Protein Plus BCT™ is now known as Nucleic Acid Plus BCT™

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Combined use of Protein Plus BCT™ and Seer Proteograph™ XT ensures reliable sample integrity for extensive plasma proteome analysis

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Introduction

While plasma proteomics has the potential to aid in the diagnosis, prognosis, and monitoring of various diseases, the development of clinically validated assays using plasma proteomics has been limited by the vast dynamic range of proteins in plasma and the impact of pre-analytical variation on the plasma proteome. The plasma proteome has an immense dynamic range (>10 log), with the most abundant plasma proteins representing more than 90% of the total protein content in plasma. This obstacle has impeded the identification and quantification of low-abundance proteins, many of which are of biological interest. With the recent development of robust and reproducible plasma protein enrichment strategies, such as the Seer Proteograph XT platform, in-depth plasma proteomic analysis by mass spectrometry has become obtainable. However, pre-analytical variables such as which anticoagulant is used, and the duration of whole blood storage before plasma isolation can significantly alter the proteomic landscape of the sample from the donor's circulating plasma proteome. Currently, blood for proteomic analysis is collected into conventional anticoagulant tubes such as EDTA. Following collection, plasma must be isolated as close to draw time as possible to preserve the integrity of the plasma proteome, but this is not always feasible. As such, delays in processing can impact the plasma proteome. During blood collection and whole blood storage, *ex vivo* blood cell activation and lysis can occur, releasing proteins into the plasma that mask the true abundance of potential biomarkers and interfere with downstream biological interpretation. To address this issue, Streck developed Protein Plus BCT, a stabilizing blood collection tube specific for proteomics. Here we demonstrate the ability of the Protein Plus BCT to minimize changes in plasma proteome following prolonged whole blood storage and examine the depth of coverage that can be obtained with Seer Proteograph XT.

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

Blood Plasma Processing

Please refer to the Protein Plus BCT Instructions For Use (IFU) for additional details.

Whole blood from five donors was collected into EDTA, ACD-A and Protein Plus BCT and centrifuged at 1800 xg for 15 minutes at room temperature immediately following draw (within 2 hrs), or after 24, 72 and 120 hrs of ambient temperature storage. The plasma fraction was carefully removed, 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 carefully removed, divided into 250 µL aliquots and frozen at -80 °C until use.

Proteomic Sample Preparation

Five technical replicates were analyzed for each donor and condition resulting in a total of 450 plasma samples ran across 14 Proteograph XT kits using the Seer SP100 Automation Instrument following the manufacturer's instructions.

Liquid Chromatography-Mass Spectrometry Analysis

Samples were analyzed using an LC-MS/MS system consisting of a Vanquish™ Neo HPLC coupled to an Orbitrap™ Astral™ mass spectrometer (Thermo Scientific™). Peptides were eluted over 22.6 min at a flow rate of 1µL/min, where buffer consists of A (H_2O, 0.1% formic acid) and B (acetonitrile, 0.1% formic acid): time 0.5 min, 4% buffer B; time 0.6 min, 8% buffer B; time 13.9 min, 22.5% buffer B; time 20.8 min, 35% buffer B; time 21.2 min, 55% buffer B; time 21.9 min, 99% buffer B; time 22.6 min, 99% buffer B.

The nanospray source voltage was set to 3000 V. Full-mass profile scans were performed in the Orbitrap™ between 380 and 980 m/z at a resolution of 240,000 with a normalized AGC target of 500%. MS1 scans were followed by HCD DIA scans in the Astral mass analyzer with a precursor isolation window width of 3 m/z and 199 scan events at 25% relative collision energy.

Data Analysis

Spectral files were analyzed on the Proteograph Analysis Suite (PAS) software platform and searched with DIA-NN (v1.8.1, library-free with match between runs enabled).

