Development of a Rapid Clinical Flow Cytometric Assay of Neutrophil Cell-Surface Biomarkers

by

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Abstract

Rationale: Neutrophils are upregulated in infectious processes. They can be measured as surrogates for the presence of an infection. Flow cytometry is a common tool that uses fluorochrome-conjugated antibodies specific to cell-surface biomarkers to identify cells or biomarkers of interest. Many clinical flow cytometry protocols exist that assess lymphocytes, but fewer protocols exist for rapid clinical evaluation of neutrophils. Expression of neutrophil cell-surface markers integrin α9β1 and CD11b has been shown to increase in elderly patients with bacterial pneumonia. A rapid clinical test for these neutrophil cell-surface markers could help to expedite appropriate antibiotic therapy to patients suffering from complex critical illnesses complicated by bacterial pneumonia for which other diagnostic methods are rendered inadequate.

Methods: We developed a rapid lyse, no-wash clinical protocol for flow cytometric analysis of neutrophil cell-surface biomarkers integrin α9β1 and CD11b from whole blood samples. Assay development included selecting appropriate neutrophil-specific biomarkers and their corresponding fluorochrome profile, determining the most consistent blood acquisition method (BAM) for obtaining samples for analysis, and evaluating the effect of preservative type and time on neutrophil absolute count (ANC), integrin α9β1 mean fluorescence intensity (MFI), and CD11b MFI.

Results: There were significant differences in ANC, CD11b MFI, and integrin α9β1 MFI over time for each BAM and Preservative type. ANC demonstrated the least variability in Citrate, while CD11b MFI and integrin α9β1 MFI were most consistent in Heparin. Therefore, Heparin was selected as the BAM of choice. Streck preservative solution and Streck Blood Collection Tube (BCT) demonstrated the least variability in ANC, integrin α9β1 MFI, and CD11b MFI.

Conclusions: Pneumonia is difficult to detect in patients with critical illness, where the usual diagnostic tools are rendered inadequate secondary to multi-system organ failure. More accurate methods of diagnosis for pneumonia can enhance targeted antibiotic therapy and are of paramount public health importance to improve patient care. Development of a rapid clinical flow cytometric protocol is the first step toward precision methods of pneumonia diagnosis; therefore, a rapid lyse/no-wash clinical flow cytometric assay for whole blood specimens was developed for identification of neutrophil cell-surface biomarkers CD11b and integrin α9β1. Heparin was the least variable BAM for assessing CD11b MFI and integrin α9β1 MFI, while Streck preservative solution and Streck BCT demonstrated the least variability over 24 hours from specimen collection. Heparin is a common type of venipuncture tube in hospitals, it is not as cost effective or feasible to purchase a separate blood collection tube (e.g. Streck BCT) for a single laboratory test. Given these real-world concerns, Streck solution added to samples collected in heparin venipuncture tubes is the most reasonable method by which to perform the rapid lyse/no-wash neutrophil flow cytometric assay.

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1.0 Introduction

Bacterial pneumonia is a common infection resulting in over 544,000 annual Emergency Department visits and killing 48,632 people annually in the United States[1,2]. Certain patient populations have a higher risk of developing pneumonia, such as elderly people who are at risk for aspiration following a stroke or other disease process resulting in impaired swallowing or an impaired gag reflex[3]. Oftentimes, the diagnosis of pneumonia is not straightforward due to confounding complex medical illness and non-infectious inflammation that obscures radiographic findings. This is especially true in patients resuscitated from cardiac arrest and is related to the sepsis-like inflammatory cascade they develop following ischemia and reperfusion from the arrest and subsequent resuscitation. Therefore, more sensitive and specific biomarkers are needed to diagnose pneumonia in multiple patient populations. A prior study in elderly patients with aspiration pneumonia demonstrated differential expression of neutrophil cell-surface markers integrin α9β1 and CD11b, as assessed by flow cytometry, in patients with pneumonia as compared to those without[3]. Presence of differential expression of these neutrophil biomarkers in complex critical illness states (e.g. patients after resuscitation from cardiac arrest) could specify the diagnosis of pneumonia, allowing clinicians to more rapidly identify the disease and effectively allocate antibiotic therapy.

1.1 Neutrophil Cell-Surface Biomarkers and the Immunology of Pulmonary Infection

Neutrophils are known to be upregulated in infectious processes, and specific neutrophil cell-surface biomarkers could serve as a target for further study as surrogates of pulmonary infection. The biomarkers of interest in this study were chosen based on their physiologic relation to the development of infectious pulmonary inflammation in pneumonia[3-8]. Neutrophils are upregulated via the T_H17 pathway, through activation by Interleukin 17A (IL-17A), in pulmonary infections[3,11,12]. T_H17 cells are a subset of $CD4^+$ T cells that play a key role in mucosal immunity, hence their importance in pulmonary infections and the innate immune response[11,12]. Alveolar macrophages secrete IL-23, which activates T_H17 cells to produce IL-17A, which is essential for neutrophil migration in pulmonary infection, particularly to lipopolysaccharides, which are lipoglycans and endotoxins released by bacteria[3,11-16].

Integrins facilitate the human inflammatory response by mediating neutrophil adhesion and extravasation to the site of infection[4,9]. Integrins are transmembrane glycoproteins composed of two subunits, α and β, that form a heterodimer which spans the cell membrane[9]. The β subunit generally enables cell attachment while the α9 subunit is more specific and known to be crucial to the pulmonary innate immune system response[9,10]. Research at the molecular level has demonstrated that integrin α9β1 is expressed on neutrophils and that vascular cell adhesion molecule-1 (VCAM-1) acts as a ligand for integrin α9β1, facilitating neutrophil transmigration during infectious and inflammatory states[4,10]. CD11b is a β2 integrin expressed in complex with CD18 on the surface of leukocytes including neutrophils, monocytes, and NK cells[5]. CD11b is upregulated as part of the innate immune response to infections and induces cell adhesion, it also acts as a complement receptor[5,6,7]. Prior studies have demonstrated upregulation of CD11b in

community acquired pneumonia, where it facilitates migration of neutrophils to the site of pulmonary infection[3,5].

1.2 Neutrophil Flow Cytometric Assays

Flow cytometry is a common technique to assess cell populations and cell-surface biomarker expression. Prior study in elderly patients with aspiration pneumonia demonstrated differential expression of neutrophil cell-surface markers integrin α9β1 and CD11b, as assessed by flow cytometry, in patients with pneumonia as compared to those without[3]. However, this study did not analyze specimens in real-time; rather, patient samples were stored and frozen at -80°C, which could affect the integrity of neutrophils and the expression of integrin α9β1 and CD11b. There is evidence in the literature that increasing the temperature from 4°C to 37°C can upregulate CD11b expression while hypothermia can inhibit expression, so it is recommended that samples either be kept on ice for procedures or maintained at room temperature[17,18,22]. Prior studies also suggest that cell damage can result in non-specific binding of antibodies to cell membranes, which can alter the fluorescence profiles for both cells and their membrane biomarkers[17,20].

The literature concerning how citrate, heparin, and EDTA anticoagulants influence CD11b expression is conflicting. Heparin was found to significantly elevate cytokines as compared to citrate or EDTA; however, these assays used plasma, whereas this study is analyzing biomarkers from whole blood[19]. Henno *et al.* determined that serum collected in EDTA, heparin, and citrate resulted in higher cytokine levels than plasma, while storage of whole blood at room temperature resulted in elevated levels of some cytokines due to *in vitro* release from cells[20,23]. Henno *et al.* and Patil *et al.* noted varied expression among different cytokines depending on the anticoagulant

used, but none of the cytokines in these studies overlap with those being evaluated in this study[20,23]. EDTA demonstrated superior elevation of neutrophils as compared to heparin or citrate for direct isolation from whole blood, but a density gradient with centrifugation was used for neutrophil isolation, which is a different approach than employed in this study[21]. No studies were identified that directly matched the protocols described herein; therefore, guidance from the literature is limited in its relationship to this study.

The goal of developing this assay was to create a clinical test to rapidly evaluate the differential expression of neutrophil cell-surface biomarkers for diagnosis of pulmonary infection. Freezing or otherwise manipulating neutrophils may not only alter the biomarkers of interest, as described previously, it also results in delay of testing and dissemination of results to clinicians. Given the lack of consensus regarding the appropriate anticoagulant venipuncture specimen collection tube as well as the most ideal cell preservative, further study is warranted to determine the optimal specimen collection and preservative conditions for neutrophils and their cell-surface biomarkers. In order to develop a rapid lyse/no-wash protocol to assess neutrophil-specific cell-surface expression of integrin α9β1 and CD11b, we evaluated the variability over time of biomarker expression between three different blood acquisition methods and three different preservative types.

2.0 Methods

2.1 Blood Donors

Verbal consent for peripheral venipuncture was obtained from 16 healthy donors associated with this project who had no known inflammatory or rheumatologic diseases, cancer, or immunosuppression. A total of six subjects provided specimens for the blood acquisition method experiment and 10 subjects provided blood for the preservative experiment. This project was considered by the Institutional Review Board to be a quality improvement analysis for calibration of a laboratory technique.

2.2 Blood acquisition and preparation

For analysis of the blood acquisition method (BAM), samples were collected in BD Vacutainer sodium citrate, BD Vacutainer heparin sulfate, and BD Vacutainer K2 ethylenediaminetetraacetic acid (ETDA) venipuncture tubes. For analysis of preservative types, samples were collected in BD Vacutainer heparin sulfate tubes and Streck Blood Collection Tubes (BCT).

Whole blood collected for the blood acquisition method (BAM) assays was divided evenly and Streck preservative solution was added to half of the samples in a 1:1 ratio for each BAM tested. Whole blood for the preservative assays was either left unadulterated (control), had Streck preservative solution added in a 1:1 ratio, or was collected in a Streck BCT direct-draw blood

collection tube. *4',6-Diamidino-2-phenylindole* dihydrochloride (DAPI, 10 µL of 200 µg/mL solution) stain was added to the control samples in the preservative comparison experiments just prior to flow cytometry analysis to discriminate between intact, live cells and dead cells.

Each assay was performed in triplicate with a fluorescence-minus-one control (FMO)[25]. Samples were processed immediately and at 24 hours post-collection for the BAM analyses as well as for the preservative analyses. All samples remained at ambient temperature following collection and during analysis. Samples were placed on a nutating mixer between time point assays.

2.3 Flow cytometer calibration

In a properly calibrated flow cytometer, mean fluorescence intensity (MFI) is a surrogate for the relative number of target molecules per cell and can be used to assess changes in biomarker expression over time intensity. The cytometer was calibrated before each use with Spherotech 8-peak Rainbow Particles in order to ensure quantitative comparability of MFI within and between experiments. Photomultiplying tubes (PMTs) are sensors in flow cytometers that detect specific wavelengths of light filtered within the cytometer. PMT voltages require calibration to ensure consistency of measurements; therefore, PMT voltages for all parameters were adjusted to place the seventh bead peak (derived from the Spherotech 8-peak Rainbow Particles) in a predetermined target channel prior to each experiment. Single stained controls, which are hard-stained beads for the dyes used in a flow cytometry experiment (e.g. FITC, PE, APC, antibody capture beads for tandem dyes), were run with each experiment for spectral compensation.

2.4 Measurement of cell surface chemokine receptors for blood acquisition method analyses

Whole blood (100µL), regardless of BAM type, was incubated at room temperature for 20 minutes in the dark with CD45 (5 µL, Biolegend #304017, A488, mouse IgG1 k, anti-human, 100 tests), CD16 (20µL, BD Biosciences #555408, PE-Cy5, mouse IgG1 k, anti-human, 100 tests), CD14 (5µL, Biolegend #325618, PE-Cy7, mouse IgG1 k, anti-human, 100 tests), CD33 (10µL, Miltenyi Biotec #130-111-022, APC-vio770, human, 100 tests), integrin α9β1 (5µL, Biolegend #351606, PE, mouse IgG1 k, anti-human, 100 tests), and CD11b/MAC-1 (20µL, BD Biosciences #550019, APC, mouse IgG1 k, anti-human, 100 tests). PBS (25 µL) was added to the FMO samples in order to equalize the volume. Lysing solution (2 mL, IOTest 3, Beckman Coulter #A07799) was added to each sample and incubated for 10 minutes in the dark at room temperature. Flow-count fluorospheres (100 µL, Beckman Coulter #7547053, 20mL) was added and samples were transferred to the flow cytometer (Gallios, 4 laser, Beckman Coulter).

2.5 Measurement of cell surface chemokine receptors for preservative analyses

Whole blood, regardless of BAM type, was incubated with CD45 (5 µL, Biolegend #304017, A488, mouse IgG1 k, anti-human, 100 tests), CD16 (5 µL, BD Biosciences #557758, APC-Cy7, mouse IgG1 k, anti-human, 100 tests), CD66b (5 µL, Invitrogen #25066642, Pe-Cy7, mouse IgM k, anti0human, 100 tests), integrin α9β1 (5µL, Biolegend #351606, PE, mouse IgG1 k, anti-human, 100 tests), and CD11b/MAC-1 (20µL, BD Biosciences #550019, APC, mouse IgG1 k, anti-human, 100 tests). PBS (25 µL) was added to the FMO samples in order to equalize the volume. Lysing solution (2 mL, IOTest 3, Beckman Coulter #A07799) was added to each sample

and incubated for 10 minutes in the dark at room temperature. Flow-count fluorospheres (100 µL, Beckman Coulter #7547053, 20mL) beads were added and samples were transferred to the flow cytometer (Gallios, 4 laser, Beckman Coulter).

Flow cytometry data was generated and saved in flow cytometry standard (FCS) format using Beckman Coulter Kaluza Acquisition for Gallios software. Gating was performed using Applied Cytometry VenturiOne® Analysis Software. Two parameter dot plots in log scale were used to identify populations of interest. Cells were differentiated from beads using forward-scatter intensity versus forward-scatter time-of-flight, after which cells were assessed using a forward-scatter versus side-scatter intensity plot to eliminate the lysed red blood cells. Next, a CD45 versus side-scatter intensity plot identified distinct cell populations of neutrophils, monocytes, lymphocytes, and eosinophils. In order to restrict outcomes analysis to neutrophils, all subsequent outcomes gating was defined using the neutrophil gate determined in the CD45 versus side-scatter intensity plot. Plots of the biomarker of interest versus side-scatter intensity were generated and polygonal gates were drawn around populations expressing the target molecule of interest. MFI was used to quantify the two primary biomarker outcomes of interest, integrin α9β1 and CD11b. Absolute neutrophil count (ANC) was determined using a manufacturer-specified concentration of Beckman Coulter Stem Count beads that was added in equal volume (100 µL) to the sample.

2.6 Selection of Cluster of Differentiation Molecules for Neutrophil Identification

Neutrophils can be identified by their side scatter and forward scatter properties in flow cytometry, in combination with cell-specific cluster of differentiation (CD) molecules. Positive selection, in which a cell is classified on the basis of expressing certain molecules, can be combined

with negative selection, in which a cell is categorized based on the lack of certain molecules, to identify cell types. The CD markers selected for neutrophil identification were CD45, CD16, CD14, and CD33 in the BAM analyses. In order to simplify the gating scheme, the CD markers were streamlined in the Preservative analyses to include CD45, CD16, and CD66b. CD45 is expressed on all hematopoetic cells, CD16 is expressed on neutrophils as well as macrophages and NK cells, CD14 is expressed on myelomonocytic cells, CD33 is expressed on monocytes, and CD66b is expressed on neutrophils and NK cells[24]. CD45 was used to categorize neutrophils in combination with their side scatter and forward scatter characteristics. Side scatter assesses the granularity (or internal complexity) of cells, while forward scatter separates cells based on size. Neutrophils are highly granular and display higher intensity side scatter than monocytes or lymphocytes. Neutrophils are similar in size but often slightly smaller than monocytes, but they are larger than lymphocytes; therefore, neutrophils have a higher intensity forward scatter than lymphocytes but are similar to monocytes. Hence, further discrimination between neutrophils and monocytes using CD markers demonstrates that neutrophils are $CD66b^{+}$, $CD16^{+}$, $CD33^{low}$, $CD14^{low}$ while monocytes are $CD45^{+}CD33^{+}CD14^{+}CD16^{-}CD66b^{-}$.

2.7 Statistical Analysis

Raw data was assessed for normality, using the Shapiro-Wilks test in conjunction with histogram, qnorm, and pnorm plots. Comparisons between groups were tested at baseline using the Repeated Measures Analysis of Variance (RM-ANOVA) test (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) The Bonferroni correction was used to adjust for multiple comparisons. Paired data across time points was tested using the

Student's paired t-test or Wilcoxon signed-rank test, depending on normality (Stata 15SE, StataCorp. 2017. *Stata Statistical Software: Release 15*. College Station, TX: StataCorp LLC). Sample size calculations were performed in G*Power (G*Power Version 3.1.9.2) and determined that a minimum of 6 subjects was required in the RM-ANOVA model with three groups and four measurements, assuming 80% power to detect an effect size of 2 with alpha 0.05 and a two-sided test. This sample size was also determined to be appropriate for the Wilcoxon signed-rank test and the Student's paired t-test. Six subjects provided specimens for the blood acquisition method experiment and 10 subjects provided specimens for the preservative experiment.

3.0 Results

The primary outcomes for both the blood acquisition methods (BAM) and Preservative experiments were CD11b Mean Fluorescence Intensity (MFI), Integrin α9β1 MFI, and Absolute Neutrophil Count (ANC). The 95% confidence intervals displayed in all figures depicting repeated measures ANOVA analyses represent 95% confidence intervals for the estimated marginal means of the model, not 95% confidence intervals for the sample means.

3.1 Normality Testing

Normality testing for the BAM studies demonstrated that ANC did not require transformation to achieve normality in any BAM type, time point, or preservative. The logarithm base 10 (log) transformation was necessary for CD11b MFI in Citrate at each time point and for both the No Preservative and Streck preservative assays. Integrin α9β1 MFI required log10 transformation for samples collected in Citrate and assayed at Time 24 hours. CD11b MFI also required the log transformation when collected in EDTA or Heparin in Streck preservative at Time 0 hours. For ease of interpretation, CD11b MFI and Integrin α9β1 MFI were log transformed for all the BAM experiments, which maintained normality even when the transformation was not initially necessary. Normality testing for the preservative experiments demonstrated that log transformation was required for ANC with each preservative type, Integrin α9β1 MFI in Streck preservative, and CD11b MFI at each preservative type. Therefore, for ease of interpretation the log transformation was applied to ANC, Integrin α9β1 MFI, and CD11b MFI for each preservative

type. This did not affect the normality of variables for which no transformation was initially necessary.

3.2 Repeated Measures Analysis of Variance (RM-ANOVA) Assessing Blood Acquisition Method

3.2.1 RM-ANOVA Assessing BAM, Preservative, and Time Effects on ANC

The overall RM-ANOVA model of ANC adjusted for BAM, Preservative type, and Time demonstrated a significant difference ($p<0.0001$) in ANC related to all variables and interaction terms tested in the model. The interaction between BAM*preservative*time (**Figure 1**) was also significant ($p<0.002$). Large effect sizes noted for each term in the model (partial-η^2 = 0.554 – 0.963).

The RM-ANOVA model of ANC with No Preservative (**Figure 2**), adjusted for BAM and Time, demonstrated a significant ($p <0.0001$) difference between BAM types and there was also a significant interaction between BAM and time ($p<0.004$). There was no significant difference between time points ($p = 0.759$). Large effect sizes were noted for each significant variable in the model (partial-η^2 = 0.953 and 0.629, respectively), and there was a small effect size for Time (partial-η^2 = 0.012). The RM-ANOVA model of ANC with Streck preservative (**Figure 3**), adjusted for BAM and Time, demonstrated a significant difference in ANC between BAM types ($p<0.0001$) and Time points ($p<0.0001$), and the interaction between BAM and Time was significant ($p<0.0001$). Large effect sizes were noted for each term in the model (partial-η^2 = 0.740 – 0.912).

