Addressing the challenge of pre-analytical variables in circulating protein biomarker analysis with Protein Plus BCT™ using Olink®

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Introduction

While liquid biopsy for plasma proteins is a promising avenue for disease early detection, diagnosis, prognosis, and monitoring, the clinical translation of plasma proteomics has been limited due to the complexity of the proteome and the impact of pre-analytical variation on its integrity. In recent years, advancements in plasma proteomic technologies, such as Olink Proximity Extension Assay (Olink), have helped address the challenge of proteome complexity. However, challenges with pre-analytical variables have persisted. During blood collection and whole blood storage, *ex vivo* blood cell activation and lysis can occur, releasing proteins into the plasma and obscuring the relevant *in vivo* abundances. This is especially true for diseases where immune or inflammation biomarkers are of particular interest as they are expressed in white blood cells and platelets. To address this issue, Streck developed Protein Plus BCT, a blood collection tube that stabilizes blood cells and minimizes changes in plasma proteome following prolonged whole blood storage. Here, we demonstrate this functionality and the compatibility of Protein Plus BCT with Olink assays by comparing the performance of Protein Plus BCT against the conventional anticoagulants, EDTA, and ACD-A.

Protein Plus BCT is for Research Use Only. Not for use in diagnostic procedures. Protein Plus BCT should only be used for research or the development of new assays.

Methods

Whole blood was collected from five healthy donors into EDTA, ACD-A, and Protein Plus BCT and was centrifuged at 1800 xg for 15 minutes at room temperature following draw (within 2 hours), or after 24, 72, and 120 hours of ambient temperature storage. The plasma fraction was carefully removed, so as not to disturb the buffy coat, and transferred to a fresh microcentrifuge tube for a second spin at 2800 xg for 15 minutes at room temperature. The resultant supernatant was removed carefully and stored at -80 °C until use. Plasma samples were sent to the Vanderbilt University Medical Center High-Throughput biomarker core, an Olink certified service provider for analysis with the Olink Explore 384-plex Cardiometabolic panel. This panel was selected because of the high proportion of blood cell related proteins assayed.

Results

Plasma Levels of Biomarkers of Interest are Maintained by Protein Plus BCT

To demonstrate that Protein Plus BCT stabilizes blood cells, and as a result, better maintains draw-time levels of blood cell-associated protein biomarkers than conventional anticoagulant tubes (EDTA and ACD-A), we measured draw-time plasma levels of blood cell associated protein biomarkers (IL-8, LOX-1, Azurocidin-1, and S100A11) in samples collected into EDTA, ACD-A, or Protein Plus BCT. Samples collected in EDTA or ACD-A had at least an 11-fold (3.5 NPX difference) increase in abundance of IL-8 (CXCL8, cytokine expressed in platelets and macrophages) after 72 hours and at least 64-fold (6 NPX difference) increase after 120 hours of whole blood storage. Conversely, no increase in abundance was observed for Protein Plus BCT samples (Figure 1A). We also observed at least a 13-fold increase (3.7 NPX difference) in LOX-1, another platelet-associated protein, after 72 hours of storage and a 16-fold increase (4 NPX difference) after 120 hours of storage. Less than a 15% increase (1.15-fold) was observed in samples collected into Protein Plus BCT (Figure 1B). Azurocidin-1, a neutrophil protein, displayed a 9.7-fold increase (3.3 NPX difference) after 120 hours of whole blood storage in EDTA and at least a 64-fold increase (6 NPX difference) following 72 and 120 hours of whole blood storage in ACD-A. In Protein Plus BCT, less than a 35% increase (1.35-fold) was observed after any storage duration (Figure 1C). Another WBC-associated protein, S100A11, displayed at least a 19-fold increase (4.3 NPX difference) from 24 hours onward for samples collected into EDTA. A 10-fold increase (3.4 NPX difference) at 24 hours was observed in samples collected into ACD-A, which grew to a 31-fold increase (5 NPX difference) after 120 hours of whole blood storage. For samples collected into Protein Plus BCT, a maximum of 2.2-fold increase was observed (Figure 1D). These representative examples demonstrate increased blood cell-associated protein stability during extended ambient storage for samples collected into Protein Plus BCT versus those collected into EDTA and ACD-A.