Results

Protein Plus BCT™ Maintains Protein and Peptide Identifications During Prolonged Whole Blood Storage

As whole blood is stored before plasma isolation, blood cells (RBCs, WBCs and platelets) can activate, lyse, and/or degrade releasing their protein contents into the plasma. This can not only increase the abundance of proteins already found circulating in plasma, but also further complicate an already complex plasma proteome by introducing proteins that are not present in the plasma *in vivo*. To determine whether Protein Plus BCT limits changes to the plasma proteome over time, the number of peptides and proteins identified were compared across plasma samples isolated at draw time and up to 5 days of ambient temperature, whole blood storage in EDTA, ACD-A and Protein Plus BCT. At draw time, the average number of proteins identified from plasma isolated from Protein Plus BCT, 3870, is commensurate with the average number of identifications for EDTA and ACD-A, 3885 and 4240, respectively (Figure 1A). However, as the duration of sample storage prior to plasma isolation increases, the number of identifications also increases for EDTA and ACD-A, suggesting that proteins not found at draw time are released into the plasma proteome (Figure 1A and 1B). For samples isolated from EDTA, a 38% increase in protein identifications is observed after 24 hrs of whole blood storage and a 56% increase is observed after 5 days (120 hrs) (Figure 1A). The trend is similar for samples isolated from ACD-A, with samples displaying a 25% increase in protein identifications after 24 hrs and a 40% increase after 5 days (120 hrs) of whole blood storage (Figure 1A). However, the increase in identifications in samples collected into Protein Plus BCT is no more than 12% after 5 days (120 hrs) of whole blood storage (Figure 1A). A similar trend is observed for peptides identifications (Figure 1B). These data suggest that the stabilization formulation of Protein Plus BCT minimizes the lysis and activation of blood cells and their subsequent release of proteins into the surrounding plasma.

When the overall landscape of identified proteins was examined, we observed that samples isolated from Protein Plus BCT had the largest overlap between draw time (2 hrs) and 24, 72 and 120 hrs of storage and the lowest percentage of proteins unique to the stored samples compared to samples isolated from EDTA and ACD-A (Figure 1C-E). When the proteins identified from plasma isolated from Protein Plus BCT after 5 days (120 hrs) of ambient temperature storage were compared to the proteins identified from plasma isolated from conventional anticoagulants at all storage durations (24, 72 and 120 hrs), we noted that a majority of the differentially identified proteins were unique to the conventional anticoagulants and many were related to blood cell instability (Figure 1F and 1G). Furthermore, a high degree of overlap was observed between EDTA to ACD-A after prolonged whole blood storage, indicating that both conventional blood collection tubes failed to prevent contamination of plasma proteomes by proteins leached from blood cells (Figure 1H). Taken together, these data indicate that Protein Plus BCT not only provides a consistent number of protein identifications but also high continuity in the proteins identified across prolonged whole blood storage.

