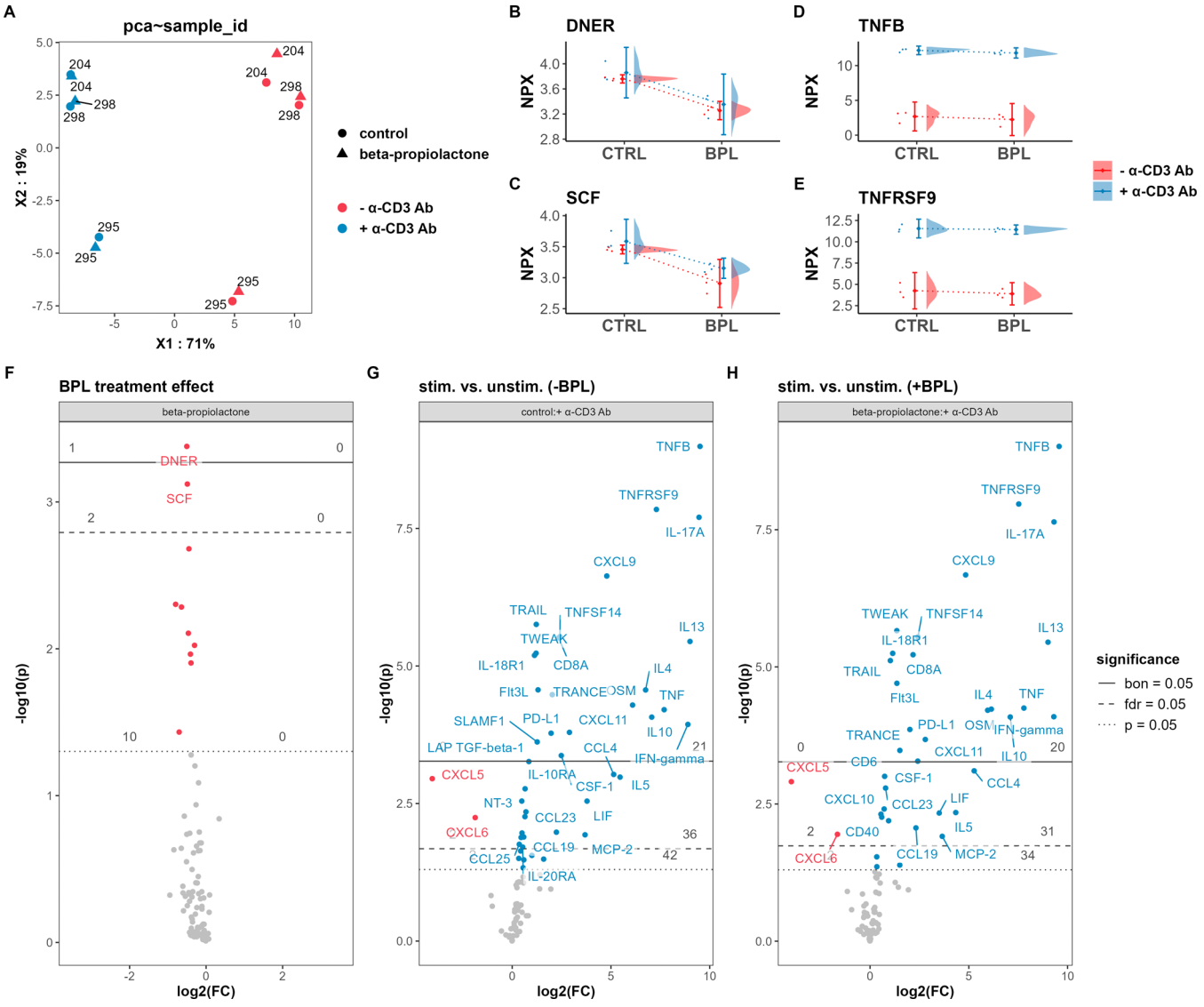


Figure 6. Olink Target data set. (A) Principal component analysis. PCA with coloring showing anti-CD3 Ab stimulation (with α-CD3 Ab = red, without α-CD3 Ab = blue); shape indicates BPL treatment (circle = untreated, triangle = BPL-treated); samples are indicted by IDs. (B, C) Raincloud plots for protein features with significant differences between BPL-treated and untreated samples. (D, E) Raincloud plots for protein features with most significant differences between α-CD3 Ab-stimulated and unstimulated samples. (F–H) Volcano plots of proteins with differential abundance in CM. (F) Volcano plot showing differences in Olink Target measurements between BPL-treated and untreated samples, assessed by limma. Two proteins (DNER, SCF) were measured at lower levels after BPL treatment (FDR < 0.05). (G) Volcano plot of distribution of differentially abundant proteins in CM of α-CD3 Ab-stimulated samples, measured without BPL treatment. (H) Differentially abundant proteins in CM of α-CD3 Ab-stimulated samples measured after BPL sample treatment.

involved in immune response and inflammation pathways are analyzed per sample. Of these, 61% percent were detected in >75% of CM samples. PCA revealed a strong separation of samples, with 71% of the variation of component one being apparently attributed to the presence or absence of anti-CD3 Ab stimulation prior to analysis. Small effects based on the BPL treatment are observable, but the much larger effect of CD3 activation dominates the data (Figure 6A). Differential expression analysis, comparing all BPL-treated with untreated samples, revealed significantly lower measurement of two proteins, DNER and SCF (Figure 6B,C,F). The effects of BPL did, however, not interfere with the analysis of the experimentally intended comparison of secretory protein expression in anti-CD3 stimulated versus unstimulated cells. Differential expression analysis of secretory proteins from CM samples with or without stimulation undergoing BPL treatment show very similar results as compared to untreated samples (Figure 6G,H). In the BPL untreated samples, 36 proteins were found upregulated and two, downregulated; in the BPL treated samples, 31 were found to be significantly increased upon CD3 stimulation, while two proteins were found to downregulated. Similar to the observations in the SomaScan analysis, differential expression detection of the top differentiating proteins between conditions (TNFB, TNFRSF9 and IL-17A) remains unaffected by BPL treatment.

The impact of BPL treatment on the effect size between observed secretory protein levels in CD3 stimulated T cells in contrast to unstimulated cells was again evaluated by calculation and comparison of Cohen's d[14] (Figure 7). Median effect size between protein levels in CM of CD3 stimulated