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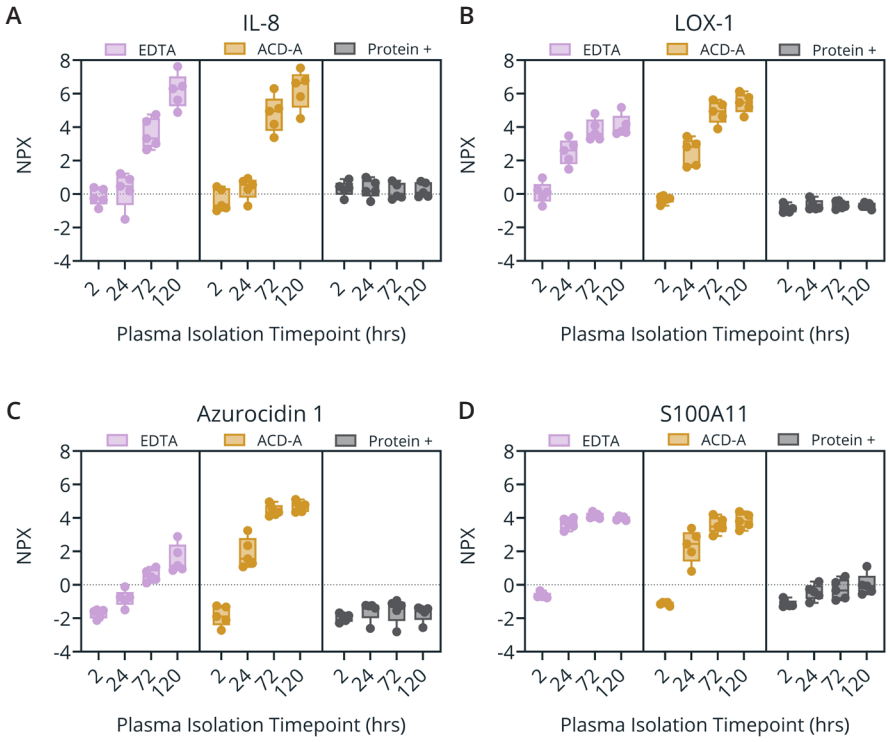

Figure 1: (A-D) Plasma levels, as represented by NPX (normalized protein expression unit reported on a Log_2 scale), of blood cell-associated protein biomarkers from samples isolated from EDTA, ACD-A, and Protein Plus BCT at draw time (2 hrs) and after 24, 72, and 120 hrs of ambient temperature whole blood storage.

Protein Plus BCT Limits Changes to the Plasma Proteome During Whole Blood Storage

To investigate all the proteins analyzed in the cardiometabolic panel, protein abundance levels in samples collected into EDTA, ACD-A, and Protein Plus BCT following prolonged whole blood storage (24 hours, 72 hours, and 120 hours) were compared to those at draw time. After only 24 hours of whole blood storage, the number of proteins that statistically significantly increased in abundance was 2.3 and 4.2 times greater in samples collected into ACD-A and EDTA, respectively, than for those collected into Proteins Plus BCT (Figure 2 A-D). For all time points analyzed, the number of proteins statistically significantly increasing in abundance was greater in samples collected into EDTA and ACD-A than those collected into Protein Plus BCT (Figure 2 A, and E-J). Additionally, the average NPX difference for all proteins, relative to draw time, was at least 100% greater for samples collected into EDTA and ACD-A and stored for 72 hours or longer than for samples collected into Protein Plus BCT and stored for the same time. The largest difference was between EDTA and Protein Plus BCT, where the average NPX difference between samples analyzed after 72 hours of storage and those analyzed at draw time was 183% greater in EDTA than Protein Plus BCT. These data suggest that Protein Plus BCT better maintains draw-time levels of protein abundance during prolonged whole blood storage than EDTA or ACD-A for the proteins measured.