Variability in ANC, adjusted for Time and Preservative type, was assessed for each specific BAM. There was no significant difference in ANC related to Time ($p = 0.072$) or the interaction between Preservative and Time ($p = 0.538$) for Citrate (**Figure 4**). Preservative had a significant effect on ANC in the model ($p = 0.044$). The effect size was large for Time (partial-$\eta^2 = 0.349$), small for the interaction term (partial-$\eta^2 = 0.049$), and large for Preservative (partial-$\eta^2 = 0.416$). There was a significant difference ($p<0.0001$) in ANC between Preservative types and Time points for both the Heparin (**Figure 5**) and EDTA models (**Figure 6**). Large effect sizes were noted for all main effect variables and the interaction term in the Heparin model (partial-$\eta^2 = 0.854 – 0.959$) as well as in the EDTA model (partial-$\eta^2 = 0.909 – 0.969$).

Variability of ANC, adjusted for BAM and Preservative type, was assessed for each individual Time point. At Time 0 Hours (**Figure 7**), there was a significant difference in ANC between the three BAM ($p<0.0001$), both Preservative types ($p<0.001$), and the interaction between BAM and Preservative was also significant ($p<0.001$). There was a large effect size for each of these variables (partial-$\eta^2 = 0.767 – 0.968$). Similarly, at Time 24 hours (**Figure 8**) there was a significant difference in ANC between BAM ($p<0.008$), Preservative type ($p<0.0001$), and the interaction between BAM and Preservative was also significant ($p<0.0001$). There was a large effect size for each of these variables (partial-$\eta^2 = 0.750 – 0.983$).

Further analysis of the change in raw ANC over time demonstrated that ANC declined an average of 7.31 – 12.86% over time in Citrate, with Streck preservative mitigating this effect (**Appendix A, Table 1**). Heparin demonstrated an average ANC increase of 16.34 – 52.39% over time, which was not mitigated by Streck preservative. EDTA had the greatest increase in ANC over time of 22.29 – 122.64%, made more pronounced with Streck preservative. The coefficient

of variation for ANC was overall most favorable in Heparin with Streck preservative, Citrate with No Preservative, and EDTA with No Preservative (**Appendix A, Table 2**).

3.2.2 RM-ANOVA Assessing BAM, Preservative, and Time Effects on CD11b MFI

The overall RM-ANOVA model (**Figure 9**) for log CD11b MFI was adjusted for BAM, time points, and preservative types. The model demonstrated a significant difference in log CD11b MFI for BAM ($p<0.0001$), preservative ($p<0.0001$), and the interaction terms BAM*preservative ($p<0.0001$), BAM*time ($p<0.0001$), time*preservative ($p<0.0001$), and BAM*time*preservative ($p<0.004$). Time had no significant effect on log CD11b MFI in the model ($p = 0.373$). Large effect sizes were noted for each variable and interaction term in the overall RM-ANOVA model for CD11b MFI (partial-$\eta^2 = 0.100 – 0.919$).

The RM-ANOVA model with No Preservative (**Figure 10**), adjusted for BAM and Time, demonstrated a significant ($p <0.001$) difference in log CD11b MFI due to Time, BAM ($p <0.001$), and the BAM*time interaction term was also significant in the model ($p= 0.002$). Large effect sizes were noted for each variable (partial-$\eta^2 = 0.546 – 0.751$). The RM-ANOVA model with Streck preservative (**Figure 11**), adjusted for BAM and Time, demonstrated that BAM ($p <0.0001$), Time ($p < 0.0001$), and the interaction of BAM and Time ($p <0.0001$) all significantly impacted log CD11b MFI. Large effect sizes were appreciated for each variable in the model (partial-$\eta^2 = 0.765 – 0.982$).

Variability of log CD11b MFI, adjusted for Time and Preservative type (**Figure 12**), was assessed in Citrate. The main effects of Preservative and Time, as well as the interaction between Preservative and Time, imparted significant ($p<0.0001$) differences in log CD11b MFI in Citrate. The variables and interaction term each demonstrated large effect sizes (partial-$\eta^2 = 0.871 –$

0.968). Change in log CD11b MFI in Heparin, adjusted for Time and Preservative type (**Figure 13**), demonstrated a significant effect of Time ($p = 0.014$), Preservative ($p < 0.0001$), and the interaction between Time and Preservative ($p = 0.003$) on log CD11b MFI. Large effect sizes were found for each variable and interaction term (partial-$\eta^2 = 0.549 – 0.932$). The change in log CD11b MFI in EDTA, adjusted for BAM and Preservative type (**Figure 14**), demonstrated a significant effect of Preservative ($p < 0.0001$) and the interaction between Time and Preservative ($p < 0.0001$) on log CD11b MFI. Time did not significantly impact log CD11b MFI in the EDTA model ($p = 0.181$). There was a large effect size for each variable and the interaction term (partial-$\eta^2 = 0.212 – 0.934$).

The variability of log CD11b MFI, adjusted for BAM and preservative type, was assessed for each individual time point. In the Time 0 hours model (**Figure 15**), BAM and Preservative demonstrated significant main effects ($p<0.0001$) on log CD11b MFI, while the interaction between the two variables was not significant ($p = 0.063$). There were large effect sizes for each variable and the interaction term (partial-$\eta^2 = 0.293 – 0.953$). Both BAM ($p = 0.023$) and Preservative ($p < 0.0001$) demonstrated significant main effects on log CD11b MFI, and the interaction between BAM and Preservative ($p < 0.0001$) was also significant in the Time 24 hours model (**Figure 16**). Large effect sizes were shown for each variable and the interaction term (partial-$\eta^2 = 0.376 – 0.953$).

Further analysis of the change in the non-transformed value of CD11b MFI over time demonstrated that CD11b MFI in Citrate increased by 3.71% over 24 hours with No Preservative and decreased 33.38% with Streck preservative (**Appendix B, Table 1**). CD11b MFI in Heparin increased 78.93% over time with No Preservative, whereas CD11b MFI decreased 12.12% over time with Streck preservative. CD11b MFI in EDTA showed the greatest increase over time,

112.40%, with No Preservative. Adding Streck preservative resulted in a 34.98% decrease in CD11b MFI in EDTA over time. The coefficient of variation for CD11b MFI was smallest for Heparin with Streck Preservative and Citrate with Streck Preservative (**Appendix B, Table 2**).

3.2.3 RM-ANOVA Assessing BAM, Preservative, and Time Effects on Integrin α9β1 MFI

The overall RM-ANOVA model of log Integrin α9β1 MFI (**Figure 17**), adjusted for the main effects of BAM, Preservative type, and Time, demonstrated a significant difference in log Integrin α9β1 MFI related to the main effects of BAM and Preservative ($p < 0.0001$). Time was not a significant main effect in the model ($p = 0.285$). The interactions between BAM and Time ($p < 0.0001$), BAM and Preservative ($p < 0.0001$), and Time and Preservative ($p = 0.025$) were also significant. The interaction between BAM, Time, and Preservative was not significant ($p = 0.193$). Large effect sizes noted for each variable and interaction term in the model (partial-$\eta^2 = 0.141 – 0.970$).

The RM-ANOVA model of log Integrin α9β1 MFI with No Preservative (**Figure 18**), adjusted for BAM and Time, demonstrated a significant ($p < 0.0001$) difference between BAM types, a significant effect of Time ($p = 0.021$), and there was also a significant interaction between BAM and Time ($p < 0.001$). Large effect sizes were noted for each main effect variable as well as for the interaction term in the model (partial-$\eta^2 = 0.507 – 0.805$). The RM-ANOVA model of log Integrin α9β1 MFI with Streck preservative (**Figure 19**), adjusted for BAM and Time, demonstrated a significant ($p < 0.0001$) difference in log Integrin α9β1 MFI between BAM types, and the interaction between BAM and Time was significant ($p < 0.011$). Time did not show a significant effect on log Integrin α9β1 MFI in the model ($p = 0.104$). Large effect sizes were noted for each term in the model (partial-$\eta^2 = 0.296 – 0.851$).

The variability in log Integrin α9β1 MFI, adjusted for Time and Preservative type, was assessed for each specific BAM. For the model of log Integrin α9β1 MFI in Citrate (**Figure 20**), there was a significant difference in log Integrin α9β1 MFI related to Time ($p = 0.009$) and Preservative ($p < 0.0001$), but the interaction between Preservative and Time was not significant ($p = 0.433$). The effect size was large for Time (partial-$\eta^2 = 0.598$) and Preservative (partial-$\eta^2 =$ 916), but small for the interaction term (partial-$\eta^2 = 0.079$). There was a significant difference ($p < 0.0001$) in log Integrin α9β1 MFI between Preservative types and Time points for the Heparin model (**Figure 21**), and the interaction between Time and Preservative was also significant in this model ($p = 0.001$). Large effect sizes were noted for all main effects and the interaction term (partial-$\eta^2 = 0.757 – 0.945$). The EDTA model, adjusted for Preservative and Time (**Figure 22**), revealed significant differences in log Integrin α9β1 MFI related to the main effects of Time ($p = 0.015$) and Preservative ($p < 0.0001$). The interaction between Time and Preservative was not significant in the model ($p = 0.145$). The effect sizes for each main effect variable and the interaction term were large (partial-$\eta^2 = 0.246 – 0.949$).

Variability of log Integrin α9β1 MFI, adjusted for BAM and Preservative type, was subsequently assessed for each individual Time point. At Time 0 hours (**Figure 23**), there was a significant difference in log Integrin α9β1 MFI related to the main effects of BAM ($p<0.0001$) and Preservative type ($p<0.0001$), and the interaction between BAM and Preservative was also significant ($p<0.018$). There was a large effect size for each of the variables and the interaction term (partial-$\eta^2 = 0.482 – 0.859$). Similarly, at Time 24 hours (**Figure 24**) there was a significant difference in log Integrin α9β1 MFI related to BAM ($p<0.001$) and Preservative type ($p<0.0001$), and the interaction between BAM and Preservative was also significant ($p<0.0001$). There was a large effect size for each of these variables (partial-$\eta^2 = 0.731 – 0.960$).

Further analysis of the change in the raw Integrin α9β1 MFI over time demonstrated that in Citrate, Integrin α9β1 MFI increased an average of 8.56% over time with No Preservative. When Streck was added, Integrin α9β1 MFI in Citrate increased only 3.75% over time (**Appendix C, Table 1**). Integrin α9β1 MFI in Heparin decreased by an average of 2.22% over time with No Preservative. Integrin α9β1 MFI in Heparin with Streck Preservative resulted in an average decrease of 24.62% over time. Integrin α9β1 MFI in EDTA with No Preservative increased by 33.86% over time, Streck mitigated this effect with an average increase of Integrin α9β1 MFI of 0.17% over time. The coefficient of variation for Integrin α9β1 MFI was lowest for Citrate with Streck, Heparin with either preservative type, and EDTA with No Preservative (**Appendix C, Table 2**).

3.3 Student's Paired T-Test and Wilcoxon Signed-Rank Test Analysis of Main Effects from Blood Acquisition Method RM-ANOVA Models

The overall and reduced RM-ANOVA models above demonstrated that significant differences existed due to both main effects and interaction terms within the models. Specific pairwise comparisons to evaluate each main effect were completed using either the Student's Paired T-Test or the Wilcoxon Signed-Rank Test, depending on normality.

3.3.1 Student's Paired T-Test Comparison of each Main Effect on ANC

The two-sided Student's paired t-test was used to individually assess the main effects of BAM on ANC, controlling for Time and Preservative. There were non-significant differences in ANC between Citrate and Heparin at Time 0 with Streck preservative and between Heparin and

EDTA at Time 24 hours with Streck preservative. All other pairwise comparisons were significant (**Table 1**).

Next, the two-sided Student's paired t-test was used to assess the effect of each Time point on ANC, controlling for BAM and Preservative. There were non-significant differences in ANC between Time 0 hours and Time 24 hours in Citrate with Streck and Heparin with No Preservative. All other differences in ANC between Time points were significant (**Table 2**).

A two-sided Student's paired t-test was also used to assess the effect of each Preservative type on ANC, controlling for BAM and Time. There were non-significant differences in ANC between Preservative types with Citrate at Time 24 hours and with EDTA at Time 0 hours. All other differences in ANC between Preservative types were significant (**Table 3**).

3.3.2 Wilcoxon Signed-Rank Test Analysis of each Main Effect on Log CD11b MFI

A two-sided Wilcoxon Signed-Rank test was used to individually evaluate the RM-ANOVA model main effects (BAM, Time, Preservative) on log CD11b MFI since the non-transformed variable was not normal and log transformation did not achieve normality across all parameters. There were non-significant differences in log CD11b MFI between Citrate and Heparin at Time 24 hours with No Preservative and between Citrate and EDTA at Time 24 hours with No Preservative. Additionally, there was a non-significant difference in log CD11b MFI between Heparin and EDTA at Time 0 hours with Streck preservative. All other differences in log CD11b MFI between BAM types were significant, controlling for Time and Preservative variables (**Table 4**).

The two-sided Wilcoxon Signed-Rank test was then used to determine the effect of Time on log CD11b MFI, controlling for BAM and Preservative. There was a non-significant difference

in log CD11b MFI between time points at Citrate with No Preservative. All other comparisons between Time points were significant for changes in log CD11b MFI (**Table 5**).

The two-sided Wilcoxon Signed-Rank test was also used to determine the effect of Preservative on log CD11b MFI, controlling for BAM and Time. There were non-significant differences in log CD11b MFI between Preservative types at Time 0 hours in Citrate and at Time 0 hours in Heparin. All other differences in log CD11b MFI between Preservative types were significant (**Table 6**).

3.3.3 Student's Paired T-Test Analysis of Each Main Effect on Log Integrin α9β1 MFI

Finally, a two-sided Student's paired t-test was used to individually assess the main effects of BAM, Time, and Preservative from the RM-ANOVA model on log Integrin α9β1 MFI. There was a non-significant difference in log Integrin α9β1 MFI between Citrate and EDTA at Time 0 hours with Streck. All other BAM comparisons, controlling for Time and Preservative, demonstrated significant changes in log Integrin α9β1 MFI (**Table 7**).

The two-sided Student's paired t-test was also used to assess the effect of each Time point on log Integrin α9β1 MFI, controlling for BAM and Preservative. Non-significant differences in log Integrin α9β1 MFI between Time 0 hours and Time 24 hours were generally revealed, with the exception of Heparin with Streck and EDTA with No Preservative (**Table 8**).

The two-sided Student's paired t-test was again used to assess the effect of each Preservative type on log Integrin α9β1 MFI, controlling for BAM and Time. All differences in log Integrin α9β1 MFI between the Preservative types were found to be significant (**Table 9**).

3.4 Evaluation of the Effect of Preservative Types and Time Points on ANC, CD11b MFI, and Log Integrin α9β1 MFI

3.4.1 RM-ANOVA Model of ANC Adjusted for Preservative and Time Effects

The overall RM-ANOVA model of ANC, adjusted for the main effects of Preservative and Time (**Figure 25**) and including the interaction between Preservative and Time, revealed significant differences in log ANC for the main effect of Preservative type ($p < 0.0001$). The interaction between Preservative and Time was also significant ($p < 0.0001$). The model did not demonstrate significant differences in log ANC due to Time ($p = 0.453$) and there was a relatively small effect size for this main effect (partial-$\eta^2 = 0.020$). Large effect sizes were noted for Preservative and the interaction term (partial-$\eta^2 = 0.780$ and 0.348, respectively).

The change in log ANC over time was explored for each Preservative type. The average change in log ANC declined over time with No Preservative (**Figure 26**) but increased modestly with Streck (**Figure 27**) and BCT (**Figure 28**). The non-transformed values of ANC were assessed for change over Time at each Preservative type and demonstrated the same trends (**Appendix D**).

Paired sample testing was performed using Wilcoxon Signed-Rank test or Student's Paired T-Test, based on normality testing as described previously, in order to determine the effect of each discrete covariate in the RM-ANOVA model on the change in Log ANC. The effect of Preservative type at each Time point was assessed and all differences in log ANC were found to be significant, apart from non-significant differences in log ANC between Streck and BCT at both time points (**Table 10**).

The effect of Time at each Preservative was evaluated and revealed significant differences in log ANC between Time points for Streck and BCT; however, there was a non-significant difference in log ANC over time with No Preservative (**Table 11**).

3.4.2 RM-ANOVA Model of CD11b MFI Adjusted for Preservative and Time Effects

The overall RM-ANOVA model of CD11b MFI, adjusted for the main effects of Preservative and Time (**Figure 29**) and including the interaction between Preservative and Time, displayed significant differences in log CD11b MFI for the main effects of Preservative type ($p < 0.0001$) and Time ($p < 0.0001$). The interaction between Preservative and Time was also significant ($p < 0.0001$). Large effect sizes were observed for the main effects and the interaction term (partial-$\eta^2 = 0.580 – 0.930$).

The change in log CD11b MFI over time was explored for each Preservative type. The average change in log CD11b MFI increased over time with No Preservative (**Figure 30**), Streck (**Figure 31**) and BCT (**Figure 32**). The non-transformed values of CD11b MFI were assessed for change over Time at each Preservative type and demonstrated the same trends (**Appendix E**). Of note, CD11b MFI was markedly elevated with No Preservative as compared to Streck and BCT.

Paired sample testing was performed in order to determine the effect of each discrete covariate in the RM-ANOVA model on the change in log CD11b MFI. The effect of Preservative type at each Time point was assessed and all differences in log CD11b MFI were found to be significant, apart from a non-significant difference in log CD11b MFI between Streck and BCT at Time 24 hours (**Table 12**).

The effect of Time at each Preservative was evaluated and revealed significant differences in log CD11b MFI between Time points for No Preservative, Streck, and BCT (**Table 13**).

3.4.3 RM-ANOVA Model of Integrin α9β1 MFI Adjusted for Preservative and Time Effects

The overall RM-ANOVA model of Integrin α9β1 MFI, adjusted for the main effects of Preservative and Time (**Figure 33**) and including the interaction between Preservative and Time, exhibited significant differences in log Integrin α9β1 MFI for the main effects of Preservative type ($p < 0.0001$) and Time ($p < 0.0001$). The interaction between Preservative and Time was also significant ($p < 0.0001$). Large effect sizes were observed for the main effects and the interaction term (partial-$\eta^2 = 0.461 – 0.897$).

The variability in log Integrin α9β1 MFI over time was evaluated for each Preservative type. The average change in log Integrin α9β1 MFI increased over time with No Preservative (**Figure 34**) and declined modestly over time with Streck (**Figure 35**) and BCT (**Figure 36**). The non-transformed values of Integrin α9β1 MFI were assessed for change over Time at each Preservative type and showed the same trends (**Appendix F**).

Paired sample testing was performed in order to determine the effect of each discrete covariate in the RM-ANOVA model on the change in log Integrin α9β1 MFI. The effect of Preservative type at each Time point was assessed and all differences in log Integrin α9β1 MFI were found to be significant, except for a non-significant difference in log Integrin α9β1 MFI between Streck and BCT at Time 0 hours (**Table 14**).

The effect of Time at each Preservative was evaluated and displayed significant differences in log CD11b MFI between Time points for No Preservative, Streck, and BCT (**Table 15**).