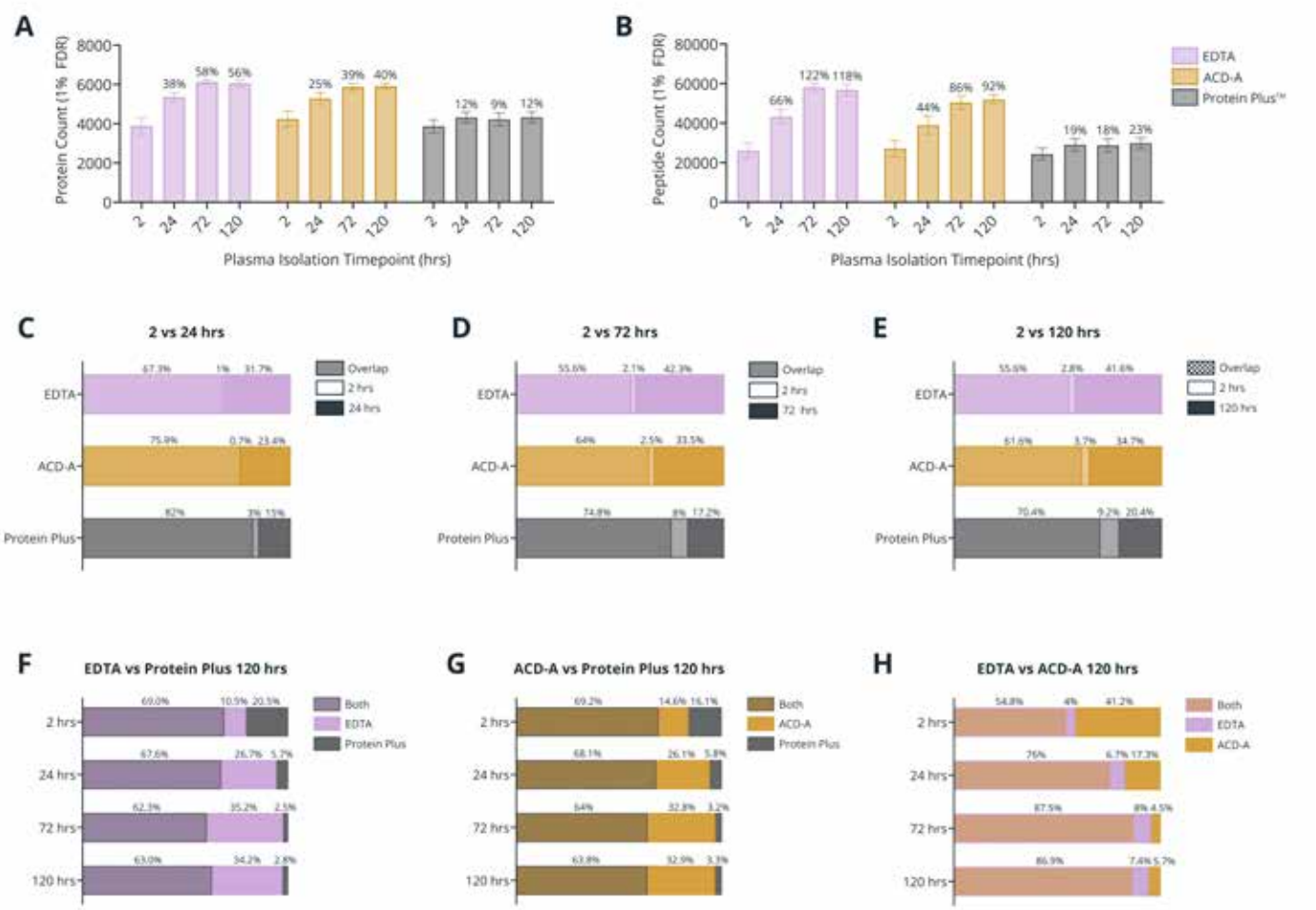

Figure 1: (**A-B**) Average number of proteins (**A**) and peptides (**B**) identified at 1% FDR in plasma isolated from samples collected into EDTA, ACD-A or Protein Plus BCT at draw time (2 hrs) or after 24, 72 or 120 hrs of whole blood storage at ambient temperature. The percent increase of identifications relative to draw time (2 hrs) is displayed above each additional plasma isolation timepoint. (**C-E**) Protein identification overlap between draw time (2 hrs) and 24 (**C**), 72 (**D**), 120 (**E**) hrs for EDTA, ACD-A and Protein Plus BCT. (**F-G**) Protein identification overlap between plasma isolated from samples in EDTA (F) and ACD-A (G) at draw time (2 hrs) and after 24, 72 and 120 hrs of storage and plasma isolated from samples in Protein Plus BCT after 120 hrs of storage. (**H**) Protein identification overlap between plasma isolated from samples in EDTA at draw time (2 hrs) and after 24, 72, or 120 hrs of storage and plasma isolated from samples in ACD-A after 120 hrs of storage. When determining identification overlap, a protein was considered identified if it was present in at least 60% of samples for any one condition.

Protein Plus BCT Minimizes Proteome-Wide Changes in Protein Abundance During Whole Blood Storage

To examine proteome-wide changes in protein abundance during whole blood storage, the draw time abundances of quantifiable proteins (2730 proteins quantified in ≥60% of samples for each condition) were compared to those obtained following delayed plasma isolation (24, 72 and 120 hrs) from EDTA, ACD-A and Protein Plus BCT. After 24 hours of whole blood storage in EDTA, 486 proteins (17.8% of the quantifiable plasma proteome) significantly increased in abundance (Log_2 fold change > 2, -Log(p-value) > 1.3 and permutation-based FDR ≤ 1%) relative to draw time. Conversely, only 79 proteins (2.9% quantifiable plasma proteome) significantly increased in abundance in plasma isolated from Protein Plus during the same storage time (Figure 2A, 2B and 2D). After 120 hours of whole blood storage, 49% (1339 proteins) and 28.9% (788 proteins) of the quantifiable proteome isolated from EDTA and ACD-A tubes, respectively, significantly increased in abundance whereas fewer than 10% of proteins from plasma isolated from Protein Plus BCT significantly increased in abundance (Figure 2A and 2H-J). Taken together, these data suggest that Protein Plus BCT better maintains draw-time levels of protein abundance during prolonged whole blood storage than either EDTA or ACD-A (Figure 2 A-J).