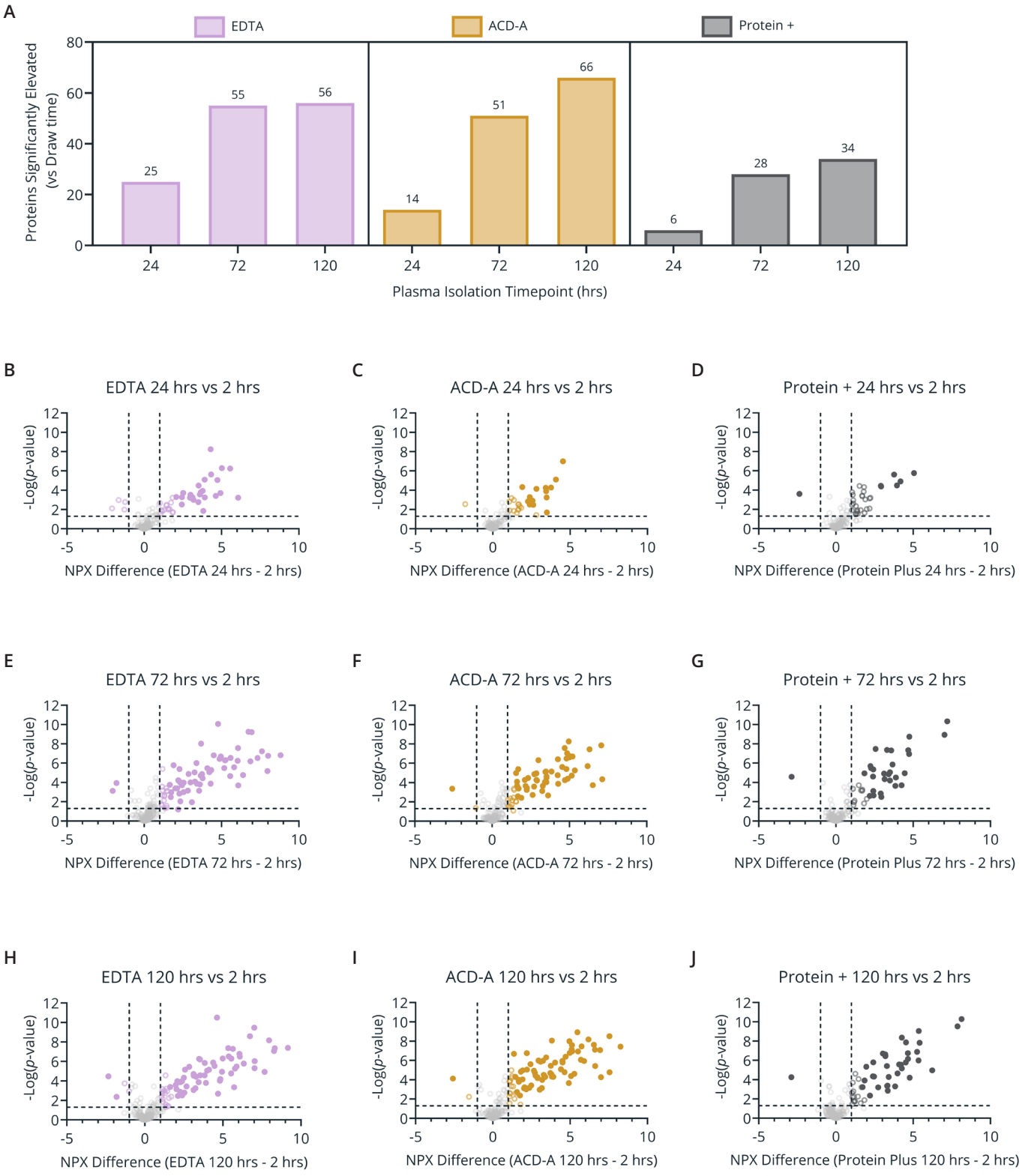

Figure 2: (**A**) Number of proteins significantly elevated in abundance after 24, 72, or 120 hrs of whole blood storage in EDA, ACD-A, and Protein Plus BCT relative to draw time (2 hrs).(**B-J)** Differential analysis of plasma protein abundances between plasma isolated from blood drawn into EDTA, ACD-A, or Protein Plus BCT at draw time (2 hrs) versus after 24 hrs (**B-D**), 72 hrs (**E-G**), or 120 hrs (**H-J**) of storage at ambient temperature. Colored outlines or markers indicate an NPX different >1 or <-1 with a -Log (*p*-value) > 1.3 and filled markers indicate FDR ≤ 1%.

Minimizes Abundance Changes in Proteins Associated with Blood Cell Activation, Movement, and Vesicles

To determine which biological functions and pathways were associated with the proteins that displayed significant increases in abundance following whole blood storage, gene ontology (GO) enrichment analysis was performed (Figure 3). We observed that changing proteins were generally associated with blood cells and their activation, cellular movement, and vesicles. For the 35 GO terms highlighted, only 11 terms were enriched for proteins that significantly changed in abundance in samples collected and stored in Protein Plus BCT, indicating many terms (69%) were enriched in samples collected and stored in EDTA or ACD-A, but not in Protein Plus BCT. This included terms such as neutrophil degranulation, azurophil granule, and tertiary granule, which are associated with white blood cells stabilized by the Protein Plus BCT formulation. For GO terms enriched in samples collected and stored in Protein Plus BCT, the magnitude of change in protein abundance was less than that for those collected and stored in EDTA or ACD-A.

Figure 3: Gene Ontology (GO) enrichment analysis of the proteins with statistically significant changes in abundance after 120 hrs of whole blood storage in EDTA, ACD-A, and Protein Plus BCT. Of the 35 GO terms highlighted as enriched in conventional anticoagulants, samples collected into Protein Plus BCT display enrichment in only 11. For those terms where Protein Plus BCT is enriched, the magnitude of change is less than observed in EDTA and ACD-A. Less overall enrichment and lower magnitude of changes demonstrate that Protein Plus BCT better maintains draw-time protein abundances. The dashed line indicated the adjusted *p*-value of 1.3 (adj *p*-value of 0.05).

Conclusions

Taken together, these data indicate that plasma isolated from Protein Plus BCT is compatible with proteomic profiling with Olink technology and that the stabilization formulation of Protein Plus BCT reduces the impact of prolonged whole blood storage on the plasma proteome by limiting *ex vivo* activation, lysis, and degradation of blood cells compared to samples collected and stored in conventional anticoagulants (EDTA, ACD-A). Protein Plus BCT ensures sample integrity and minimizes the impact of pre-analytical variables, providing researchers greater flexibility in sample handling and improved confidence in downstream biological analysis of plasma proteomic results.