4.0 Discussion

Flow cytometry is an established method to identify cell types from whole blood or plasma samples, as well as for cell-surface biomarker specification. Despite this, there exists a lack of consensus in the literature regarding the optimal specimen acquisition and preparation methodology for rapid, real-time neutrophil analysis. Many existing protocols utilize density gradients and fixatives that can alter cell-surface biomarker expression and bias counts of target populations. CD11b is a commonly measured neutrophil cell-surface biomarker; however, no existing protocol fit our specific needs for a rapid clinical test. Furthermore, Integrin α9β1 is a much less frequently measured neutrophil cell-surface biomarker; therefore, it was difficult to find protocols in the literature describing an approach to analyzing this biomarker and no existing protocol was suitable for our needs. Consequently, we developed a rapid lyse/no-wash method for clinical flow cytometry identification and enumeration of neutrophils, as well as the neutrophil cell-surface biomarkers CD11b and Integrin α9β1. The first stage of protocol development entailed testing and selecting an appropriate blood acquisition venipuncture tube (BAM) while the second stage of protocol development assessed the effects of three preservative states on our outcomes: CD11b MFI, Integrin α9β1 MFI, and absolute neutrophil count. The initial intent for this protocol was to incorporate a preservative to reduce sample degradation expected to occur in real-world clinical situations where there are varied intervals of time between sample collection and sample processing. Neutrophils have a short life span *in vivo*, approximately 24 hours, so preserving neutrophil integrity prior to sample processing is important for a clinical test reliant on neutrophils.

The BAM experiment did not reveal a clearly superior choice of venipuncture tube; no preservative type or BAM reliably demonstrated greater consistency over time. There were

statistically significant interactions between BAM, Time, and Preservative for the overall RM-ANOVA model of ANC. Reduced RM-ANOVA model testing for ANC was subsequently performed for each specific BAM and it revealed that Citrate had fewer significant differences in ANC attributable to the main effects and interaction terms in the model as compared to Heparin or EDTA. Further matched-pairs analysis of each main effect demonstrated the least ANC variability over time in Citrate with Streck or Heparin with No Preservative. Raw ANC values demonstrated a decline over time in Citrate, which was expected to occur due to cell degradation. This decline was mitigated by addition of Streck preservative; however, there were wide 95% confidence intervals surrounding the point estimate of the mean ANC in both preservative arms. ANC in Heparin or EDTA increased over time regardless of preservative type, and this was particularly marked in EDTA with Streck preservative, but Streck greatly narrowed the 95% confidence intervals for both Heparin and EDTA. Overall, EDTA with Streck preservative demonstrated the most variability in ANC over time. Since ANC cannot objectively increase with time, even in the absence of preservative, this must be interpreted as artifact.

These findings suggest that for ANC, Citrate with Streck preservative is the least variable specimen collection and preservation option. However, the user should note that ANC declines by 7.31% over time in this configuration and the 95% confidence intervals are wide for the point estimate of ANC at each time point and overlap the 95% confidence intervals of Heparin with Streck at Time 0 hours (**Appendix A**). Despite statistical significance of the changes in ANC noted over time and between BAM and preservative types, no changes were clinically significant. Streck preservative is a proprietary solution that contains EDTA, among many other ingredients. EDTA is a calcium chelating agent used to prevent clotting and preserve cell morphology, but it is possible that the composition of Streck interacts with the EDTA present in venipuncture tube resulting in

untoward neutrophil membrane changes and non-specific antibody binding. It is difficult to fully evaluate this possible confounding factor without a thorough understanding of the composition of Streck preservative solution.

The overall RM-ANOVA model of CD11b MFI adjusted for the main effects of BAM, Time, and Preservative demonstrated that all main effects, except for Time, contributed to significant changes in CD11b MFI. The interaction terms for each main effect were also found to be significant. Analysis of each main effect using the Wilcoxon Signed-Rank test did not reveal superiority of any one BAM. Citrate with no preservative had the least absolute change in raw CD11b MFI over time, followed by Heparin with Streck solution, but the 95% confidence intervals were much wider for the point estimate of CD11b MFI in Citrate without preservative as compared to Heparin with Streck. EDTA demonstrated the most CD11b MFI variability overall. CD11b MFI declined by only 12.12% in Heparin with Streck, which was the smallest change in CD11b MFI over time compared to Citrate or EDTA with Streck (**Appendix B**). CD11b MFI increased over time in each BAM when no preservative was added. Streck mitigated this increase likely by reducing non-specific binding that occurs over time as the sample degrades. Based on this data, Heparin with Streck demonstrated the least variability for CD11b MFI.

The overall RM-ANOVA model of Integrin α9β1 MFI, adjusted for the main effects and interaction terms, demonstrated that all main effects, except for Time, were significant in the model and all interactions between the main effects were significant, apart from the interaction between BAM, Time, and Preservative. Paired sample testing of each main effect revealed non-significant differences in log Integrin α9β1 MFI over time for Citrate with and without Streck as well as in Heparin without Streck, all other differences over time being significant. However, assessment of the raw Integrin α9β1 MFI showed that the point estimates of the mean had wide 95% confidence

intervals for Citrate with Streck, Heparin without Streck, and EDTA with either preservative. Additionally, Integrin α9β1 MFI increased over time for all BAM and preservative combinations except Heparin. Streck did reduce the increase in Integrin α9β1 MFI over time, likely by preventing non-specific binding. Heparin with Streck was determined to be the best specimen collection and preservation option for Integrin α9β1 because Integrin α9β1 MFI in Heparin exhibited a biologically plausible decline over time, as well as the least variability around the point estimate for Integrin α9β1 MFI (**Appendix C**).

Heparin was chosen as the BAM method for this protocol because it demonstrated the most biologically plausible trajectory over time for CD11b and Integrin α9β1, as well as the least variable point estimates for ANC, CD11b MFI, and Integrin α9β1 MFI - particularly with the addition of Streck. Due to changes over time in each outcome of interest, samples should be processed as close to collection time as possible, although predictable increases in ANC as well as decreases in CD11b MFI and Integrin α9β1 MFI can be accounted for when interpreting results of these tests as long as they are performed within a 24-hour window. In order to verify the results of the preservative effect seen in the BAM analyses, subsequent experiments were conducted to more thoroughly evaluate the effects of preservative type and time on ANC, CD11b MFI, and Integrin α9β1 MFI. Heparin was used as the venipuncture blood collection tube for the No Preservative and Streck arms of the study, a specialized Streck Blood Collection Tube was also assessed.

The overall RM-ANOVA model for variability of ANC adjusted for Preservative and Time revealed that only the Preservative main effect and the interaction between Preservative and Time were significant in the model. Paired sample testing of each main effect showed that ANC changed significantly between No Preservative and the two preservative types, but there was a non-significant change between Streck and BCT. Additionally, although there was a significant change

in ANC over time for Streck and BCT, the actual change in ANC over time was only 8.36% for Streck, 6.89% for BCT, and 6.30% for No Preservative (**Appendix D, Table 1**). Clinically, these changes were not significant. The main effects and interaction term were significant in both overall RM-ANOVA models for change in CD11b MFI and Integrin α9β1 MFI. Changes in CD11b MFI and Integrin α9β1 MFI with Streck and BCT followed similar trends over time, which differed from the trends noted in the No Preservative arm. The MFI of CD11b was markedly different with No Preservative as compared to Streck and BCT, this could relate to non-specific binding. No such discrepancies were seen in the BAM analyses, so it is less likely that CD11b expression is being suppressed by the preservatives. CD11b MFI increased modestly over time in Streck and BCT, while there was a large increase over time in No Preservative. Integrin α9β1 MFI did not demonstrate such wild fluctuations between preservative types. Integrin α9β1 MFI declined over time in both Streck and BCT but increased in No Preservative. Streck preservative solution and Streck BCT were selected as the most optimal preservative methods due to their minimal variability over time for CD11b and Integrin α9β1 MFI. Additionally, the clinically insignificant changes in ANC over time negated the statistically significant differences noted over time for Streck and BCT.

Streck and BCT proved to be the least variable preservative methods for the outcomes of interest. Streck solution is added in a 1:1 ratio to whole blood sample following specimen collection, while BCT is a separate venipuncture tube. Heparin is a common anticoagulant in hospitals and heparinized venipuncture tubes are widely available, whereas it is less cost effective and feasible for a hospital to purchase a separate blood collection tube (such as the Streck BCT) for a single laboratory test. Given these real-world concerns, Streck preservative solution added to

heparin venipuncture tubes is the most reasonable method by which to perform the rapid lyse/no-wash neutrophil flow cytometric assay in a clinical setting.

We did make slight adjustments to the biomarker panel between the BAM and Preservative experiments to streamline the gating scheme, this did not alter our outcomes of interest (**Appendix G**). CD11b and Integrin α9β1 biomarkers remained the same, but we incorporated CD66b PE-Cy7 as a confirmatory neutrophil biomarker and removed CD33 APC-vio770 and CD14 PE-Cy7. CD16 was changed from PE-Cy5 to APC-Cy7 for compatibility with the new biomarker panel. Another limitation of the study are the discrepancies between the BAM, particularly related to change in ANC over time. Although Heparin with Streck showed less point estimate variability than Citrate or EDTA for all three outcomes of interest, the changes over time assessed by paired samples testing were more often significant in Heparin and EDTA. This emphasizes the important difference between statistical and clinical significance. Clinically, the differences in ANC are not impressive and the numerical variability can be accounted for over time. Further evaluation of any potential biological interactions between the preservatives tested in this study and CD11b and Integrin α9β1 would be useful to determine if the depressed MFIs are real or due to chemical interactions with the preservatives. Alternatively, the increased MFIs noted in the No Preservative samples could relate to non-specific binding that is prevented by the preservatives. Unfortunately, the preservatives are proprietary commercial products and the company has declined to share the ingredient list with the author; therefore, formal analysis into potential biological interactions cannot be completed at this time.

In the future, replication of these studies in healthy subjects would be useful to confirm the findings herein. Additionally, extension of these studies in diseased populations would be useful to confirm the effects of BAM, Preservative type, and Time on ANC, CD11b MFI, and Integrin

α9β1 MFI and evaluate their diagnostic and prognostic value during an infectious challenge. This study demonstrates that specimen collection, preservation, and time to sample processing can result in significant changes in cell count and cell-surface membrane biomarker fluorescence. The results of this study highlight the importance of testing and standardizing sampling procedures and protocols in flow cytometric assays in advance of clinical or research applications.

5.0 Figures

The 95% confidence intervals displayed in all figures depicting RM-ANOVA analyses represent 95% confidence intervals for the estimated marginal means in the model, not the 95% confidence intervals for the actual sample means.

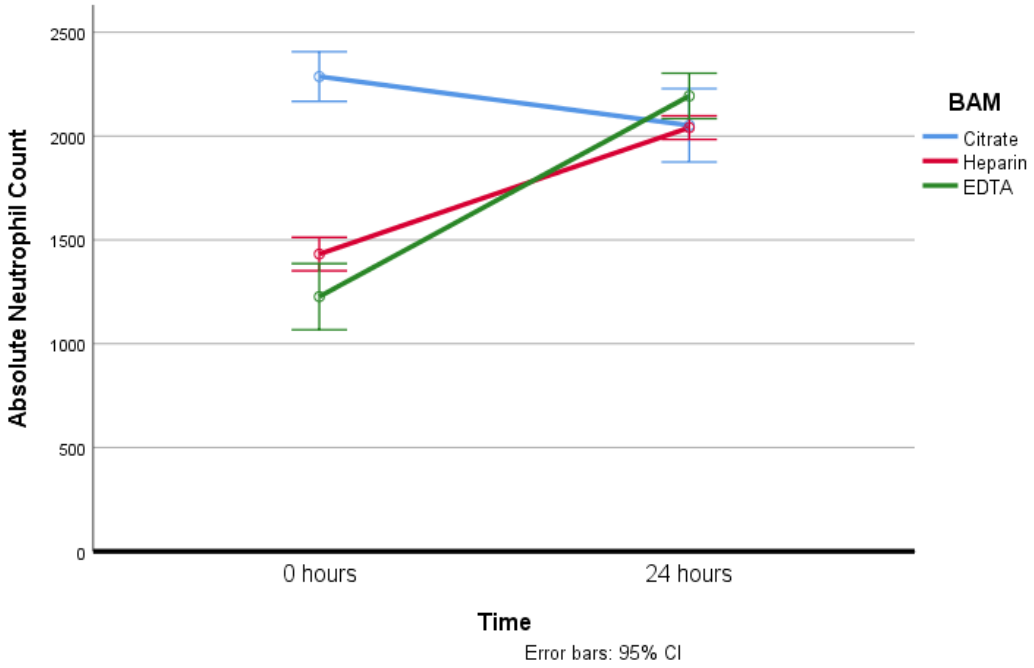


Figure 1. Overall RM-ANOVA Model for ANC Adjusted for BAM, Time Points, and Preservative Types

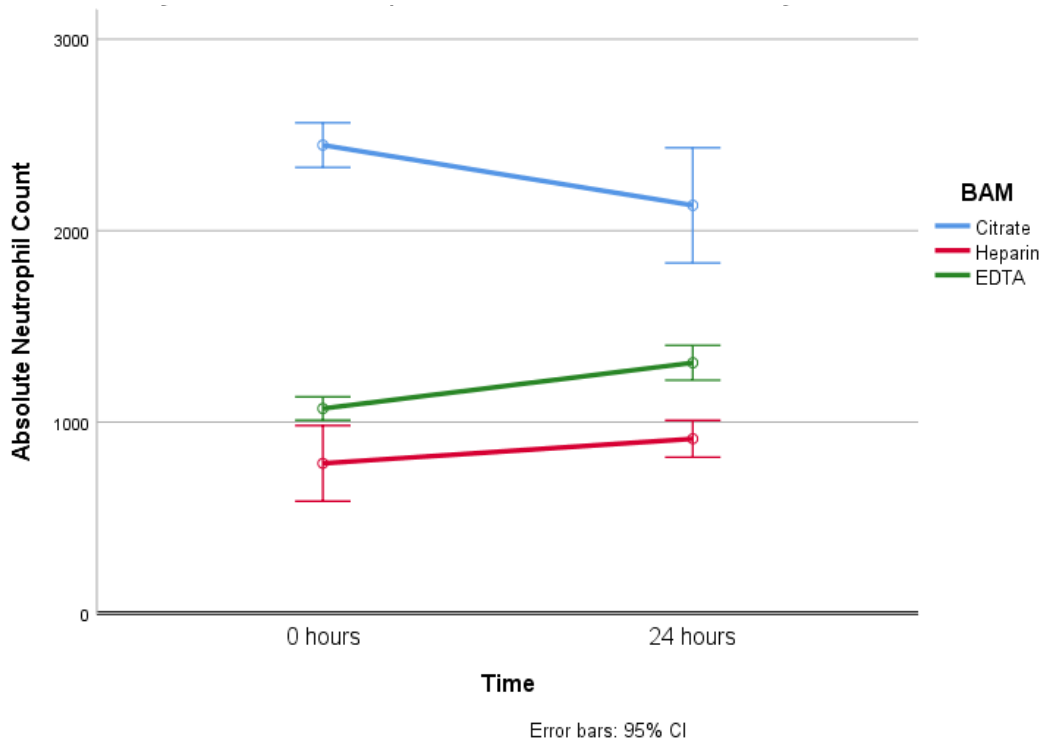


Figure 2. RM-ANOVA Model for ANC Adjusted for BAM and Time Points, with No Preservative

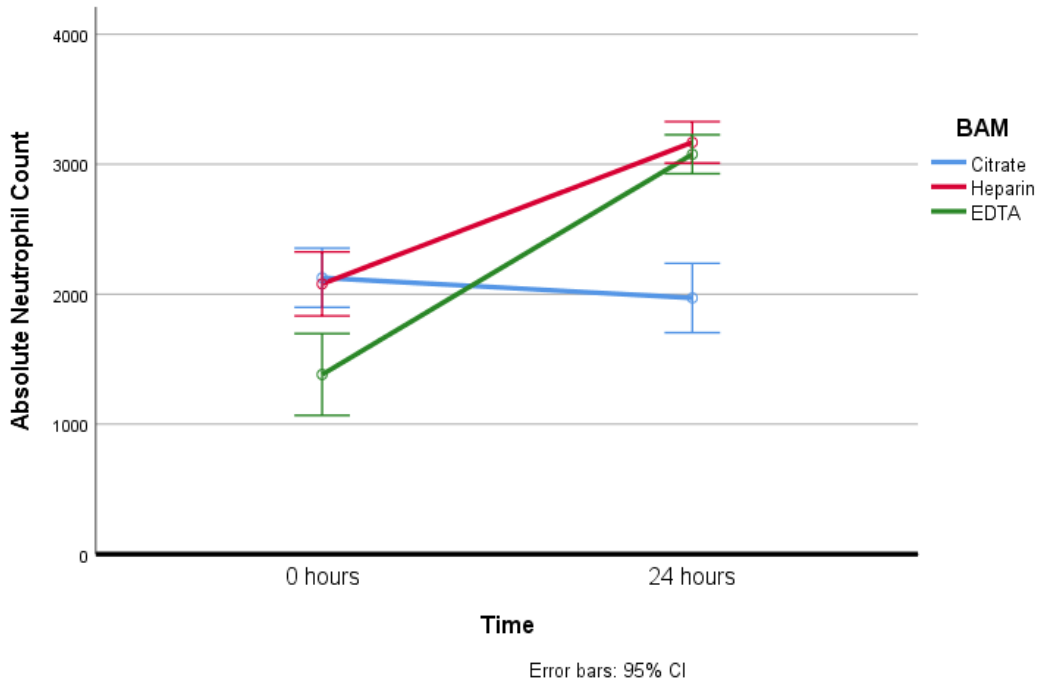


Figure 3. RM-ANOVA Model for ANC Adjusted for BAM and Time Points, with Streck Preservative

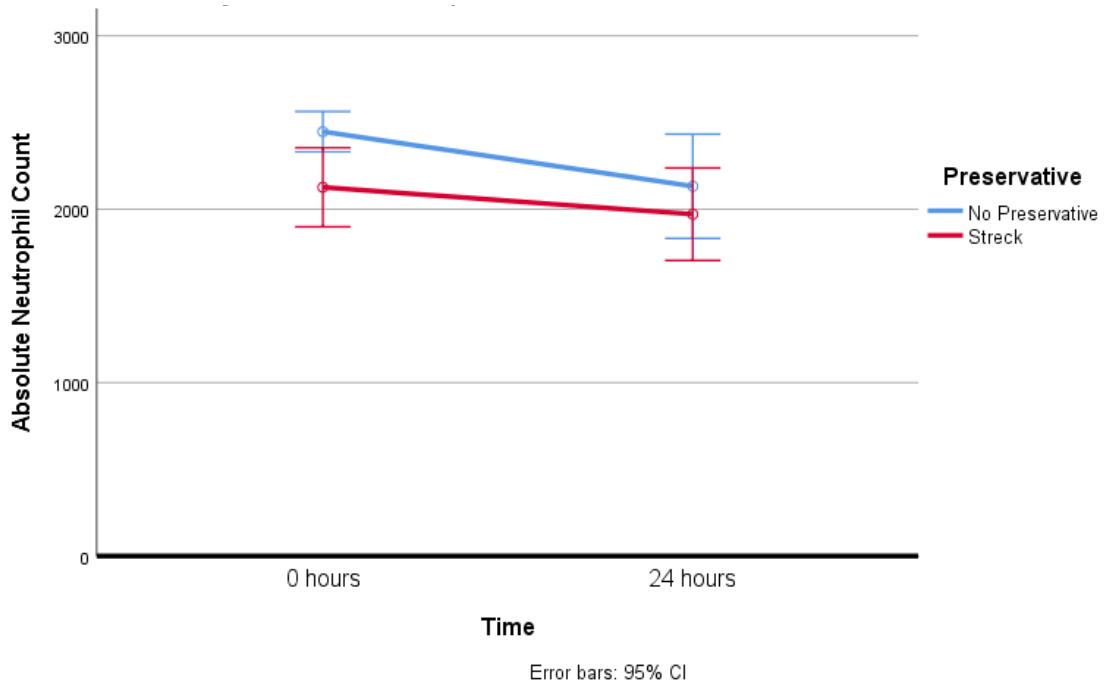


Figure 4. Variability of ANC in Citrate Adjusted for Preservative Types and Time Points

