Ex vivo Platelet Activation: An Underestimated Challenge for Plasma Protein Biomarker Research

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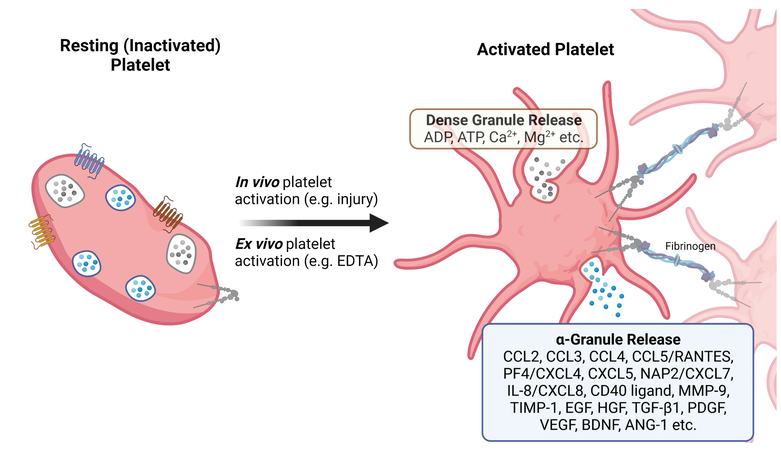
Introduction

Circulating soluble plasma proteins, such as cytokines, play a crucial role in the clinical diagnosis and prognosis of various cancers and inflammatory disorders. A significant number of these protein biomarkers are located within the α-granules of platelets **(Scheme 1)**. When platelets are activated, these α-granules undergo translocation and fuse with the platelet's plasma membrane, leading to the release of their contents into the plasma. This process occurs rapidly and irreversibly, triggering further platelet activation and aggregation, and mediating biological processes like inflammation, wound healing, angiogenesis, tumor migration, and metastasis.

Clinically, *in vivo* platelet activation and the subsequent release of proteins from α-granules have been associated with various disease states. However, *ex vivo* platelet activation can occur during blood collection (e.g., when using EDTA tube, which is commonly used for protein biomarker research), and during post-draw processing (agitation and cold temperature storage). This can result in a significant elevation in the concentration of platelet-associated proteins immediately after a blood draw. Such an increase has the potential to mask true *in vivo* platelet activation and disease-associated biomarker elevation in patient samples and deliver inaccurate and inconsistent assay results. While citrate-based blood collection tubes like ACD-A can effectively limit platelet activation during collection, samples must be processed immediately and platelet-poor plasma isolation protocol should be followed, as ongoing platelet activation and release of α-granule proteins occur over time without stabilization. Delays in plasma isolation or inadequate removal of platelets in ACD-A tubes lead to the contamination of platelet-associated proteins in plasma as a result of *ex vivo* platelet activation. Therefore, careful consideration must be given to the choice of blood collection tubes and processing protocols.

Streck is developing a novel formulation (Streck X) that minimizes *ex vivo* platelet activation during blood collection and ensures the accurate assessment of proteins of interest including cytokines in the plasma for up to 5 days of whole blood storage at ambient temperature.



Scheme 1. Platelet activation and granule release. Upon platelet activation (either *in vivo* or *ex vivo*), platelet morphology changes, and secretory granules release their cargo into the plasma. In particular, α-granules fuse with the plasma membrane and release various chemokines, cytokines, and growth factors (created using BioRender).