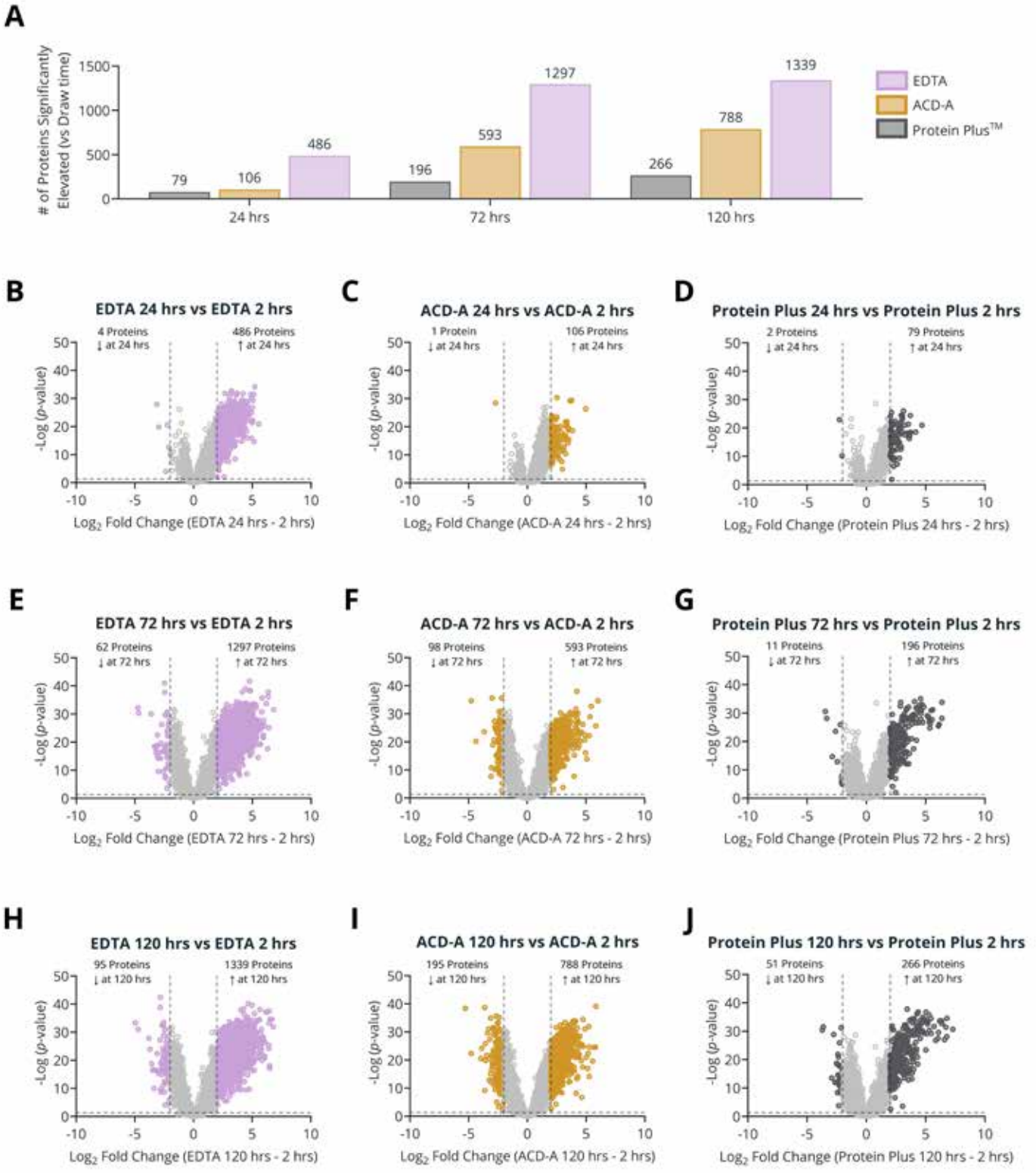

Figure 2: (**A**) Number of proteins that are significantly elevated in abundance after 24, 72 or 120 hrs of whole blood storage in EDTA, ACD-A and Protein Plus BCT relative to draw time (2 hrs). (**B-J**) Differential analysis of plasma protein abundances between plasma isolated from blood drawn into EDTA, ACD-A or Protein Plus BCT at draw time (2 hrs) or after 24 hrs (**B-D**), 72 hrs (**E-G**), or 120 hrs of whole blood storage at ambient temperature (**H-J**). Colored markers: Log_2 fold change > 2 or <-2. -Log(p-value) > 1.3 and permutation-based FDR ≤ 1%. All quantitative analysis was performed for proteins which were quantified in at least 60% of samples in each collection tube and plasma isolation timepoint (2730 plasma proteins).

Protein Plus BCT™ Maintains Draw-Time Abundance Levels of Blood Cell Associated Proteins

To further assess whether the *ex vivo* blood cell stability conferred by Protein Plus BCT minimizes plasma proteome contamination, we investigated changes in the abundance of platelet- and neutrophil-associated proteins following prolonged blood storage relative to draw time. After 24 hours of whole blood storage, platelet- and neutrophil-associated proteins increase in abundance with mean Log_2 fold change values of 1.17 and 1.44 for samples collected into EDTA and 0.74 and 0.94 for samples collected into ACD-A (Figure 3A and 3D). In contrast, samples isolated from Protein Plus BCT after 24 hours had mean Log_2 fold change of 0.21 for platelet-associated proteins (82% lower than EDTA and 72% lower than ACD-A) and 0.23 for neutrophil-associated proteins (84% lower than EDTA and 76% lower than ACD-A) (Figure 3A and 3D). After 120 hrs of whole blood storage, the mean Log_2 fold change was more than 10 times greater for samples isolated from EDTA (2.94) and ACD-A (1.95) than that for samples isolated from Protein Plus BCT for platelet-associated proteins and at least 5 times greater (2.53 and 1.49, respectively) for neutrophil-associated proteins (Figure 3A and 3D). Further, key platelet and neutrophil biomarkers showed a sharp increase in abundance during whole blood storage in EDTA and ACD-A but not in Protein Plus BCT (Figure 3B, 3C, 3E and 3F).