https://doi.org/10.1021/acs.analchem.3c04116
Anal. Chem. 2024, 96, 9332−9342

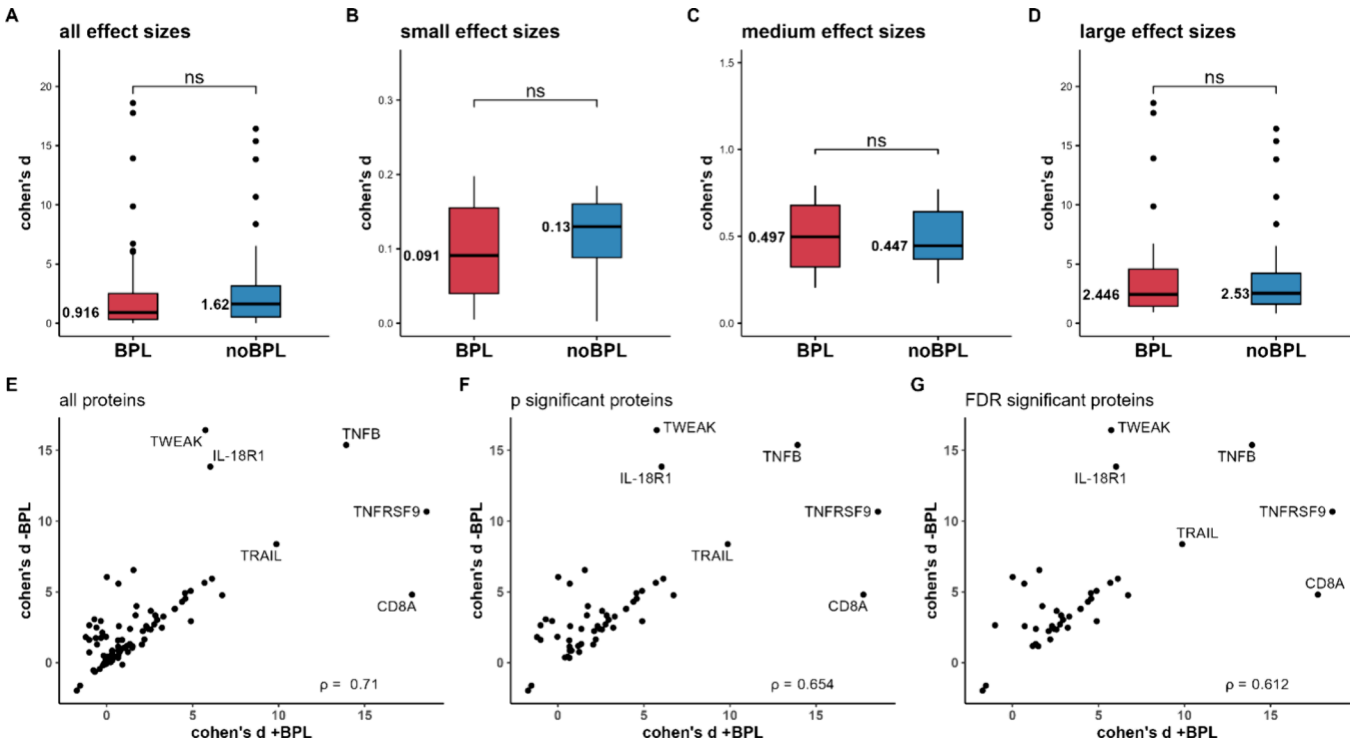


Figure 7. Analysis of changes in Cohen's d effect sizes between Olink Target assays upon BPL treatment. (A) Cohen's d over all protein features. Comparison of effect sizes between α-CD3 Ab-stimulated CM samples and unstimulated samples for features showing small effect sizes ($d < 0.2$) (B), medium effect sizes ($d > 0.2$ and $d < 0.8$) (C), and large effect sizes ($d > 0.8$) (D). (E−G) Correlation of Cohen's d effect sizes between α-CD3 Ab-stimulated CM sample and unstimulated sample protein levels with and without BPL treatment. Pearson correlation of all (E, $\rho = 0.71$), p significant (F, $\rho = 0.654$), and FDR significant (G, $\rho = 0.612$) proteins.

versus unstimulated T cells was $d = 0.916$ in BPL-treated samples and $d = 1.62$ in untreated samples. The difference in effect size $\Delta d = 0.704$ is not statistically significant, and differentiated consideration of effect size categories (small, medium, large) also failed to show significant differences (Figure 7A−D). Overall, the effect sizes for each analyzed protein target in CD3 stimulated vs unstimulated samples show a very good correlation ($\rho = 0.71$), although some outliers are observed, particularly in proteins with high values for Cohen's d (Figure 7E−G). Also, no interaction effects of BPL treatment and CD3 antibody stimulation on measured protein levels were observed with two-factor limma-based analysis (~BPL_treatment*α-CD3 Ab; BPL: +α-CD3 Ab stimulation).

Reanalysis of the Olink Explore data set from the previous study[9] leveraging limma-based differential expression analysis used in our analysis of the Olink Target data revealed significantly increased levels of 25 proteins (FDR < 0.05) (Figure S1). All of these are also included in the Olink Target panel used here and a majority of 22 replicates as significantly differentially expressed. Three proteins found significantly increased in the original data (IL2, FGF5, and IL2RB) were, however, not found significantly increased here, although they show the same trend, at least in the untreated samples (Supporting Information File 2).

DISCUSSION

In this study, two different affinity proteomic platforms were evaluated for their potential to analyze samples that underwent virus-inactivating treatment through BPL. First, plasma samples from ovarian cancer patients were compared to samples from patients with benign gynecological diseases with the primary goal to evaluate detectability of proteins after BPL treatment using the SomaScan platform. Furthermore, performance of the PEA-based Olink Target platform in BPL-treated CM samples was investigated.