Results

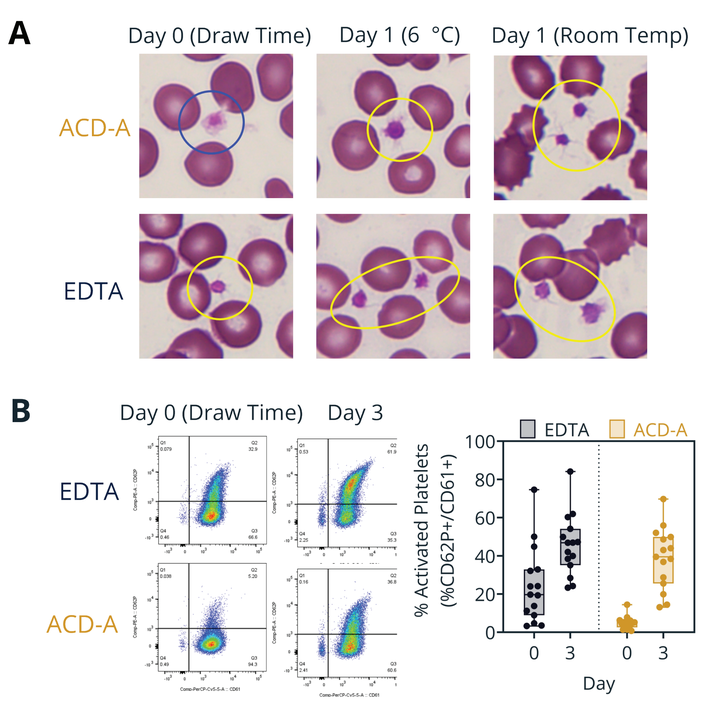


Figure 1. ***Ex vivo*** **platelet activation of samples collected into EDTA or ACD-A tubes at draw time or during whole blood storage. (A)** Samples collected into ACD-A tubes exhibit limited platelet activation at draw time (inactivated/resting platelet circled in blue) but substantial platelet activation (activated platelet circled in yellow) after one day of either cold or ambient temperature storage. Samples collected into EDTA tubes exhibit a large population of activated platelets both at draw time and during storage. Representative blood smear images shown. **(B)** Samples collected into EDTA tubes and processed at draw time show a higher population of activated platelets (CD61+CD62P+) than donor-matched ACD-A samples. EDTA samples also exhibit a dynamic donor-to-donor range in *ex vivo* platelet activation at draw time, while samples collected into ACD-A display smaller donor-to-donor variability (n=15). In both tube types, platelet activation increases after three days of storage at ambient temperature.

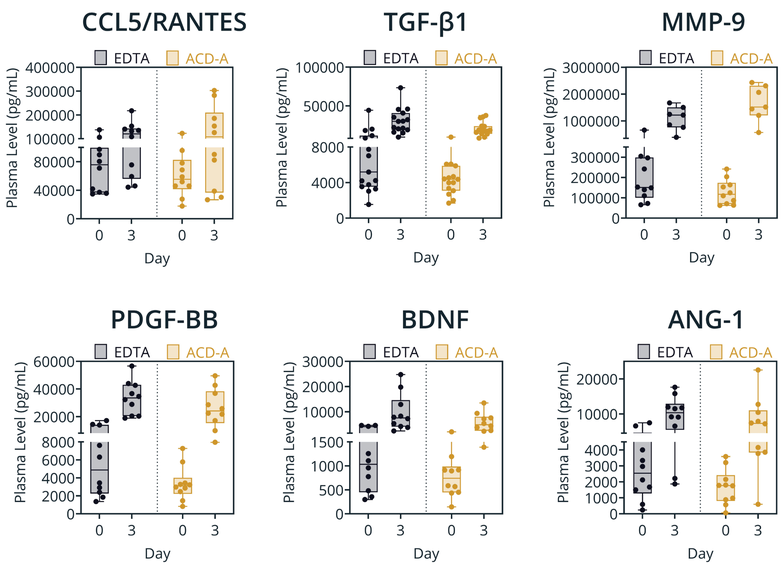


Figure 2. Plasma levels of selected soluble platelet-associated biomarkers in samples collected into EDTA or ACD-A. At draw time, the plasma concentration of all selected markers is elevated in samples collected into EDTA tubes relative to donor-matched ACD-A samples (n=10-15). After three days at ambient temperature, both EDTA and ACD-A samples exhibit increased plasma concentrations of all markers compared to draw time, suggesting neither tube can prevent the granule release of platelet-associated proteins into the plasma during whole blood storage.