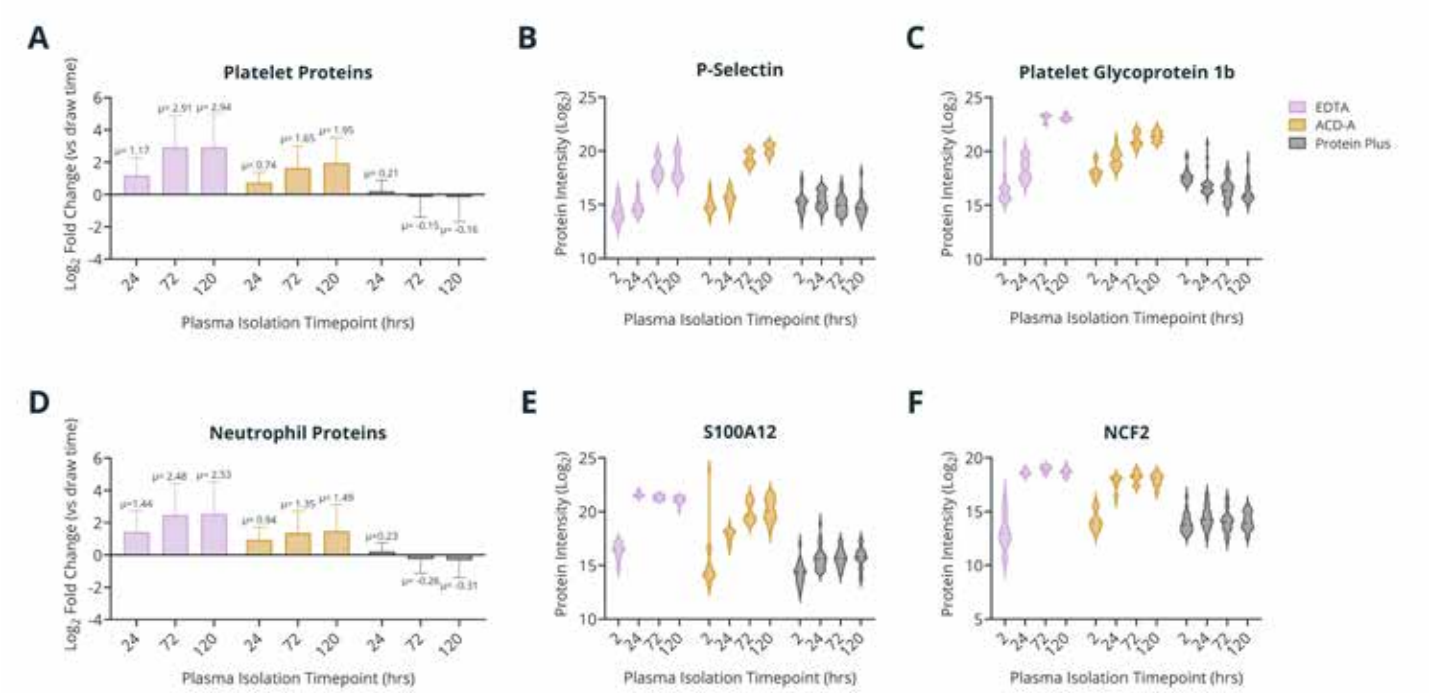

Figure 3: (**A**) Mean Log_2 fold change of platelet-associated protein abundance in plasma isolated from samples collected into EDTA, ACD-A and Protein Plus BCT after 24, 72, or 120 hrs of whole blood ambient temperature storage relative to draw time (2 hrs) protein abundance (N= 341 proteins). (**B-C**) Plasma levels (Log_2 transformed) of P-Selectin (**B**) and Platelet Glycoprotein 1b (**C**) at draw time (2 hrs) and after 24, 72 and 120 hrs of ambient temperature whole blood storage. (**D**) Mean Log_2 fold change of neutrophil-associated protein abundance in plasma isolated from samples collected into EDTA, ACD-A and Protein Plus BCT at 24, 72, or 120 hrs relative to draw time (2 hrs) protein abundance (N= 297 proteins). (**E-F**) Plasma levels (Log_2 transformed) of Neutrophil Cytosolic Factor 2 – NCF2 (**E**) and S100A12 (**F**) at draw time (2 hrs) and after 24, 72 and 120 hrs of ambient temperature whole blood storage.

Protein Plus BCT™ Stabilizes Proteins Associated Key Biological Pathways

To demonstrate that the increased proteome stabilization conferred by Protein Plus BCT is important for the downstream biological interpretation of plasma proteomics data, we examined the impact of prolonged whole blood storage on cytoskeletal proteins and proteins involved in apoptosis, protein transport, and cytokine, mTor and T-cell receptor signaling. For all investigated pathways, the mean Log_2 fold change, relative to draw time, for proteins within the pathway increased as the duration of whole blood storage increased for samples collected in EDTA and ACD-A. For samples isolated from EDTA, the mean Log_2 fold change after 24 hours was between 1.3 and 1.6 for all pathways and, after 120 hours, the Log_2 fold change was even greater with values between 2.4 and 3.3. For samples collected into the Protein Plus BCT, the overall Log_2 fold change was not only much lower than what was obtained with conventional anticoagulants, with values never exceeding 0.5, but also did not show the same degree of increase over time as was seen with conventional anticoagulants (Figure 4B-F). This further demonstrates the ability of Protein Plus BCT to minimize protein abundance changes over prolonged whole blood storage, better maintaining the draw-time plasma proteome.