Our results show that a substantial proportion of protein assays on the SomaScan platform was significantly impacted by treatment with BPL prior to analysis. Approximately one-third of assays showed altered detection, indicating a major impact of BPL treatment on the analysis (Figures 1E and 4, C1+C2). Despite this obvious effect of the BPL treatment, the crucial aspect for the assessment of suitability of SomaScan assays in its context lies in determining whether it remains possible to analyze differential protein expression, e.g., between different study groups. Our results show that the group differences in protein expression measurement are largely preserved following BPL treatment (Figures 1F,G and 4, C3−C4). The protein assays strongly affected by the treatment showed an equivalent alteration in both treatment groups, indicating that differential protein expression may still be measured even under the influence of BPL (Figure 2F−J). The ratio between protein levels in benign and cancer samples was altered in only a minute number of assays (0.6%), as indicated by significant interaction effects of treatment and disease state in linear modeling (Figures 2K−O and 4, C5). Differential expression measurements for these proteins are compromised by the BPL treatment. A slight increase in significantly changed protein levels between benign and cancer samples was observed upon BPL treatment. When investigating Cohen's d effect sizes of the measured differences between every assay from benign and cancer samples, a minor but significant increase was observed

https://doi.org/10.1021/acs.analchem.3c04116
Anal. Chem. 2024, 96, 9332−9342

in the BPL-treated samples (Figure 3A), which is also apparent in interaction effects observed using linear modeling and further reflected in the differential expression analysis, showing a higher number of significant group differences (Figure 4, C3 vs C4). This observation may indicate a slightly increased chance of false positive hits and thus require further attention in downstream validation studies. On a global scale, the differences in the means of protein levels from benign and cancer samples were maintained upon BPL treatment, as indicated by the high correlation between pre- and post-treatment effect sizes (Figure 3E−G).

As BPL is known to be most reactive with the amino acids cysteine, methionine, and histidine, while essentially non-reactive toward asparagine, glutamine, and tryptophan,[6] we hypothesized that the protein assays most affected by the treatment might target proteins with an amino acid composition reflecting this. The relative amino acid frequencies of proteins with enhanced measurements showed on average a 14% increase of isoleucine and a 10% decrease of proline compared to the expected values. Proteins with decreased measurements showed an 11% higher frequency of alanine and methionine, as well as an 11% decrease of cysteine frequency on average. While isoleucine, proline, and alanine are not known to be reactive to the reagent, BPL causes alkylation and acylation of cysteine and alkylation of methionine.[6] Modifications of these residues as well as conformational changes in the respective proteins potentially caused by such modification may influence epitope availability and consequently the binding affinity of the aptamers used in SomaScan. She et al. reported a correlation of altered protein conformation and folding with the positions of modified amino acid residues.[8] Also, BPL-modified residues may potentially mask SOMAmer-targeted epitopes by steric hindrance.[8] To fully explain BPL induced changes in the measured signal intensity, additional, individual analysis of the respective targets and knowledge of the epitopes the targeting SOMAmers rely on are necessary and beyond the scope of the present study.

BPL's use in virus inactivation is primarily rationalized by its alkylation and acetylation of guanosine and adenosine, as well as induction of DNA cross-linking.[20] The SomaScan technology is based on SOMAmers as protein binders. These molecules are single stranded deoxyoligonucleotides enhanced with protein-like functional groups, mimicking amino acid side chains that are selected from large random libraries using SELEX.[10] SOMAmers are thus potentially modified by BPL, which might alter binding affinity to their target proteins. BPL in aqueous solutions undergoes continuous hydrolysis with a half-life of 16−20 h at 4 °C.[1] The samples used in this study were incubated for 72 h at 4 °C prior to SomaScan analysis, followed by weeks of storage and transport at −20 °C and on dry ice, respectively. As it was previously recommended to allow 4 days for complete hydrolysis of BPL,[1] the presence of residual BPL in the samples cannot entirely be ruled out, yet appears unlikely.