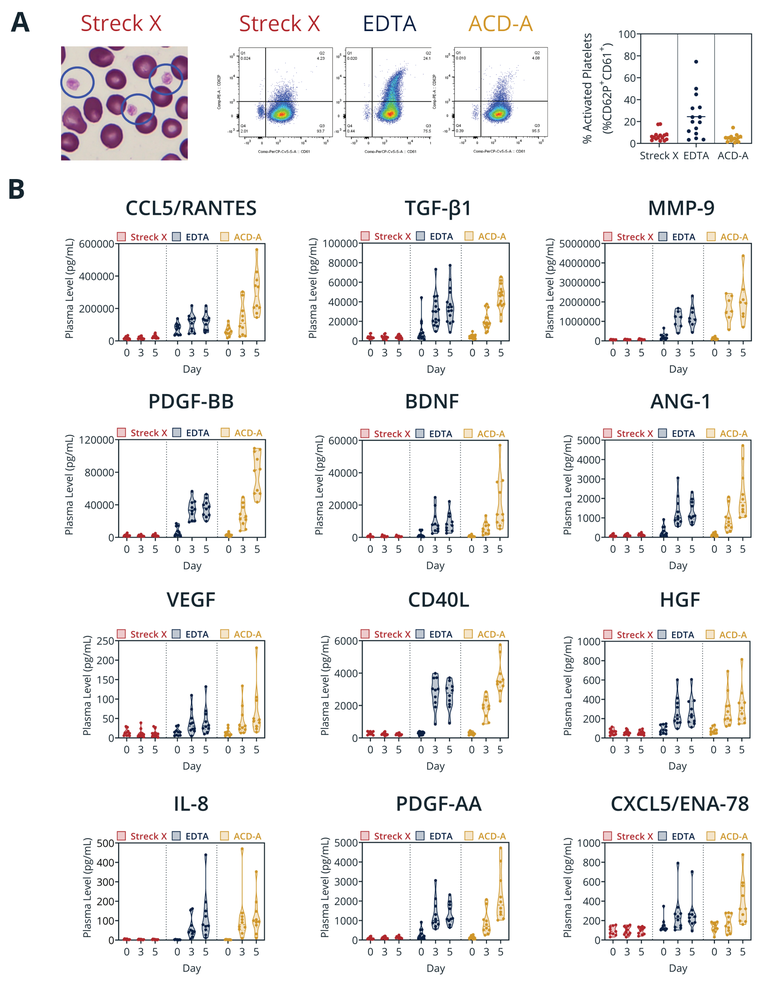


Figure 3. Platelet activation and plasma levels of platelet-associated biomarkers in samples collected into Streck X, EDTA or ACD-A. Samples collected into Streck X display **(A)** limited activation of platelets immediately after draw demonstrated by blood smear image (resting/inactivated platelet circled in blue) and flow cytometry and **(B)** stable plasma concentration of selected platelet-associated proteins for up to 5 days of whole blood storage at ambient temperature.

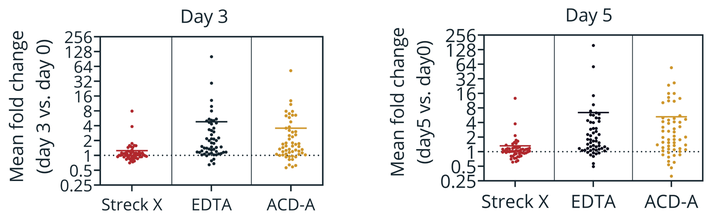


Figure 4. Stability of plasma biomarkers of interest in samples collected into Streck X, EDTA, or ACD-A during whole blood storage at ambient temperature. A total of 55 plasma protein markers of interest, including various cytokines, cancer biomarkers, and immuno-oncology checkpoint markers were analyzed. Samples collected into Streck X show increased protein stability compared to samples collected into EDTA or ACD-A after three or five days of ambient temperature storage. The mean protein fold change relative to day 0 abundance is shown (n=10).