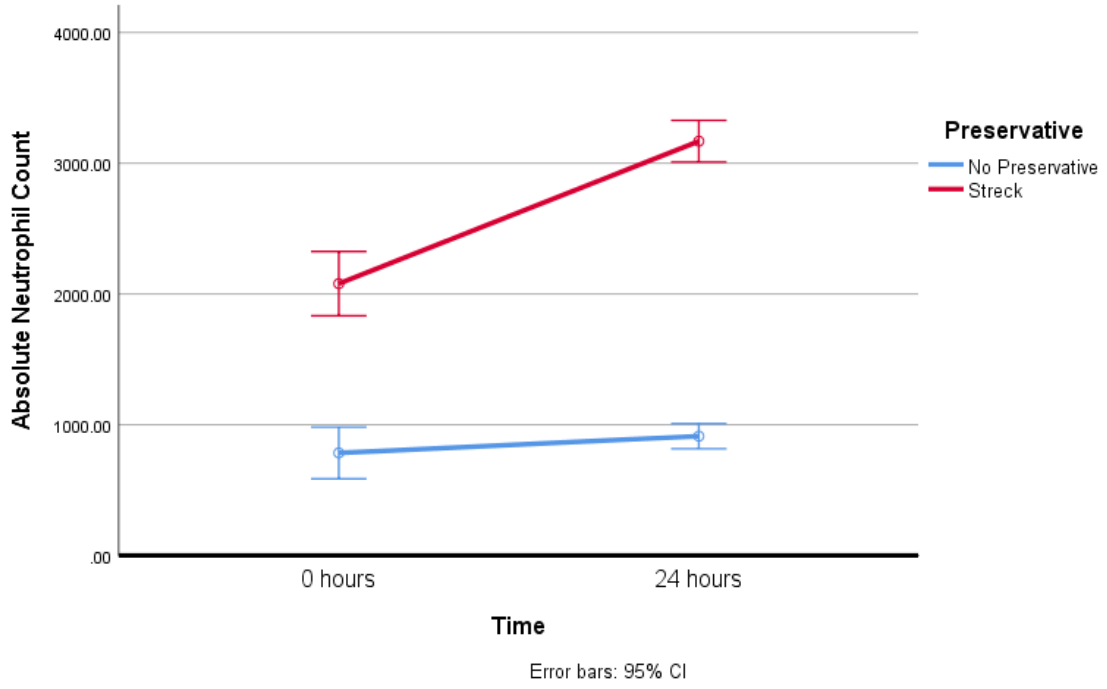


Figure 5. Variability of ANC in Heparin Adjusted for Preservative Types and Time Points

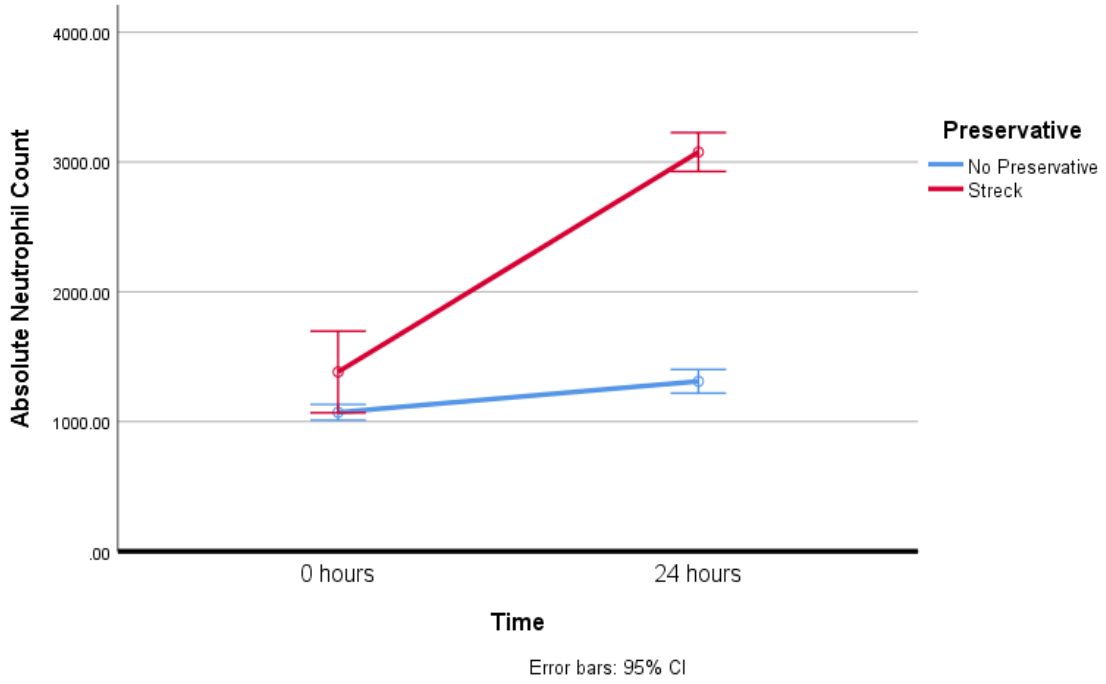


Figure 6. Variability of ANC in EDTA Adjusted for Preservative Types and Time Points

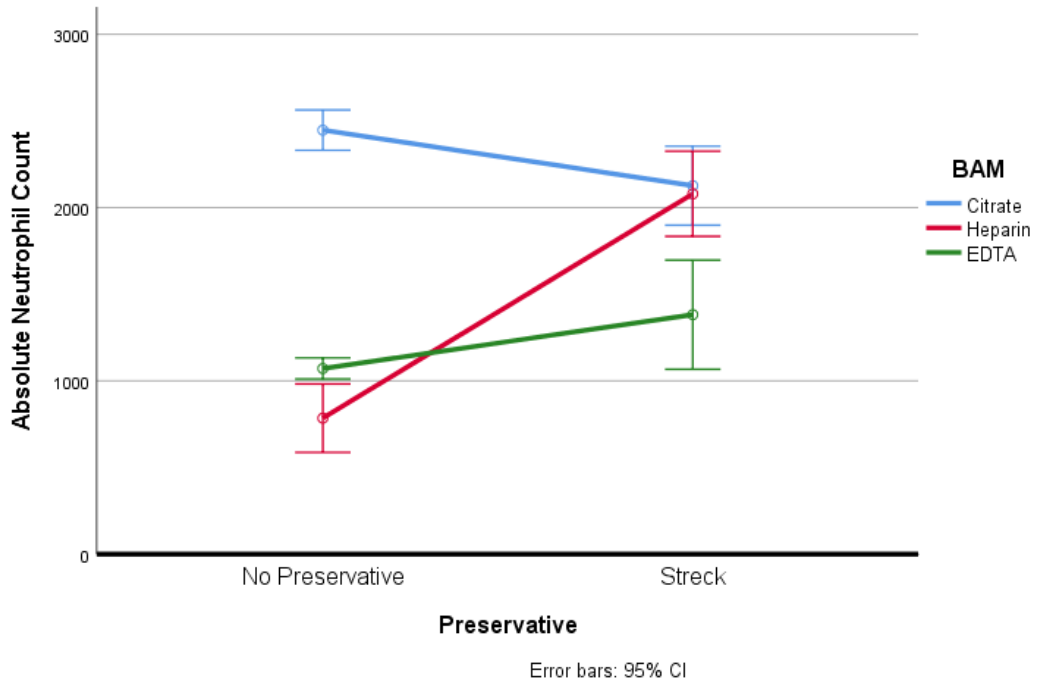


Figure 7. Variability in ANC Adjusted for BAM and Preservative Type at Time 0 Hours

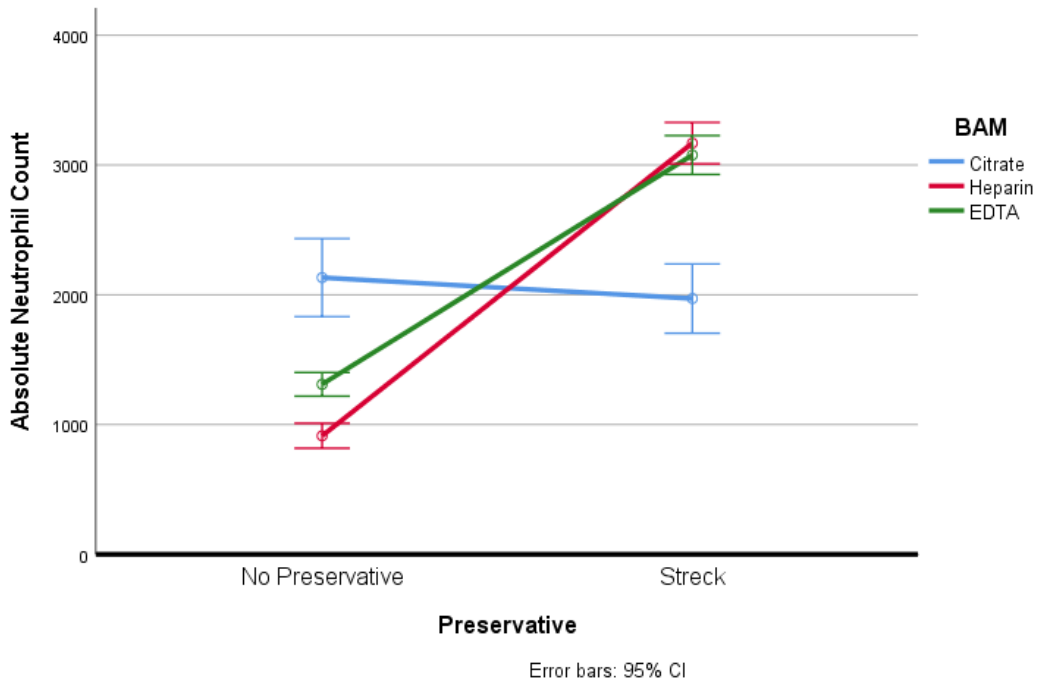


Figure 8. Variability in ANC Adjusted for BAM and Preservative Type at Time 24 Hours

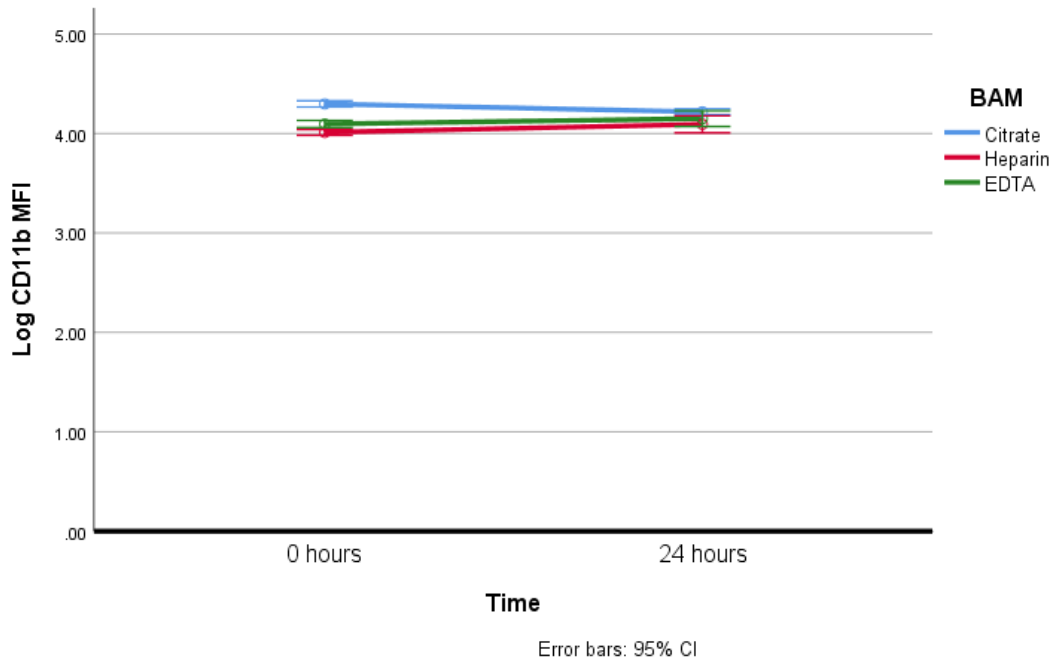


Figure 9. Overall RM-ANOVA Model for CD11b MFI Adjusted for BAM, Time Points, and Preservative Types

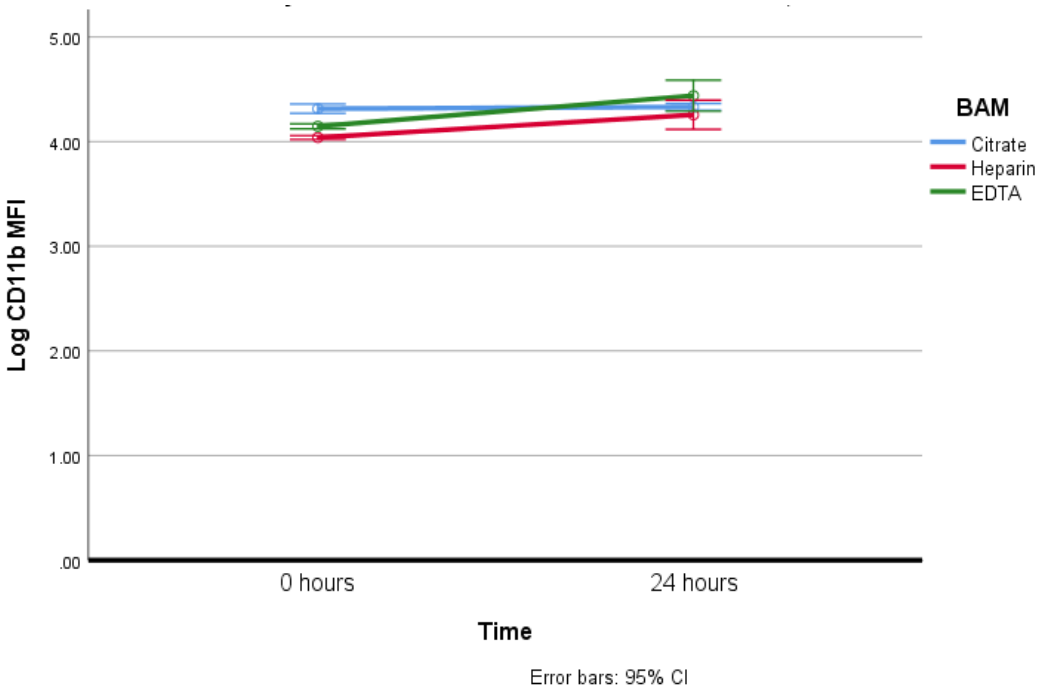


Figure 10. RM-ANOVA Model for CD11b MFI Adjusted for BAM and Time Points, with No Preservative

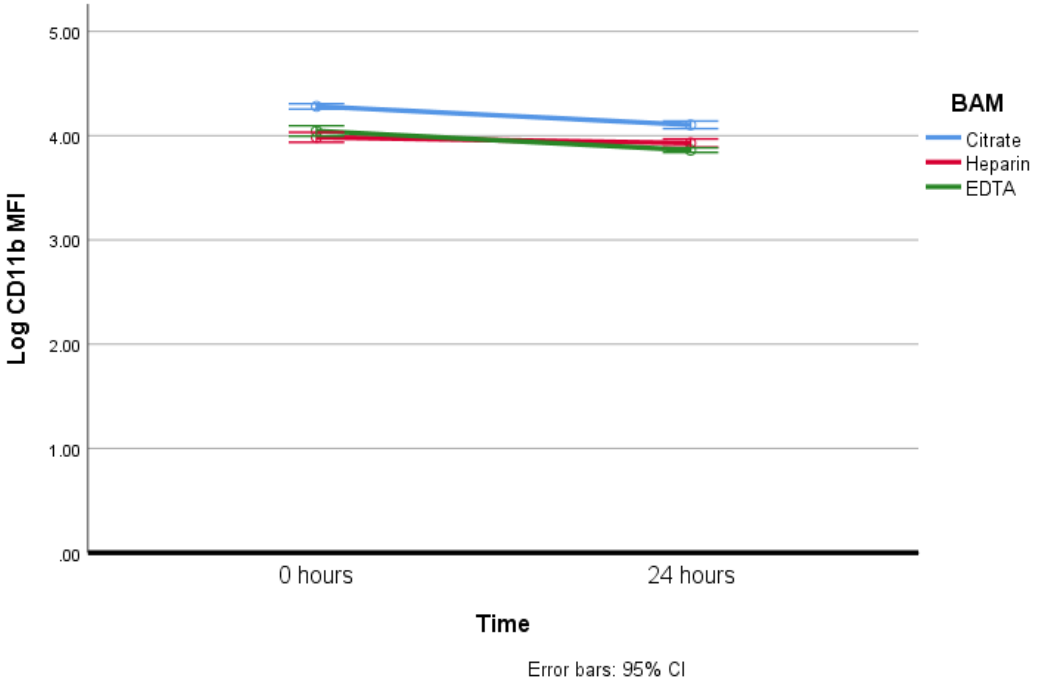


Figure 11. RM-ANOVA Model for CD11b MFI Adjusted for BAM and Time Points, with Streck Preservative

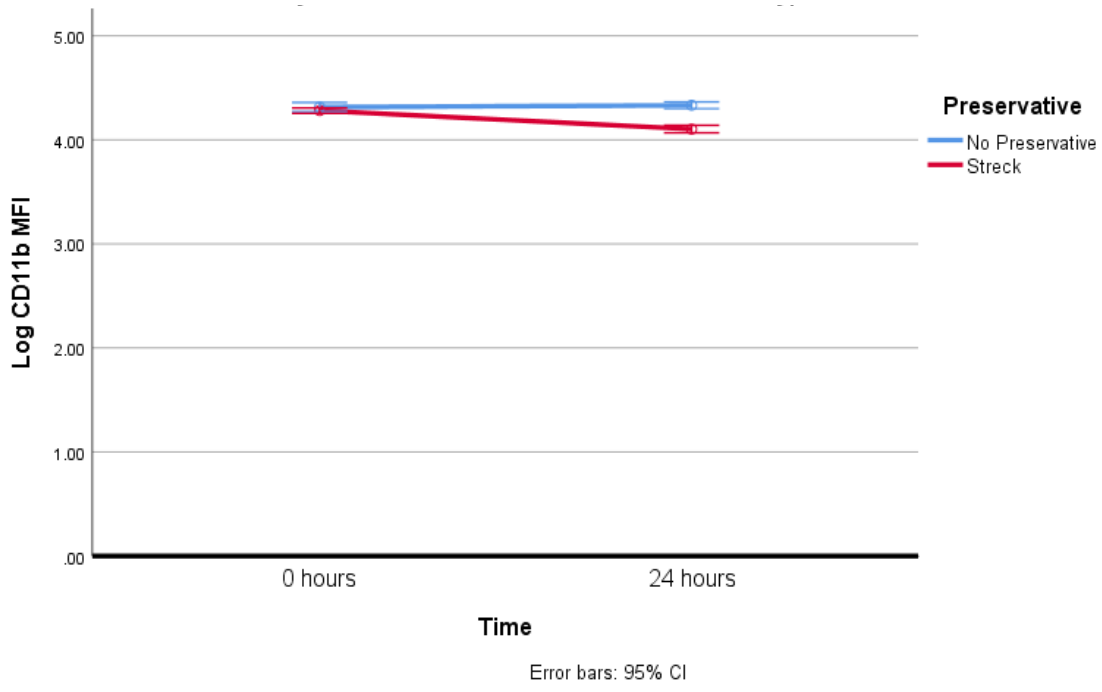


Figure 12. Variability of CD11b in Citrate Adjusted for Preservative Types and Time Points