Analysis of a different sample type on another state-of-the-art affinity proteomic platform, Olink Target, revealed similar results regarding reproducibility of differential protein expression analysis following BPL treatment in conditioned cell culture medium. Only two of 92 (~2.2%) detected proteins were found at significantly decreased levels after BPL treatment. Changes to the observed secretory protein profiles were also minor. 36 proteins were measured in significantly higher levels in the untreated CM samples, while 31 proteins showed significantly higher levels in the BPL-treated CM samples. Thus, in contrast to the SomaScan data, a higher probability for false positive hits caused by BPL treatment does not appear to be universal. Comparison of these two platforms regarding this aspect may, however, not be accurate, given the vast differences in the number of analyzed protein targets (92 in Olink Target Inflammation panel vs ~7,000 in SomaScan), and evaluation of BPL effects on protein detection may thus be more comprehensive in the SomaScan data. Either way, results obtained from the Olink panel support our main finding that virus-inactivating BPL treatment does not globally alter group-specific analysis of differential protein expression and extends it with respect to analysis platform and sample type.

Although the focus of this study is not on the differences in plasma protein levels in samples from patients with ovarian cancer and benign gynecological diseases, the results of this group comparison shall be briefly discussed here. The top markers for ovarian cancer in comparison to benign patients identified in our study are WFDC2, MUC16, and COL10A1. The former two, also known as HE4 and CA125, are well recognized plasma biomarkers for ovarian cancer and were evaluated in numerous studies,[15−19] while COL10A1 has not been proposed as an ovarian cancer biomarker candidate. This result is of great potential interest, as the PRECOG data set[12] indicates a highly significant association (z-score = 6.40) of COL10A1 gene expression with a short survival in ovarian cancer, which is consistent with the reported association of COL10A1 with other cancer entities.[21−23]

CONCLUSION

In conclusion, the BPL treatment of different sample types does not appear to significantly and globally impact the detection of differential protein expression using affinity proteomic workflows, such as SomaScan and Olink Target. A small subset of protein targets is, however, significantly affected, showing corresponding interaction effects. Based on the available data, we are unable to derive mechanistic insight into this altered detection behavior. We finally conclude that SomaScan and the PEA-based Olink platform appear to be excellent tools for the proteomic analysis of blood-derived and other samples, even in the context of special handling needs in high-risk biosafety environments.

ASSOCIATED CONTENT

Data Availability Statement

The data analyzed during the current study are available from the corresponding author on reasonable request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.3c04116.

Figure S1: Comparison of Olink Explore data from previous study with Olink Target data from reanalyzed samples; differential expression analysis (PDF)

List of SomaScan assays with significant interaction effects; amino acid frequencies of significantly in-/decreased protein levels (one sample t test) (XLSX)

AUTHOR INFORMATION

Corresponding Author

Johannes Graumann − *Institute of Translational Proteomics, Biochemical/Pharmacological Centre, Philipps-Universität*

https://doi.org/10.1021/acs.analchem.3c04116
Anal. Chem. 2024, 96, 9332−9342

Marburg, 35043 Marburg, Germany; Core Facility Translational Proteomics, Biochemical/Pharmacological Centre, Philipps-Universität Marburg, 35043 Marburg, Germany; orcid.org/0000-0002-3015-5850; Email: johannes.graumann@uni-marburg.de

Authors

Vanessa M. Beutgen − *Institute of Translational Proteomics, Biochemical/Pharmacological Centre, Philipps-Universität Marburg, 35043 Marburg, Germany; Core Facility Translational Proteomics, Biochemical/Pharmacological Centre, Philipps-Universität Marburg, 35043 Marburg, Germany;* orcid.org/0000-0002-3354-2020

Aditya M. Bhagwat − *Institute of Translational Proteomics, Biochemical/Pharmacological Centre, Philipps-Universität Marburg, 35043 Marburg, Germany; Core Facility Translational Proteomics, Biochemical/Pharmacological Centre, Philipps-Universität Marburg, 35043 Marburg, Germany*

Anna Mary Steitz − *Translational Oncology Group, Center for Tumor Biology and Immunology (ZTI), Philipps-Universität Marburg, 35043 Marburg, Germany*

Silke Reinartz − *Translational Oncology Group, Center for Tumor Biology and Immunology (ZTI), Philipps-Universität Marburg, 35043 Marburg, Germany*

Rolf Müller − *Translational Oncology Group, Center for Tumor Biology and Immunology (ZTI), Philipps-Universität Marburg, 35043 Marburg, Germany*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.analchem.3c04116

Author Contributions

J.G., S.R., and R.M. planned and designed the study. J.G., V.M.B., and A.M.S. performed experiments. V.M.B. drafted the manuscript. J.G., R.M., V.M.B., and A.M.B. analyzed and interpreted the data. V.M.B. and A.M.B. performed statistical analysis. A.M.S., S.R., and R.M. provided resources, samples, and clinical data. All authors reviewed and edited the manuscript.