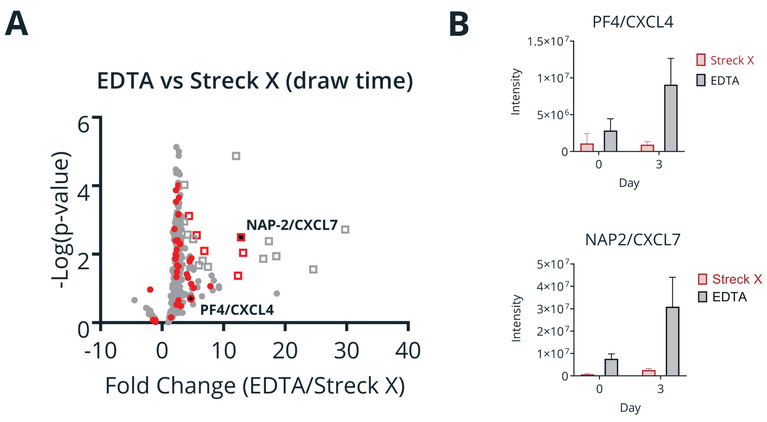


Figure 5. Quantitative LC-MS/MS analysis of plasma proteins at draw time and during whole blood storage. (A) Differential analysis of 329 plasma protein abundances between samples collected into EDTA or Streck X at draw time. Platelet-associated proteins are highlighted in red. Square markers designate proteins with a statistically significant fold change >2 (p-value < 0.05, FDR, <0.01) (n=3). In agreement with the observed increase in platelet activation for samples collected into EDTA but not Streck X, most platelet-associated proteins are elevated in plasma samples collected in EDTA compared to Streck X. **(B)** Blood samples display increased plasma levels of two key platelet activation markers when collected into EDTA tubes compared to Streck X, regardless of storage time. Further, plasma levels of both proteins remain similar from draw time to day 3 when samples are collected into Streck X, but not when collected into EDTA.

Methods

Blood Collection: Blood samples from pre-consented self-proclaimed healthy donors were drawn into evacuated blood collection tubes, containing EDTA, ACD-A or Streck X. The collected whole blood samples were processed and analyzed immediately or after 3 or 5 days of whole blood storage at ambient temperature.

Blood Smear Preparation and Imaging: Blood smear slides were prepared, dried, and stained with HARLECO® Hemacolor Stain Set (Sigma-Aldrich) following the manufacturer's protocol. Slides were imaged using an Olympus BX41 microscope (100x).

Platelet Activation Analysis by Flow Cytometry: Whole blood samples were stained with an antibody cocktail of CD61-PerCP (a platelet surface marker, BD Biosciences) and CD62P-PE (an activated platelet surface marker, BioLegend) and analyzed on a BD FACSCanto II clinical flow cytometry system. Acquisition and analysis were performed by scatter gating (forward scatter [FSC] and side scatter [SSC]) followed by fluorescent gating. Activated platelets (%) were quantified by the CD61+CD62P+ platelet population of all gated platelets (FlowJo v10.7.2). Day 3 platelet activation level may be underestimated, as aggregated platelets could be falsely gated as RBC/WBC.

Plasma Sample Preparation: Plasma was isolated using a general double-spin protocol (1800 ×g for 15 min and 2800 ×g for 15 min, both at room temperature), aliquoted, and frozen at -80 °C until use.

Analysis of Plasma Level of Protein Biomarkers: Plasma levels of proteins of interest including various cytokines, cancer markers and immuno-oncology checkpoint markers, were measured by Ella Simple Plex Assays (Bio-Techne) and Luminex xMAP® Technology Assays (Bio-Techne, samples tested by LuminexPLORE Lab) according to the manufacturer's instructions for optimal dilutions and processing.

Analysis of Plasma Proteome by LC-MS: Aliquots of plasma were depleted using High Select Top14 Abundant Protein Depletion Mini Spin Columns (Thermo Fisher) according to manufacturer's instructions. Depleted samples were subsequently prepared for proteomics analysis using S-Trap mini spin columns (Protifi). A slightly modified version of the manufacturer's protocol was followed in which Tris pH 8.5 was used in place of TEAB pH 8.5 in the lysis buffer only. The digested plasma peptides were reconstituted in 95:5 Water: ACN 0.2% FA and analyzed on a Thermo Fisher Fusion Orbitrap mass spectrometer. All raw spectra files were analyzed using MetaMorpheus (v0.0.320) and quantitative statistical analysis was performed using Perseus (v2.0.7.0).

Conclusions

Ex vivo platelet activation during blood collection and processing is an often-overlooked variable in plasma protein biomarker research. Commonly used blood collection tubes with anticoagulants like EDTA can cause platelet activation, and the release of platelet-associated proteins into the plasma occurs at draw time. While samples collected into ACD-A exhibit limited platelet activation at draw time, this inhibitory effect is not maintained during whole blood storage at ambient temperature. The novel Streck formulation (Streck X) addresses both of these issues. Samples stabilized with Streck X display minimal *ex vivo* platelet activation and α-granule release at draw time and maintain the draw time plasma levels of proteins of interest for up to 5 days of whole blood storage at ambient temperature. With this formulation, laboratories can reduce a major pre-analytical factor in plasma protein biomarker research.

Streck X is a product in development and is currently not for sale. The formulation developed, its use and/or method of manufacture may be covered by one or more patent applications.

For more information, please contact Product Manager Bill Rock at brock@streck.com.