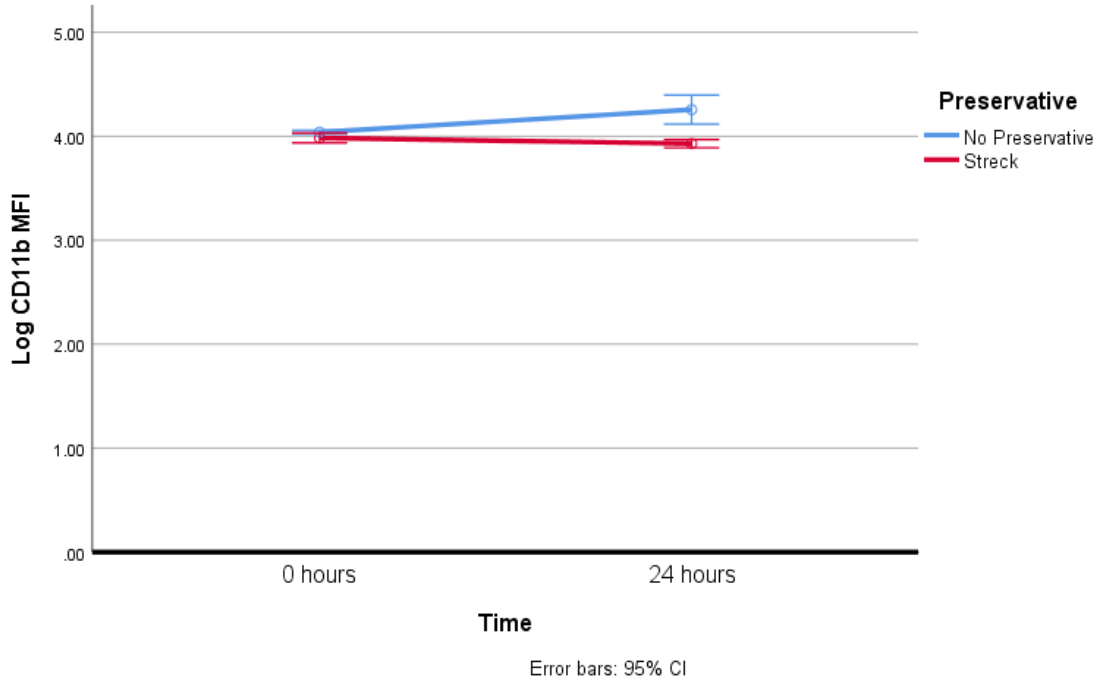


Figure 13. Variability of CD11b MFI in Heparin Adjusted for Preservative Types and Time Points

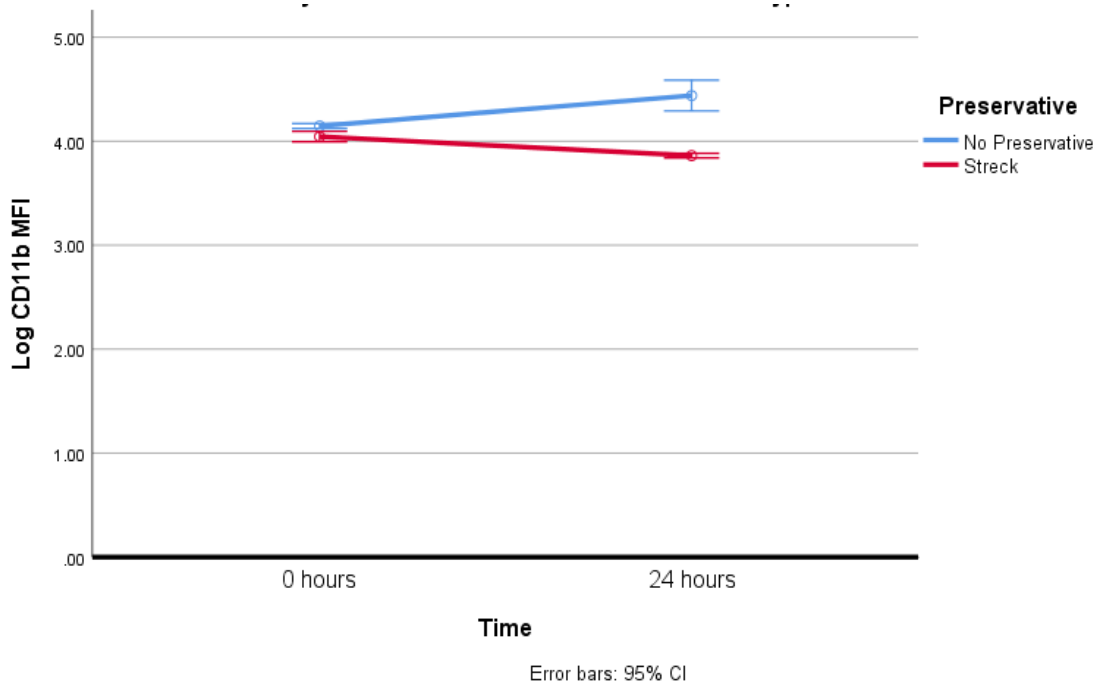


Figure 14. Variability of CD11b MFI in EDTA Adjusted for Preservative Types and Time Points

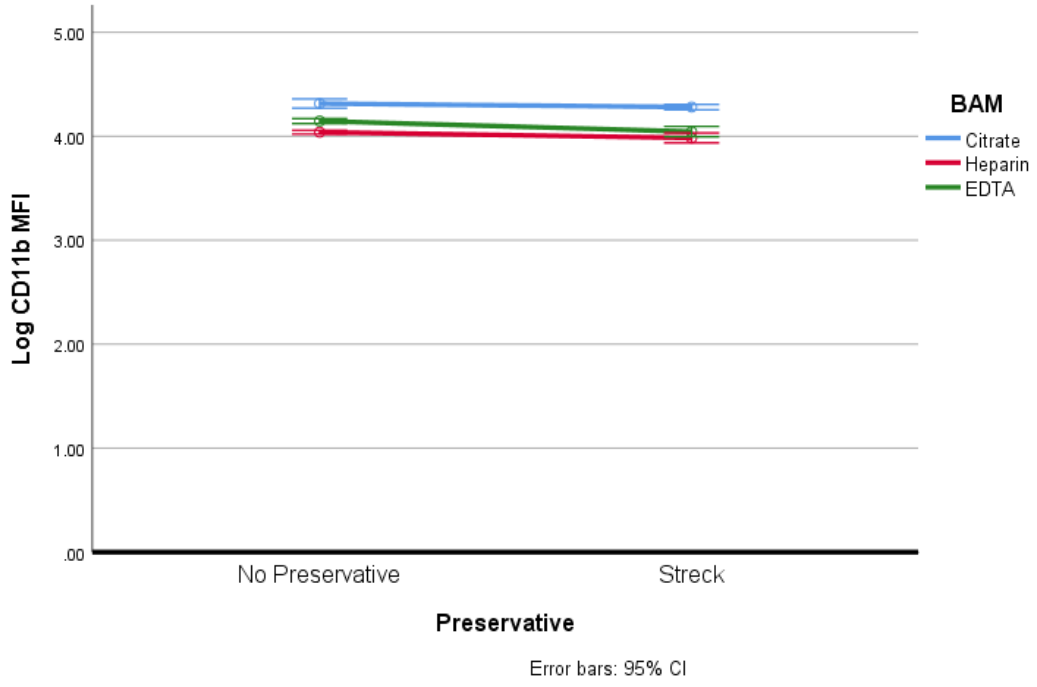


Figure 15. Variability in CD11b MFI Adjusted for BAM and Preservative Types, at Time 0 Hours

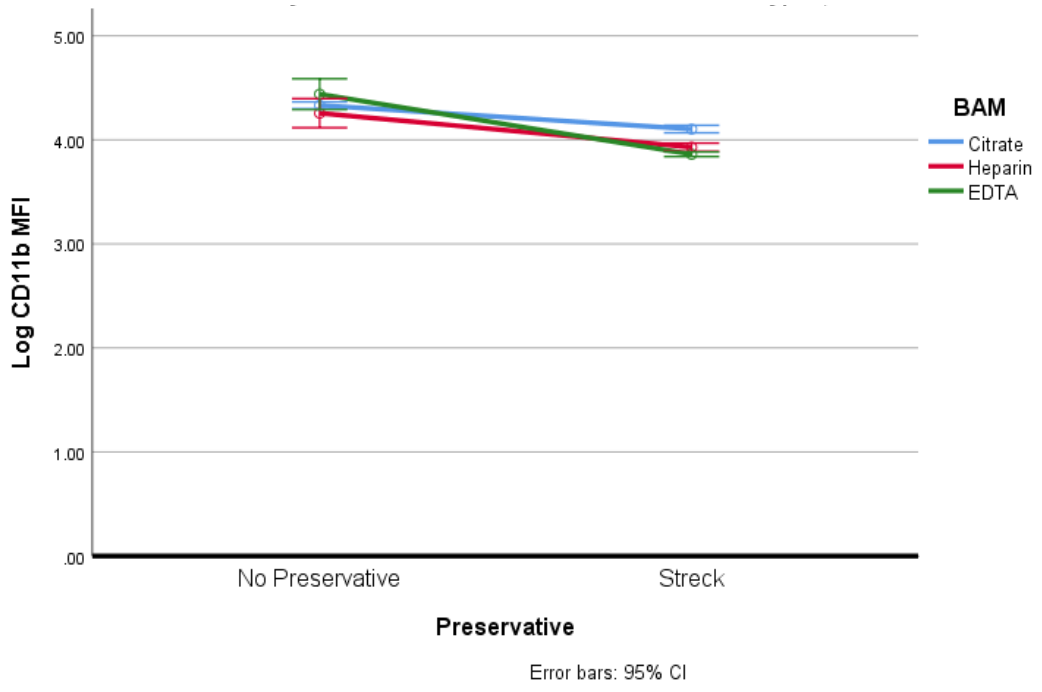


Figure 16. Variability in CD11b MFI Adjusted for BAM and Preservative Types, at Time 24 Hours

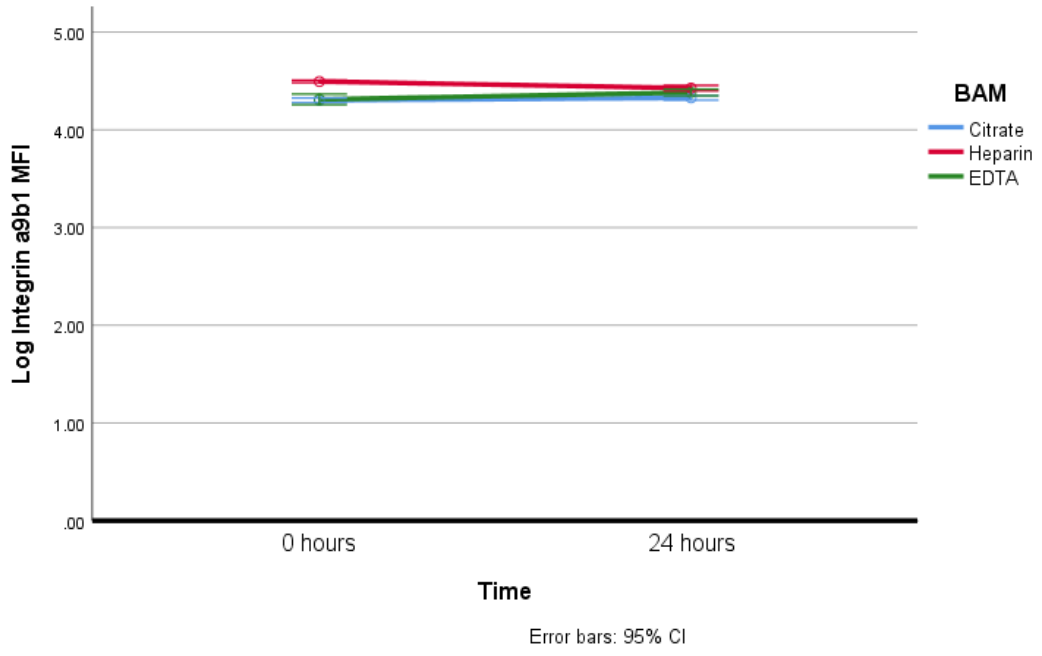


Figure 17. Overall RM-ANOVA Model for Integrin α9β1 MFI Adjusted for BAM, Time Points, and Preservative Types

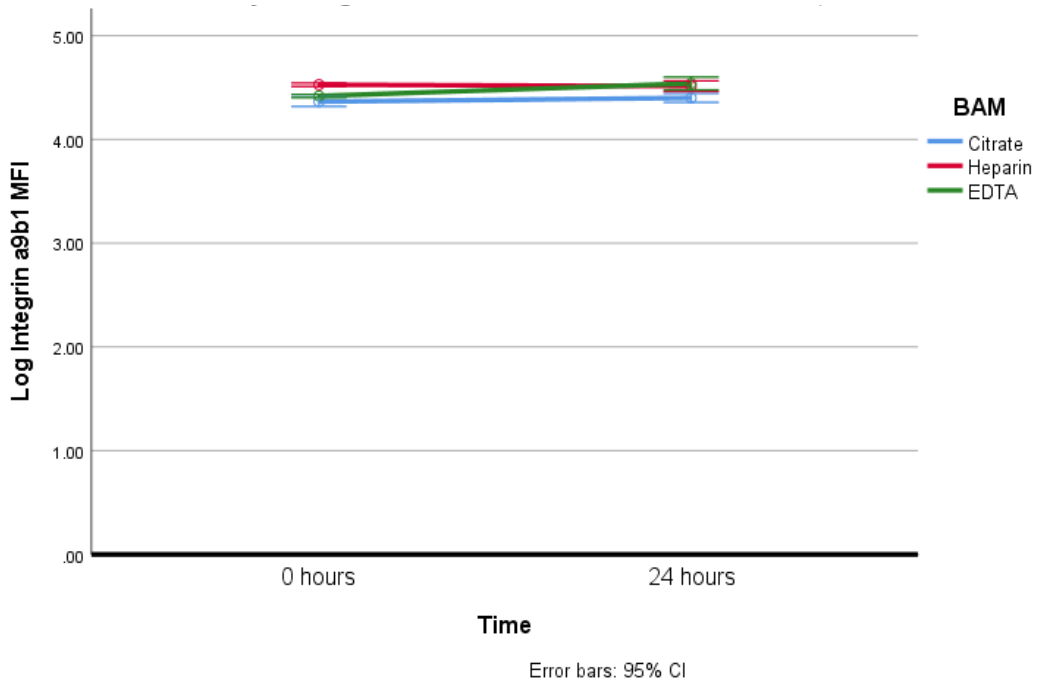


Figure 18. RM-ANOVA Model for Integrin α9β1 MFI Adjusted for BAM and Time Points, with No Preservative

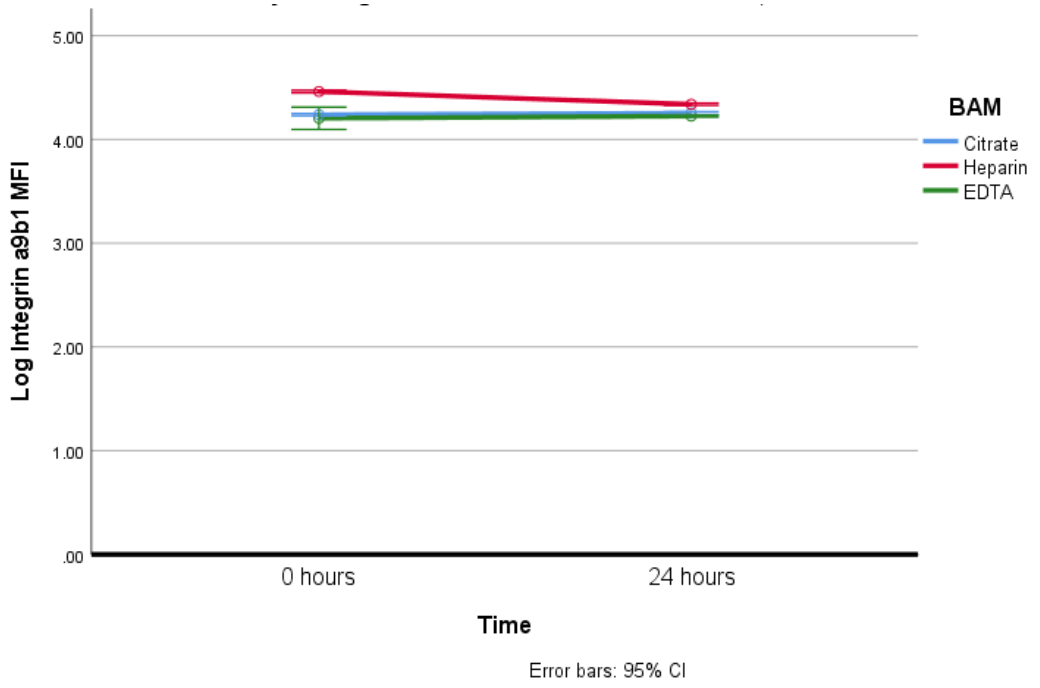


Figure 19. RM-ANOVA Model for Integrin α9β1 MFI Adjusted for BAM and Time Points, with Streck Preservative

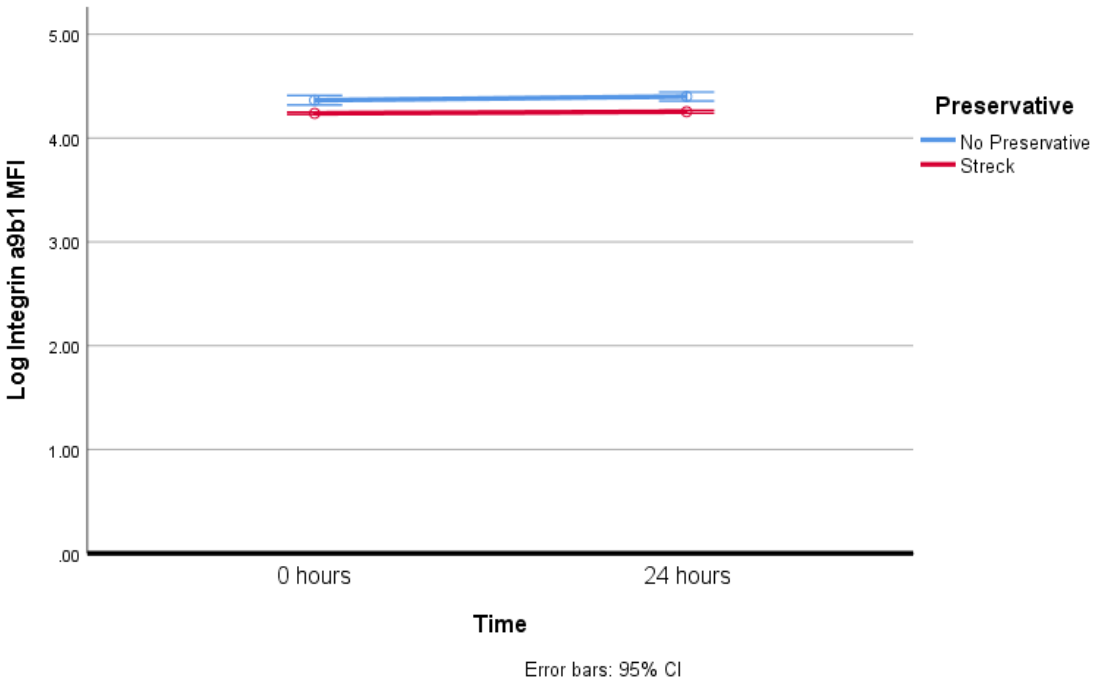


Figure 20. Variability of Integrin α9β1 MFI in Citrate Adjusted for Preservative Types and Time Points