Notes

Samples were collected according to the guidelines of the Declaration of Helsinki with the informed consent of the patients and approval by the ethics committee of Marburg University (205/10). All patients agreed in writing to the publication of pseudonymized data derived from clinical material.

The authors declare the following competing financial interest(s): Sample analysis on the SomaScan platform was provided by SomaLogic Operating Co., Inc. free of charge. The authors affirm the study to have been conducted independently and without undue influence from SomaLogic Co., Inc. The researchers involved maintained full control over the design, data analysis, and interpretation of the results, ensuring scientific rigor. The authors declare no further conflicts of interest.

■ ACKNOWLEDGMENTS

The authors are indebted to Prof. Dr. Stephan Becker, Institute for Virology, Philipps-Universität Marburg (Germany) for sharing the BPL inactivation protocol. The authors thank SomaLogic Operating Co., Inc. (Boulder, Colorado) for, in the context of the study presented here, providing access to the SomaScan platform free of charge. We also like to thank Dr. Nicole Ziegler and her team at Fraunhofer ITMP (Frankfurt a. M.) for the Olink Target analysis.

■ REFERENCES

(1) LoGrippo, G. A. *Ann. N.Y. Acad. Sci.* **1960**, *83*, 578−594.
(2) Hoffman, R. K.; Buchanan, L. M.; Spiner, D. R. *Appl. Microbiol.* **1966**, *14*, 989.
(3) Ball, M. J.; Spriggs, V.; Sutton, P. M.; Chapel, H. *J. Immunol Methods* **1986**, *95*, 113−116.
(4) Agbarakwe, A. E.; Misbah, S. A.; Griffiths, H.; Chapel, H. M. *J. Clin Pathol* **1993**, *46*, 368−369.
(5) Katona, S. J.; Bowen, M.; Kaminski, E. R. *J. Clin Pathol* **2002**, *55*, 659−661.
(6) Uittenbogaard, J. P.; Zomer, B.; Hoogerhout, P.; Metz, B. *J. Biol. Chem.* **2011**, *286*, 36198−36214.
(7) Sanders, B.; Koldijk, M.; Schuitemaker, H. In *Vaccine Analysis: Strategies, Principles, and Control*; Nunnally, B. K., Turula, V. E., Sitrin, R. D., Eds.; Springer: Berlin, Heidelberg, 2015; pp 45−80.
(8) She, Y.-M.; Cheng, K.; Farnsworth, A.; Li, X.; Cyr, T. D. *Proteomics* **2013**, *13*, 3537−3547.
(9) Steitz, A. M.; Schröder, C.; Knuth, I.; Keber, C. U.; Sommerfeld, L.; Finkernagel, F.; Jansen, J. M.; Wagner, U.; Müller-Brüsselbach, S.; Worzfeld, T.; Huber, M.; Beutgen, V. M.; Graumann, J.; Pogge von Strandmann, E.; Müller, R.; Reinartz, S. *iScience* **2023**, *26*, 108401.