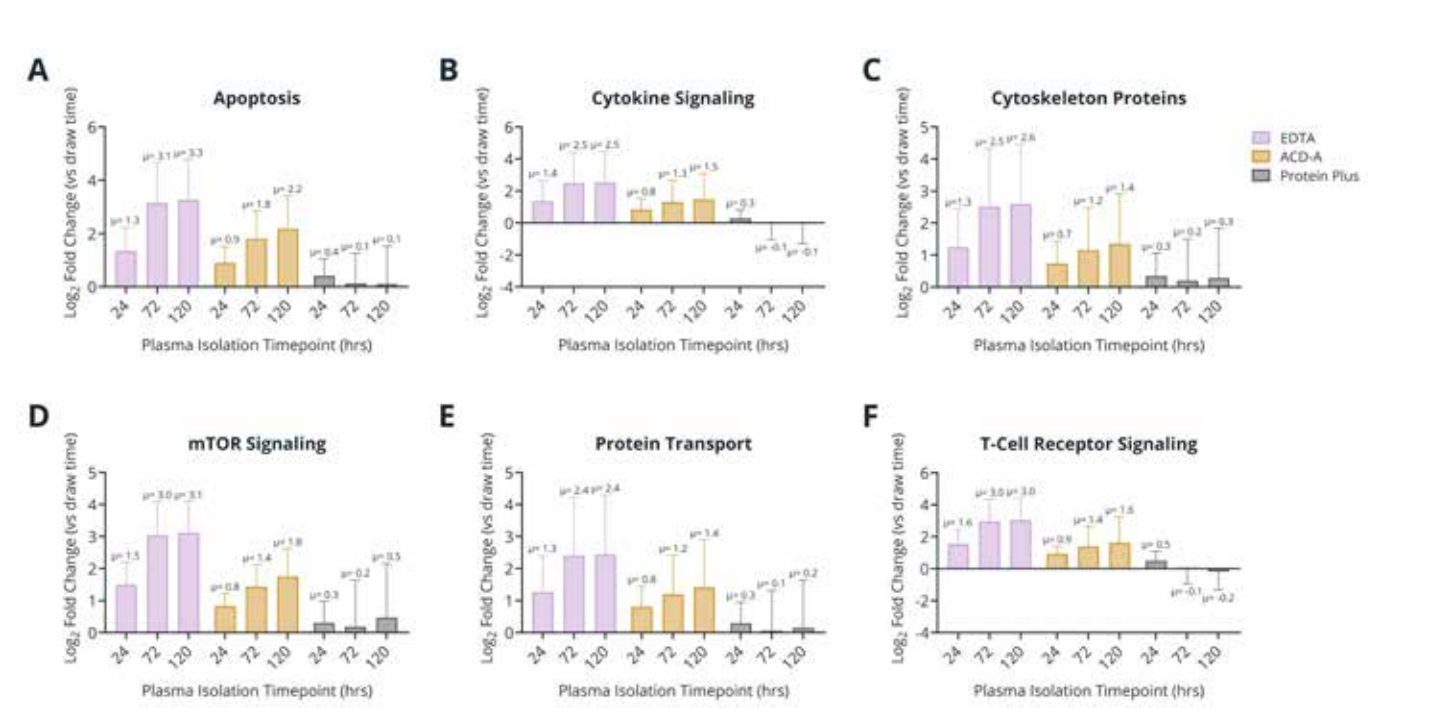

Figure 4: Mean Log_2 fold change in abundance of (**A**) apoptosis-related proteins (N= 85, Reactome R-HSA-109581), (**B**) proteins involved in the immune system's cytokine signaling (N= 220, Reactome R-HSA-1280215), (**C**) cytoskeletal proteins (N= 461, Gene Ontology GO:0005856), (**D**) proteins involved in mTOR signaling pathway (N= 16, Reactome R-HSA-165159), (**E**) proteins involved in protein transport (N= 258, Gene Ontology GO:0015031), and (**F**) proteins involved in T-cell receptor signaling pathway (N= 63, Reactome R-HSA-202403) in plasma isolated from samples collected into EDTA, ACD-A or Protein Plus BCT after 24, 72 or 120 hrs of whole blood storage at ambient temperature relative to draw-time (2 hrs) abundances.

Conclusion

The stabilization formulation in Protein Plus BCT™ greatly reduces the impact of prolonged whole blood storage on the plasma proteome by limiting the *ex vivo* activation, lysis and degradation of blood cells often seen in samples collected and stored in conventional anticoagulants (EDTA, ACD-A). Samples collected into EDTA and ACD-A show major qualitative and quantitative protein changes with delayed plasma processing, whereas those collected into Protein Plus BCT display minimal changes in the isolated plasma proteome up to five days after the draw. 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.