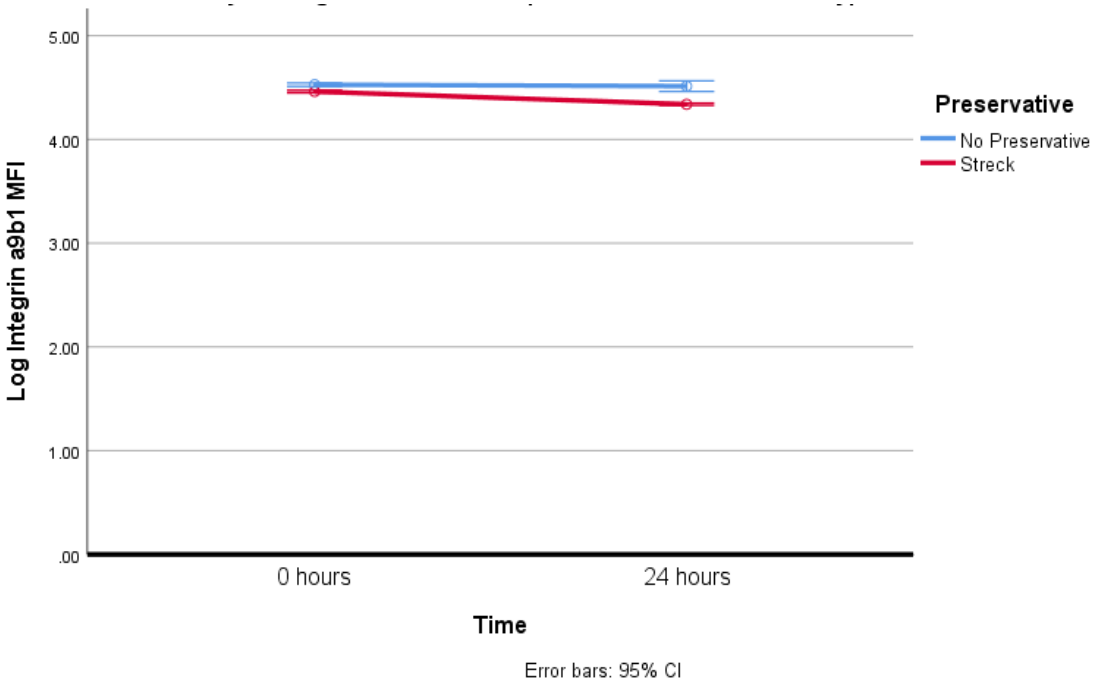


Figure 21. Variability of Integrin α9β1 MFI in Heparin Adjusted for Preservative Types and Time Points

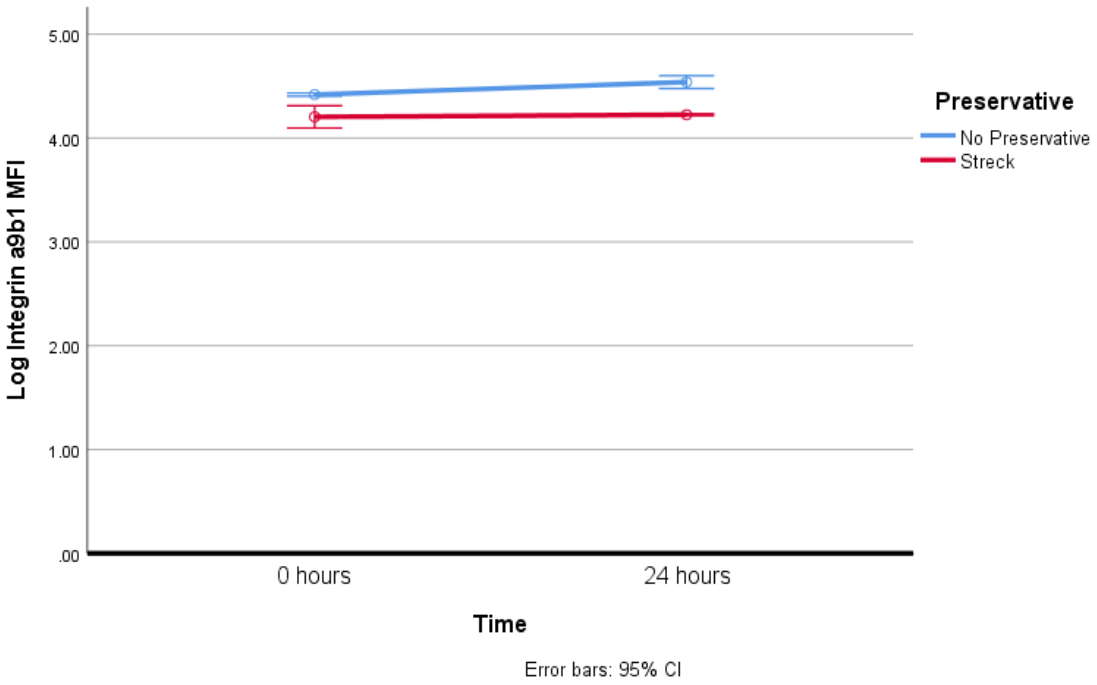


Figure 22. Variability of Integrin α9β1 MFI in EDTA Adjusted for Preservative Types and Time Points

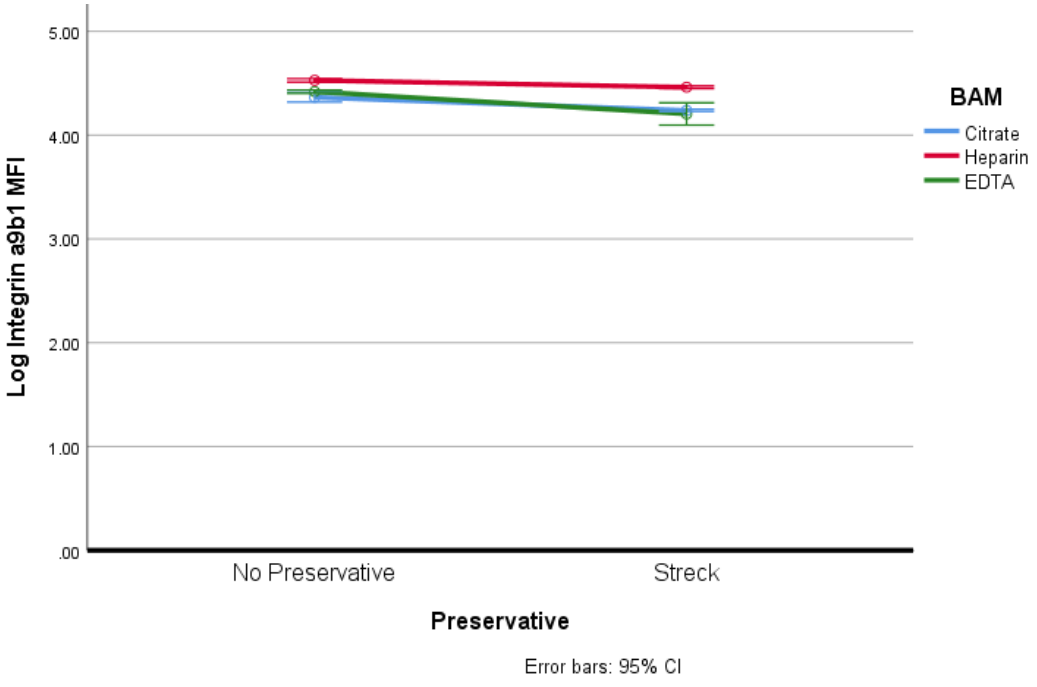


Figure 23. Variability in Integrin α9β1 MFI Adjusted for BAM and Preservative Type at Time 0 Hours

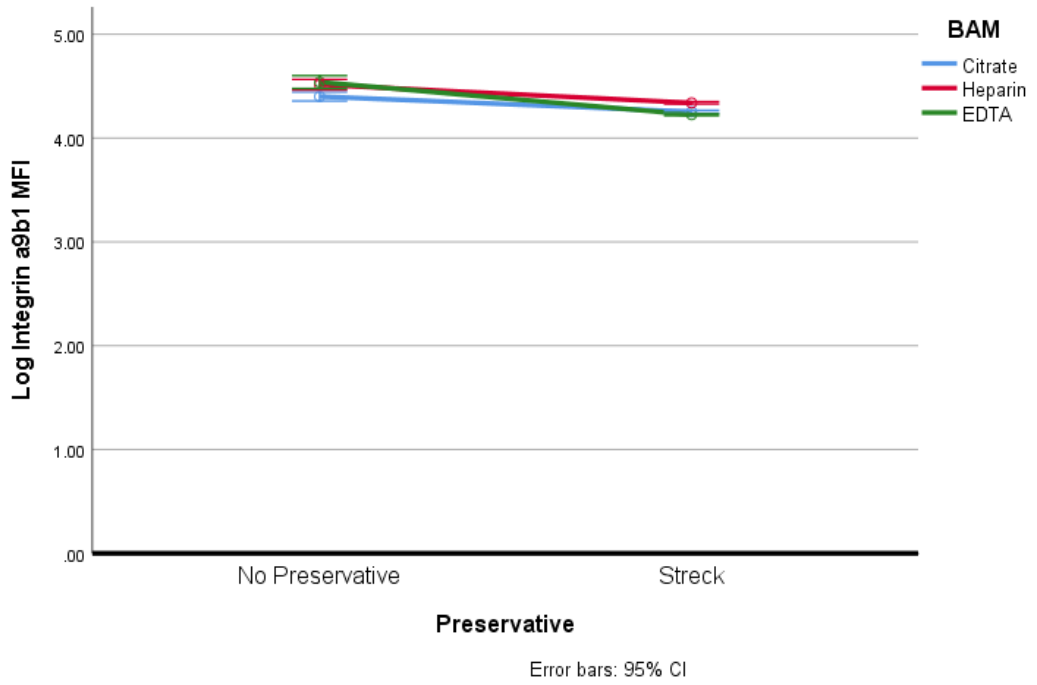


Figure 24. Variability in Integrin α9β1 MFI Adjusted for BAM and Preservative Type at Time 24 Hours

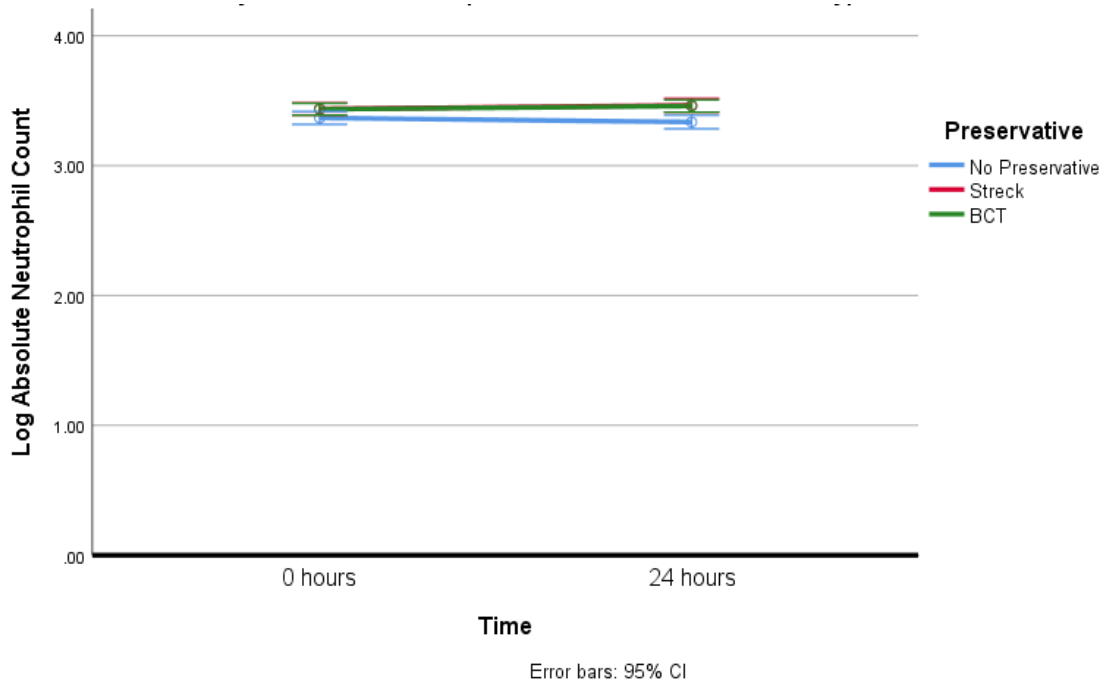


Figure 25. RM-ANOVA Model of Variability of Absolute Neutrophil Count Adjusted for Preservative Types and Time Points

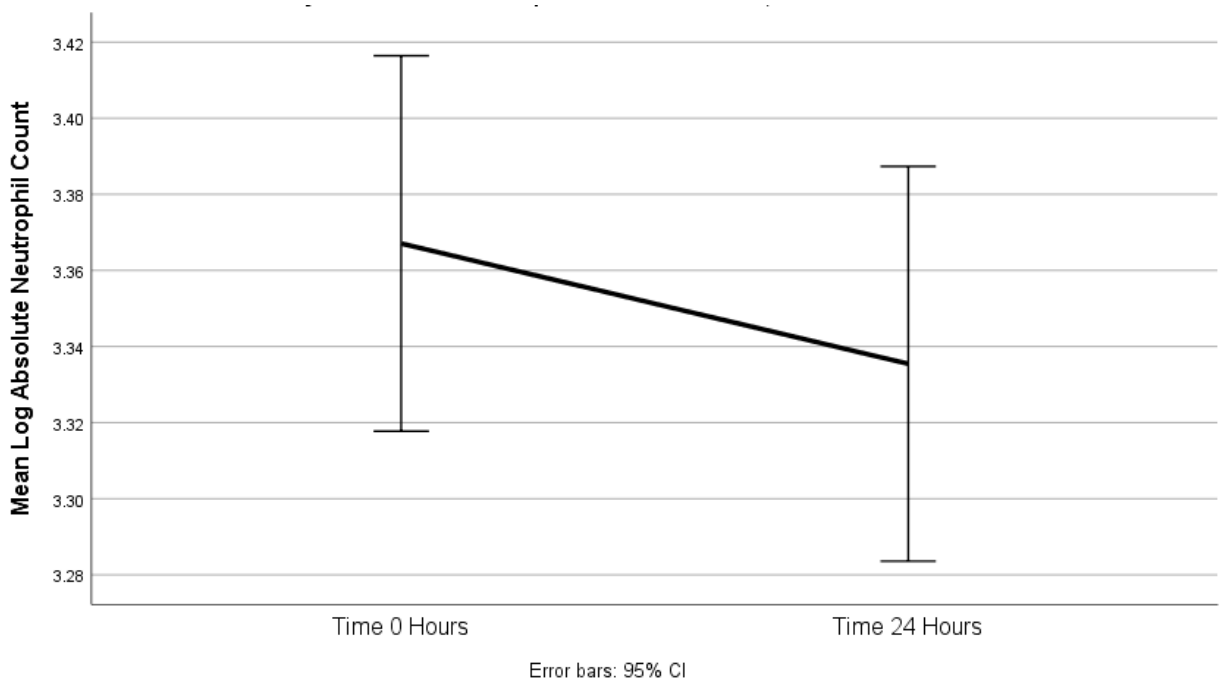


Figure 26. Variability of Absolute Neutrophil Count over Time, with No Preservative

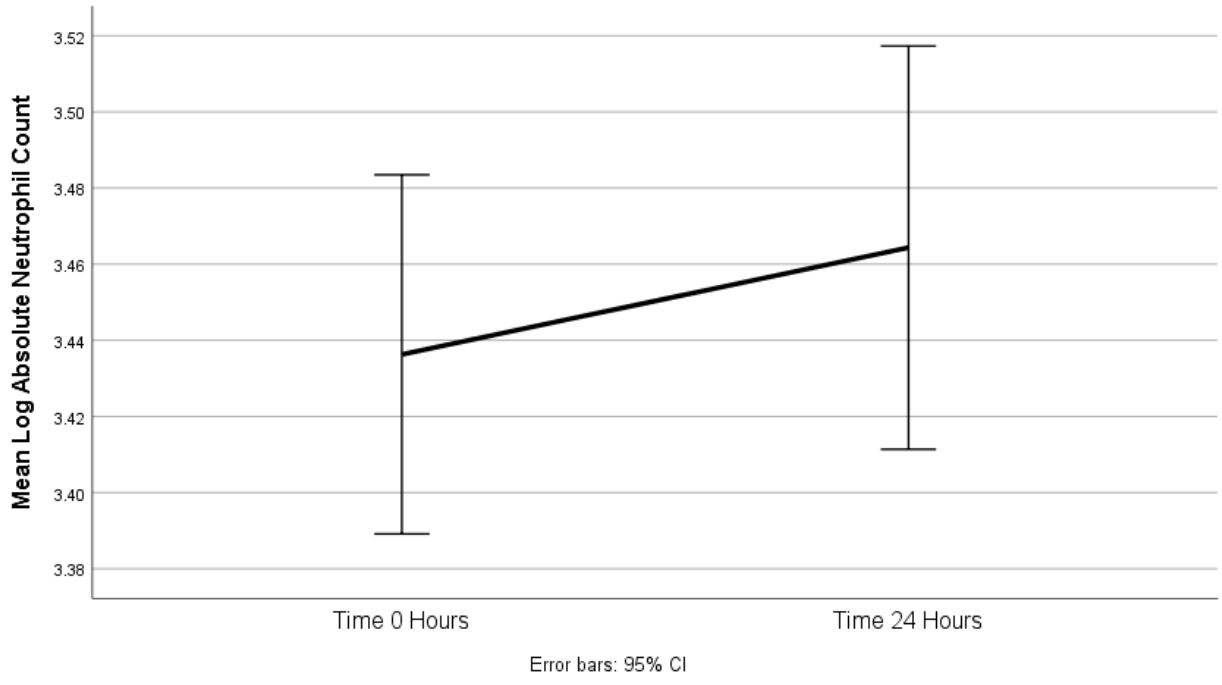


Figure 27. Variability of Absolute Neutrophil Count over Time, with Streck Preservative Solution