(10) Gold, L.; Ayers, D.; Bertino, J.; Bock, C.; Bock, A.; Brody, E. N.; Carter, J.; Dalby, A. B.; Eaton, B. E.; Fitzwater, T.; Flather, D.; Forbes, A.; Foreman, T.; Fowler, C.; Gawande, B.; Goss, M.; Gunn, M.; Gupta, S.; Halladay, D.; Heil, J.; Heilig, J.; Hicke, B.; Husar, G.; Janjic, N.; Jarvis, T.; Jennings, S.; Katilius, E.; Keeney, T. R.; Kim, N.; Koch, T. H.; Kraemer, S.; Kroiss, L.; Le, N.; Levine, D.; Lindsey, W.; Lollo, B.; Mayfield, W.; Mehan, M.; Mehler, R.; Nelson, S. K.; Nelson, M.; Nieuwlandt, D.; Nikrad, M.; Ochsner, U.; Ostroff, R. M.; Otis, M.; Parker, T.; Pietrasiewicz, S.; Resnicow, D. I.; Rohloff, J.; Sanders, G.; Sattin, S.; Schneider, D.; Singer, B.; Stanton, M.; Sterkel, A.; Stewart, A.; Stratford, S.; Vaught, J. D.; Vrkljan, M.; Walker, J. J.; Watrobka, M.; Waugh, S.; Weiss, A.; Wilcox, S. K.; Wolfson, A.; Wolk, S. K.; Zhang, C.; Zichi, D. *PLoS One* **2010**, *5*, No. e15004.
(11) Assarsson, E.; Lundberg, M.; Holmquist, G.; Björkesten, J.; Thorsen, S. B.; Ekman, D.; Eriksson, A.; Dickens, E. R.; Ohlsson, S.; Edfeldt, G.; Andersson, A.-C.; Lindstedt, P.; Stenvang, J.; Gullberg, M.; Fredriksson, S. *PLoS One* **2014**, *9*, No. e95192.
(12) Gentles, A. J.; Newman, A. M.; Liu, C. L.; Bratman, S. V.; Feng, W.; Kim, D.; Nair, V. S.; Xu, Y.; Khuong, A.; Hoang, C. D.; Diehn, M.; West, R. B.; Plevritis, S. K.; Alizadeh, A. A. *Nat. Med.* **2015**, *21*, 938−945.
(13) Ritchie, M. E.; Phipson, B.; Wu, D.; Hu, Y.; Law, C. W.; Shi, W.; Smyth, G. K. *Nucleic Acids Res.* **2015**, *43*, No. e47.
(14) Cohen, J. *Statistical Power Analysis for the Behavioral Sciences*, 2nd ed.; Routledge, NY, 1988.
(15) Graumann, J.; Finkernagel, F.; Reinartz, S.; Stief, T.; Brödje, D.; Renz, H.; Jansen, J. M.; Wagner, U.; Worzfeld, T.; Pogge von Strandmann, E.; Müller, R. *Frontiers in Oncology* **2019**, *9*, 1150.
(16) Bast, R. C.; Klug, T. L.; St. John, E.; Jenison, E.; Niloff, J. M.; Lazarus, H.; Berkowitz, R. S.; Leavitt, T.; Griffiths, C. T.; Parker, L.; Zurawski, V. R.; Knapp, R. C. *N Engl J. Med.* **1983**, *309*, 883−887.
(17) Hellström, I.; Raycraft, J.; Hayden-Ledbetter, M.; Ledbetter, J. A.; Schummer, M.; McIntosh, M.; Drescher, C.; Urban, N.; Hellström, K. E. *Cancer Res.* **2003**, *63*, 3695−3700.
(18) Kim, B.; Park, Y.; Kim, B.; Ahn, H. J.; Lee, K.-A.; Chung, J. E.; Han, S. W. *J. Clin Lab Anal* **2019**, *33*, No. e22624.
(19) Lycke, M.; Ulfenborg, B.; Kristjansdottir, B.; Sundfeldt, K. *J. Clin Med.* **2020**, *9*, 299.
(20) Hemminki, K. *Chemico-Biological Interactions* **1981**, *34*, 323−331.
(21) Huang, H.; Li, T.; Ye, G.; Zhao, L.; Zhang, Z.; Mo, D.; Wang, Y.; Zhang, C.; Deng, H.; Li, G.; Liu, H. *Onco Targets Ther* **2018**, *11*, 1571−1581.

(22) Liang, Y.; Xia, W.; Zhang, T.; Chen, B.; Wang, H.; Song, X.; Zhang, Z.; Xu, L.; Dong, G.; Jiang, F. *Frontiers in Oncology* **2020**, *10*, 1.
(23) Zhou, W.; Li, Y.; Gu, D.; Xu, J.; Wang, R.; Wang, H.; Liu, C. *Heliyon* **2022**, *8*, No. e11083.

https://doi.org/10.1021/acs.analchem.3c04116
Anal. Chem. 2024, 96, 9332−9342