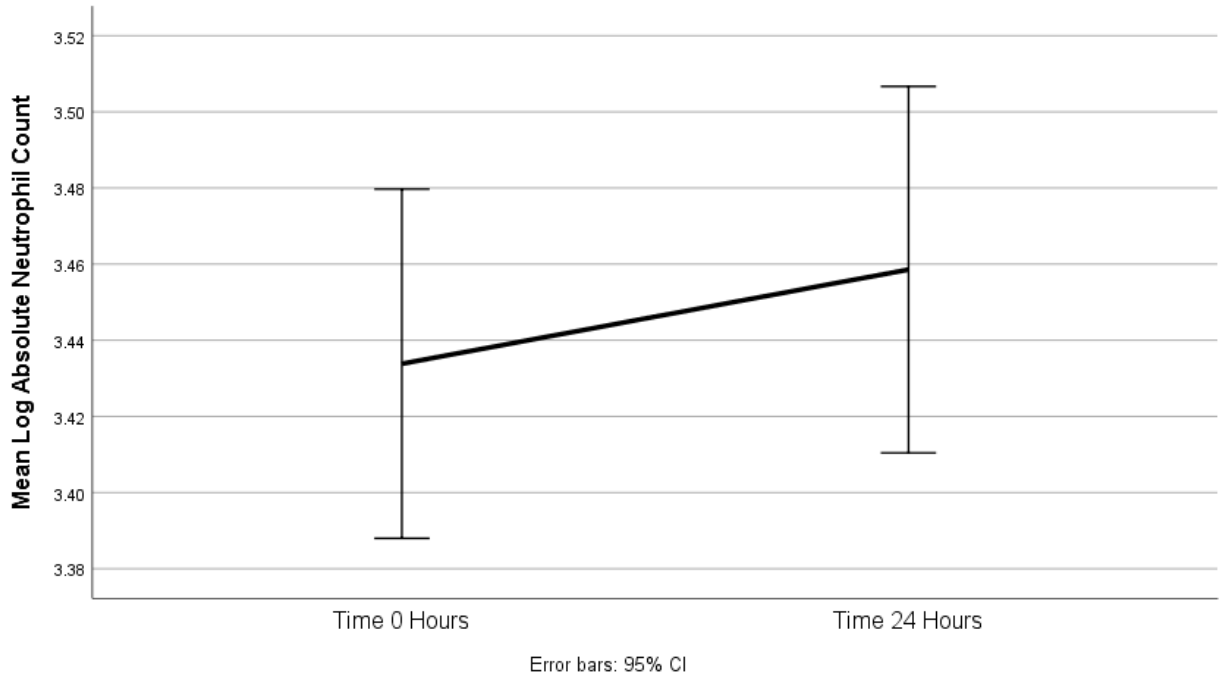


Figure 28. Variability of Absolute Neutrophil Count over Time, with Streck BCT

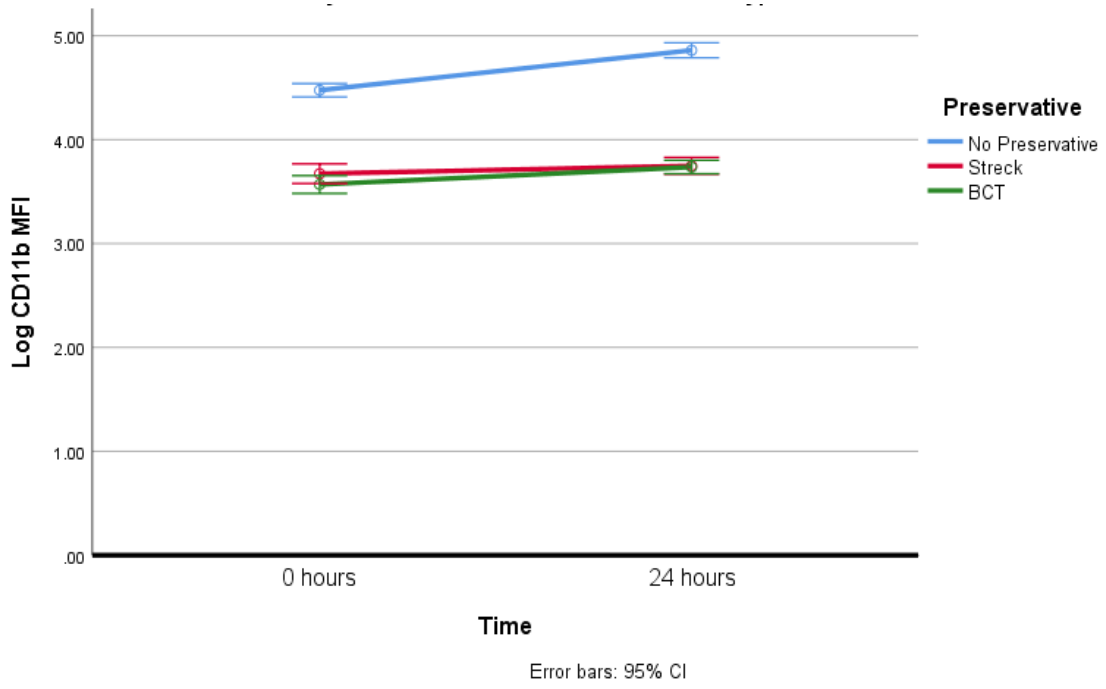


Figure 29. RM-ANOVA Model of Variability of CD11b MFI Adjusted for Preservative Types and Time Points

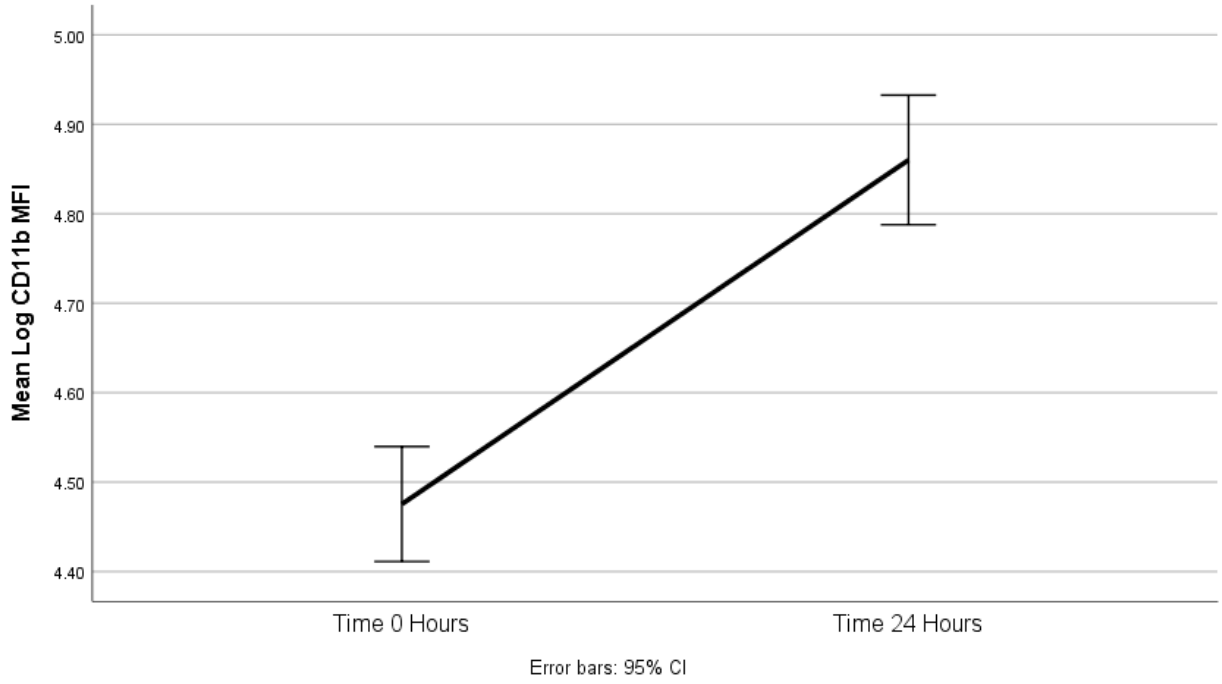


Figure 30. Variability of CD11b MFI over Time, with No Preservative

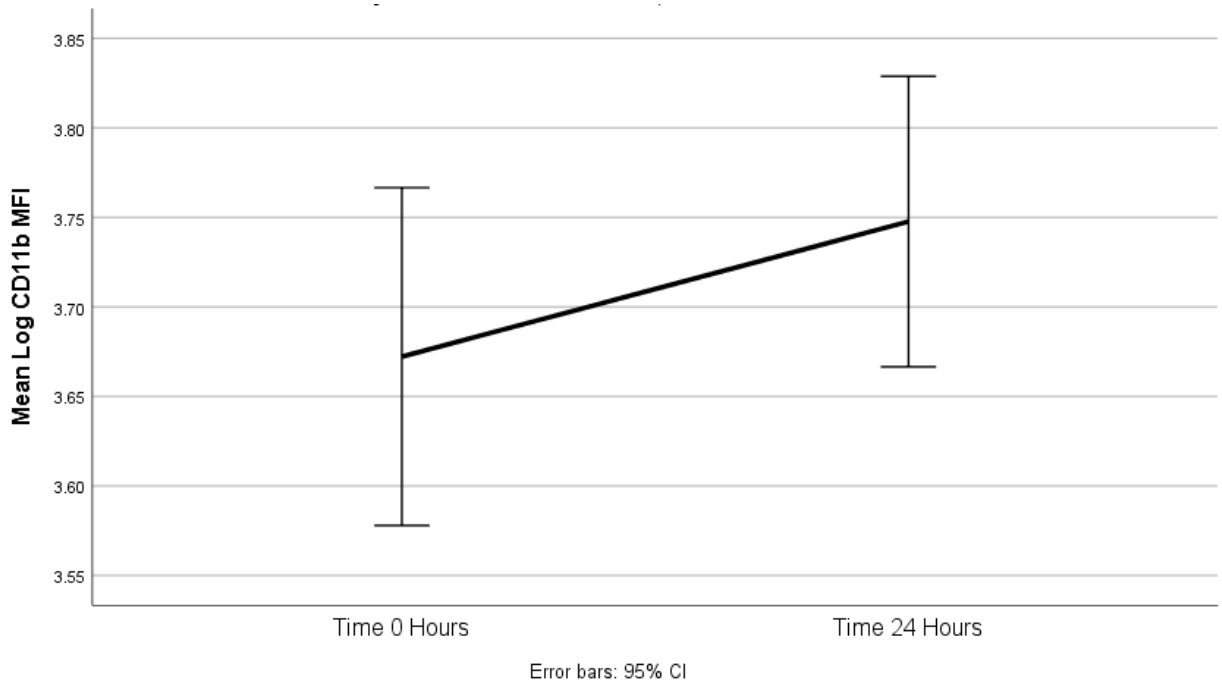


Figure 31. Variability of CD11b MFI over Time, with Streck Preservative Solution

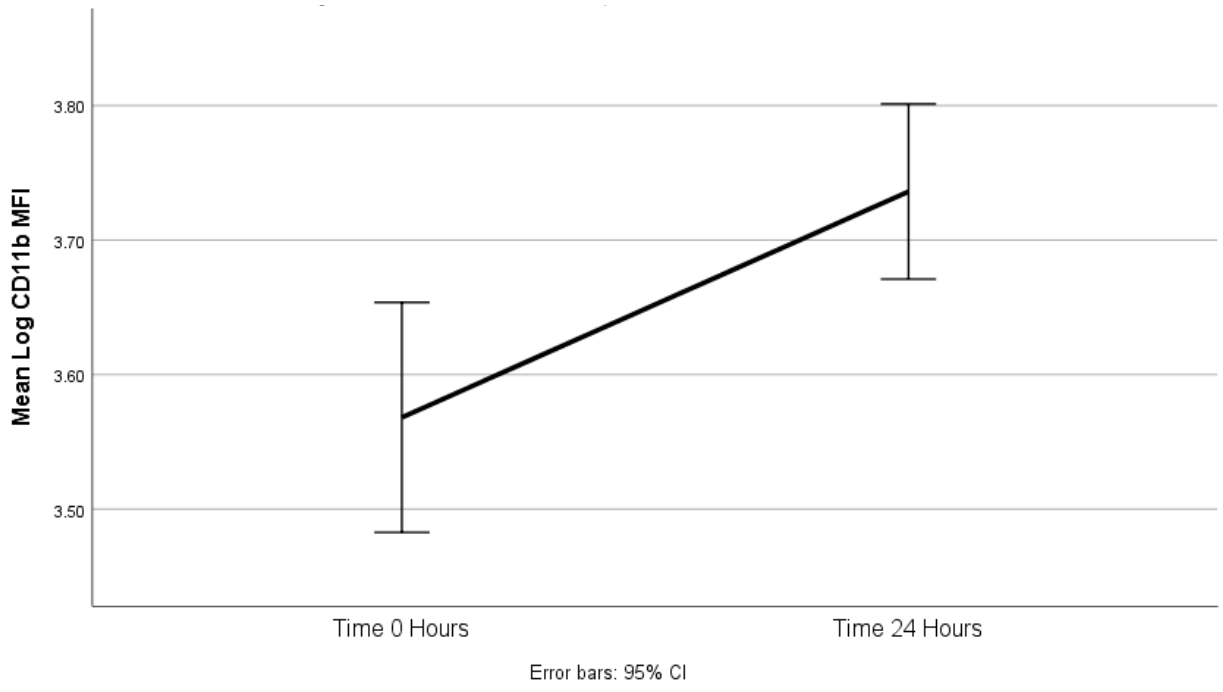


Figure 32. Variability of CD11b MFI over Time, with Streck BCT

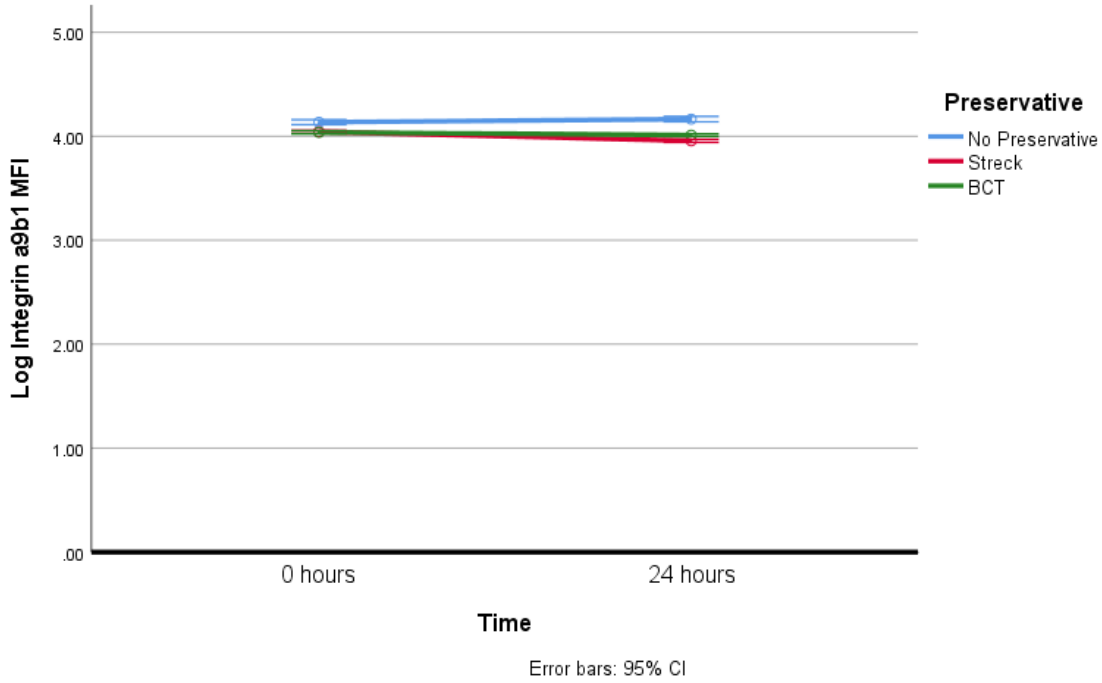


Figure 33. RM-ANOVA Model of Variability of Log Integrin α9β1 MFI Adjusted for Preservative Types and Time Points

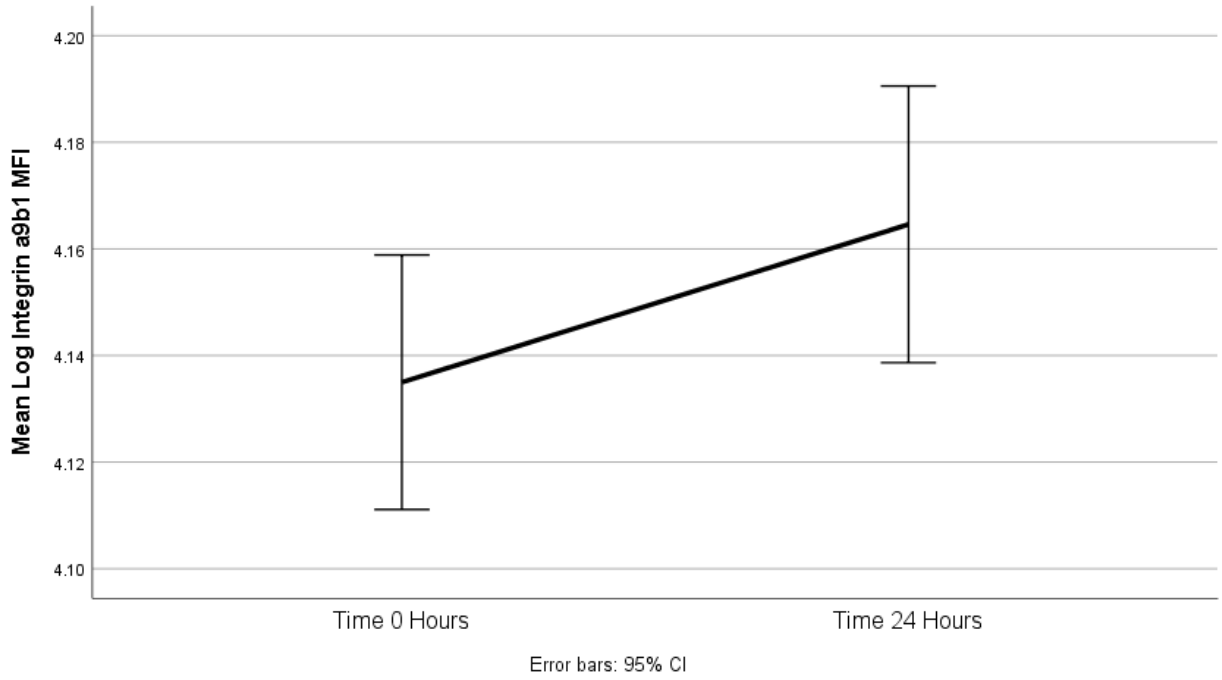


Figure 34. Variability of Log Integrin α9β1 MFI over Time, with No Preservative

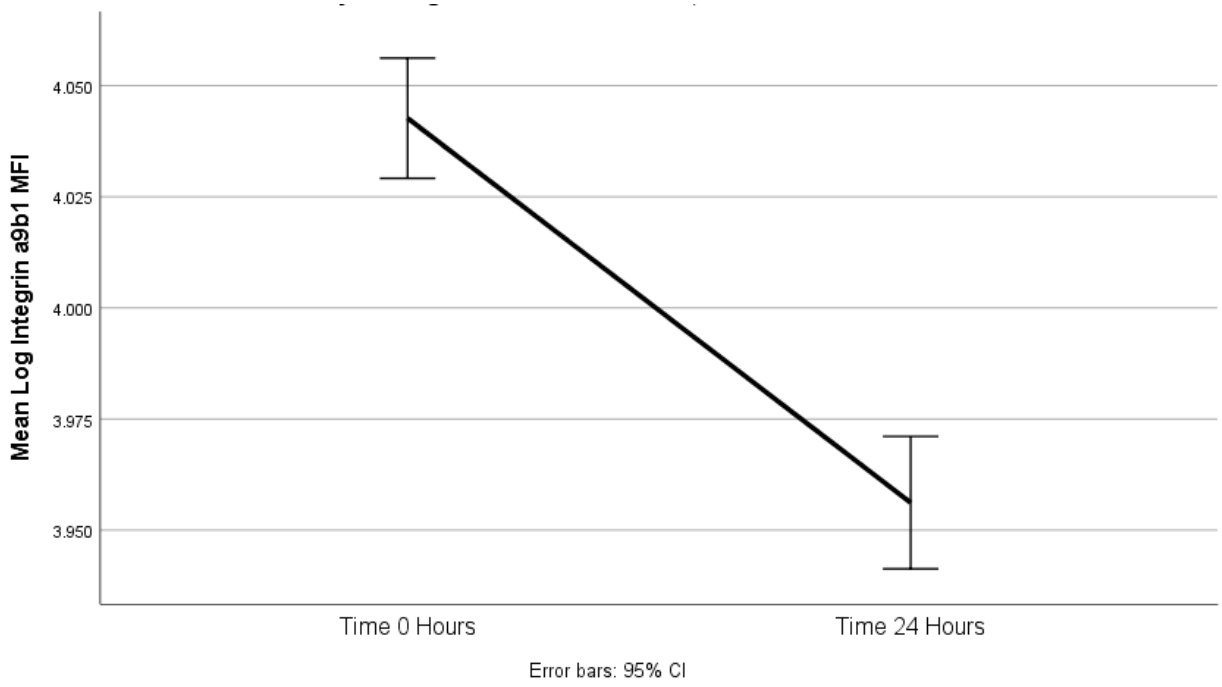


Figure 35. Variability of Log Integrin α9β1 MFI over Time, with Streck Preservative Solution

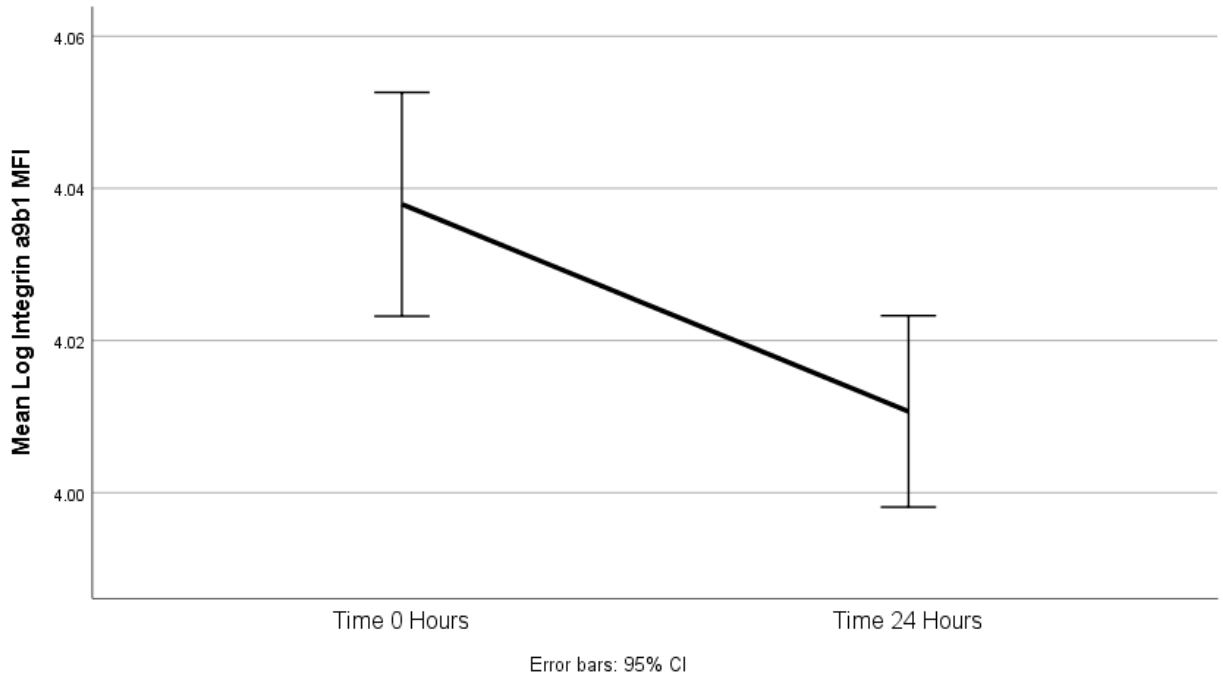


Figure 36. Variability of Log Integrin α9β1 MFI over Time, with Streck BCT

6.0 Tables

Table 1. Student's Paired T-Test Evaluating the Effect of Each BAM on ANC

BAM	No Preservative		Streck Preservative	
	Time 0 hours	Time 24 hours	Time 0 hours	Time 24 hours
Citrate vs Heparin	p <0.0001	p <0.0001	p = 0.79	p<0.0001
Citrate vs EDTA	p <0.0001	p = 0.0002	p = 0.0008	p = 0.0001
Heparin vs EDTA	p = 0.006	p = 0.0006	p = 0.004	p = 0.051

Table 2. Student's Paired T-Test Evaluating the Effect of Individual Time Points on ANC

Time	Citrate		Heparin		EDTA	
	No Preservative	Streck	No Preservative	Streck	No Preservative	Streck
0 vs 24 hours	p = 0.03	p = 0.47	p = 0.07	p <0.0001	p = 0.002	p <0.0001

Table 3. Student's Paired T-Test Evaluating the Effect of Preservative Type on ANC

Preservative	Citrate		Heparin		EDTA	
	Time 0 hours	Time 24 hours	Time 0 hours	Time 24 hours	Time 0 hours	Time 24 hours
No Preservative vs Streck	p = 0.03	p = 0.43	p = 0.0001	p <0.0001	p = 0.06	p <0.0001

Table 4. Wilcoxon Signed-Rank Test Evaluating the Effect of each BAM on Log CD11b MFI

BAM	No Preservative		Streck Preservative	
	Time 0 hours	Time 24 hours	Time 0 hours	Time 24 hours
Citrate vs Heparin	p = 0.008	p = 0.26	p = 0.008	p = 0.008
Citrate vs EDTA	p = 0.008	p = 0.14	p = 0.008	p = 0.008
Heparin vs EDTA	p = 0.008	p = 0.02	p = 0.14	p = 0.008

Table 5. Wilcoxon Signed-Rank Test Evaluating the Effect of Individual Time Points on Log CD11b MFI

Time	Citrate		Heparin		EDTA	
	No Preservative	Streck	No Preservative	Streck	No Preservative	Streck
0 vs 24 hours	p = 0.31	p = 0.008	p = 0.008	p = 0.01	p = 0.01	p = 0.008

Table 6. Wilcoxon Signed-Rank Test Evaluating the Effect of Preservative Type on Log CD11b MFI

Preservative	Citrate		Heparin		EDTA	
	Time 0 hours	Time 24 hours	Time 0 hours	Time 24 hours	Time 0 hours	Time 24 hours
No Preservative vs Streck	p = 0.09	p = 0.008	p = 0.051	p <0.008	p = 0.008	p = 0.008

Table 7. Student's Paired T-Test Evaluating the Effect of each BAM on Log Integrin α9β1 MFI

BAM	No Preservative		Streck Preservative	
	Time 0 hours	Time 24 hours	Time 0 hours	Time 24 hours
Citrate vs Heparin	p <0.0001	p = 0.009	p <0.0001	p <0.0001
Citrate vs EDTA	p = 0.042	p = 0.003	p = 0.487	p = 0.008
Heparin vs EDTA	p <0.0001	p = 0.019	p = 0.0006	p <0.0001

Table 8. Student's Paired T-Test Evaluating the Effect of Individual Time Points on Log Integrin α9β1 MFI

Time	Citrate		Heparin		EDTA	
	No Preservative	Streck	No Preservative	Streck	No Preservative	Streck
0 vs 24 hours	p = 0.089	p = 0.086	p = 0.493	p <0.0001	p = 0.005	p = 0.651

Table 9. Student's Paired T-Test Evaluating the Effect of Preservative Type on Log Integrin α9β1 MFI

Preservative	Citrate		Heparin		EDTA	
	Time 0 hours	Time 24 hours	Time 0 hours	Time 24 hours	Time 0 hours	Time 24 hours
No Preservative vs Streck	p = 0.0002	p <0.0001	p <0.0001	p <0.0001	p = 0.002	p <0.0001

Table 10. Student's Paired T-Test Evaluating the Effect of Preservative Type on Log ANC

	Time 0 Hours	Time 24 Hours
No Preservative vs Streck	p <0.0001	p <0.0001
No Preservative vs BCT	p <0.0001	p <0.0001
Streck vs BCT	p = 0.492	p = 0.387

Table 11. Student's Paired T-Test and Wilcoxon Signed-Rank Test* Evaluating the Effect of Time on Log ANC

	No Preservative	Streck*	BCT*
Time 0 vs Time 24 hours	p = 0.055	p = 0.0003	p = 0.020

*Wilcoxon Signed-Rank Test used for Streck and BCT analyses

Table 12. Wilcoxon Signed-Rank Test Evaluating the Effect of Preservative Type on Log CD11b MFI

	Time 0 Hours	Time 24 Hours
No Preservative vs Streck	p <0.0001	p <0.0001
No Preservative vs BCT	p <0.0001	p <0.0001
Streck vs BCT	p = 0.013	p = 0.720

Table 13. Wilcoxon Signed-Rank Test Evaluating the Effect of Time on Log CD11b MFI

	No Preservative	Streck	BCT
Time 0 vs Time 24 hours	p <0.0001	p = 0.007	p <0.0001

Table 14. Student's Paired T-Test Evaluating the Effect of Preservative Type on Log Integrin α9β1 MFI

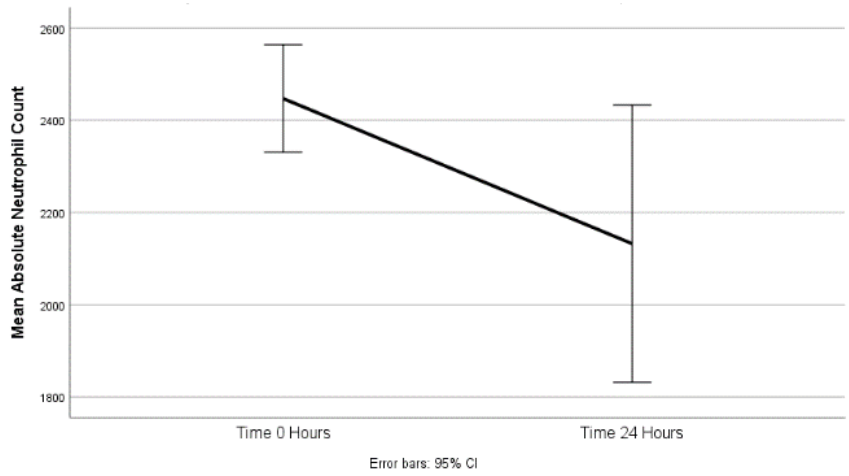
	Time 0 Hours	Time 24 Hours
No Preservative vs Streck	p <0.0001	p <0.0001
No Preservative vs BCT	p <0.0001	p <0.0001
Streck vs BCT	p = 0.538	p <0.0001

Table 15. Student's Paired T-Test and Wilcoxon Signed-Rank Test* the Effect of Time on Log Integrin α9β1 MFI

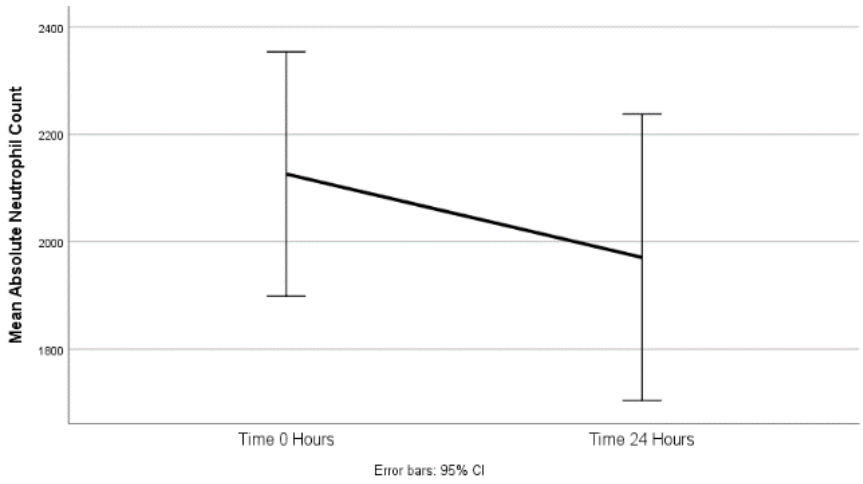
	No Preservative	Streck*	BCT
Time 0 vs Time 24 hours	p = 0.006	p <0.0001	p = 0.002

**Wilcoxon Signed-Rank Test used for Streck analysis

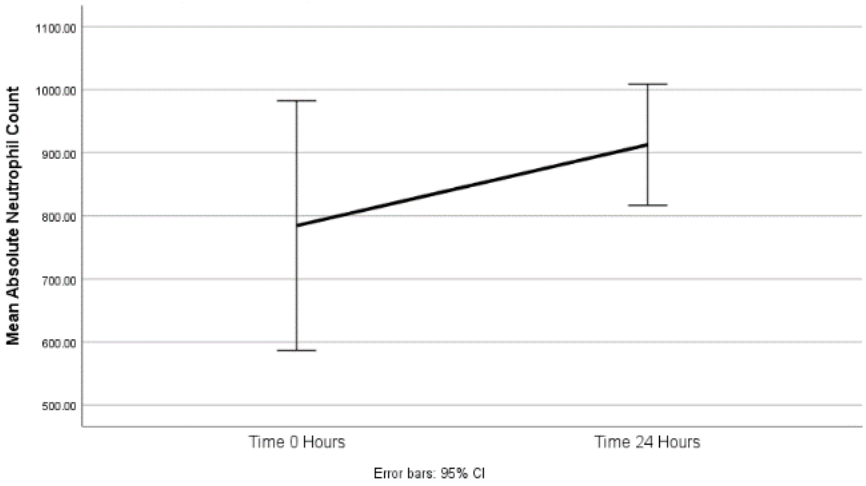
Appendix A Change in ANC over time for each BAM



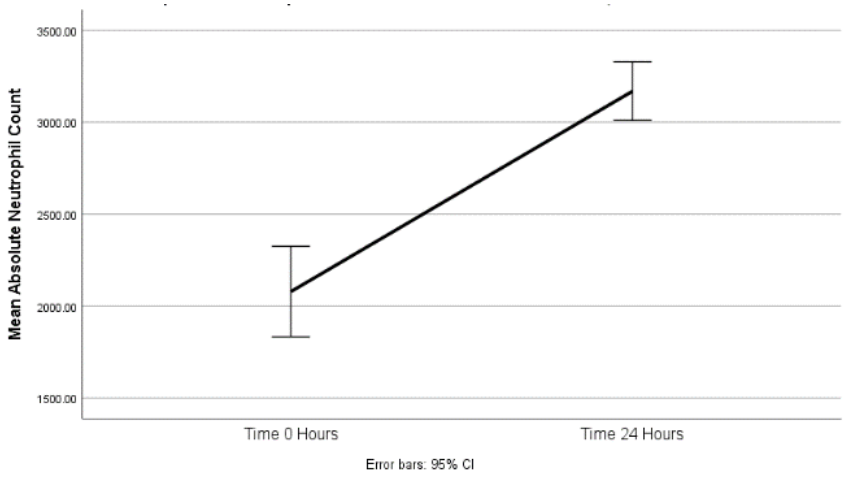
Appendix A, Figure 1. Absolute Neutrophil Count in Citrate from Time 0 Hours to Time 24 Hours, with No Preservative



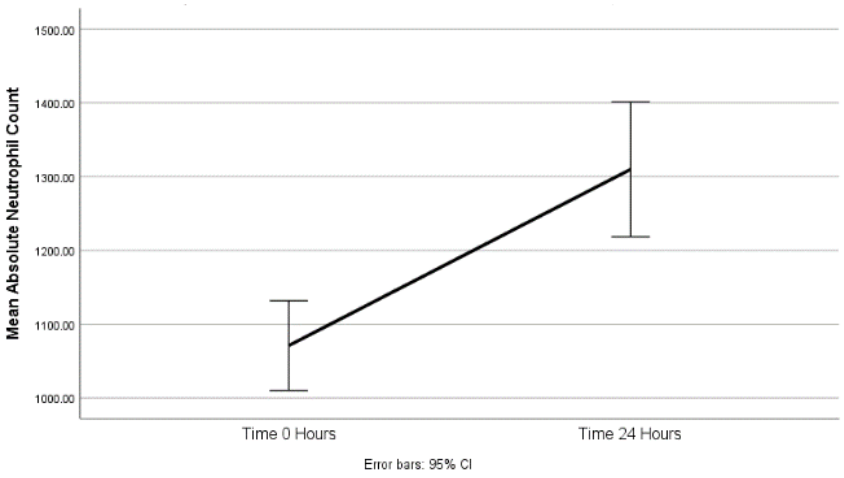
Appendix A, Figure 2. Absolute Neutrophil Count in Citrate from Time 0 Hours to Time 24 Hours, with Streck Preservative



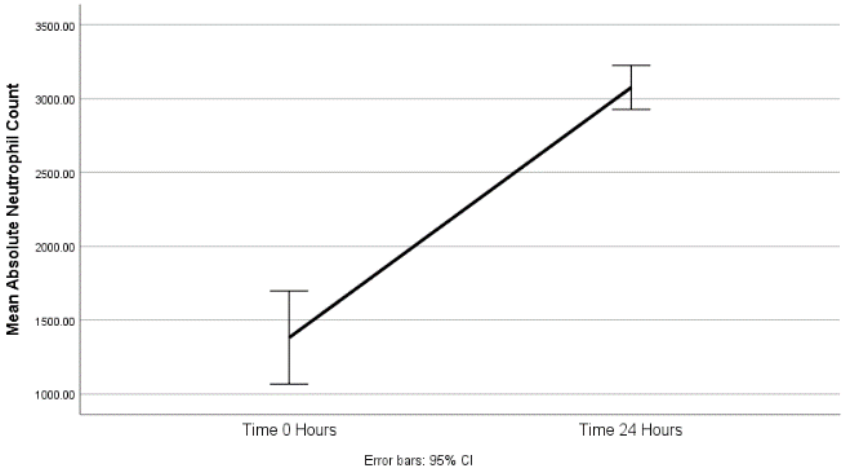
Appendix A, Figure 3. Absolute Neutrophil Count in Heparin from Time 0 Hours to Time 24 Hours, with No Preservative



Appendix A, Figure 4. Absolute Neutrophil Count in Heparin from Time 0 Hours to Time 24 Hours, with Streck Preservative



Appendix A, Figure 5. Absolute Neutrophil Count in EDTA from Time 0 Hours to Time 24 Hours, with No Preservative



Appendix A, Figure 6. Absolute Neutrophil Count in EDTA from Time 0 Hours to Time 24 Hours, with Streck Preservative

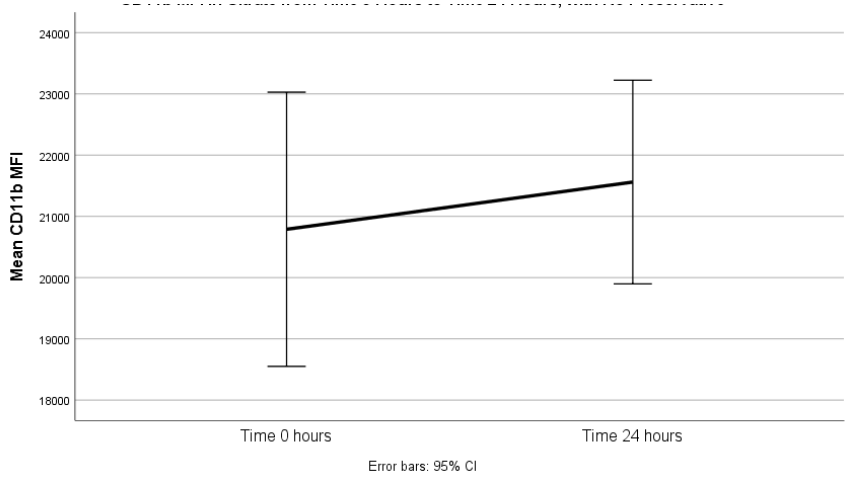
Appendix A, Table 1. Average Change in ANC over Time for each BAM

BAM		Time 0	Time 24	Total Change	Percent Change
Citrate	No Preservative	2447	2132.33	-314.67	12.86%
	Streck	2126.22	1970.89	-155.33	7.31%
Heparin	No Preservative	784.5171	912.7462	+128.23	16.34%
	Streck	2079.1851	3168.4546	+1089.27	52.39%
EDTA	No Preservative	1071.0542	1309.7691	+238.71	22.29%
	Streck	1381.8499	3076.5728	+1694.72	122.64%

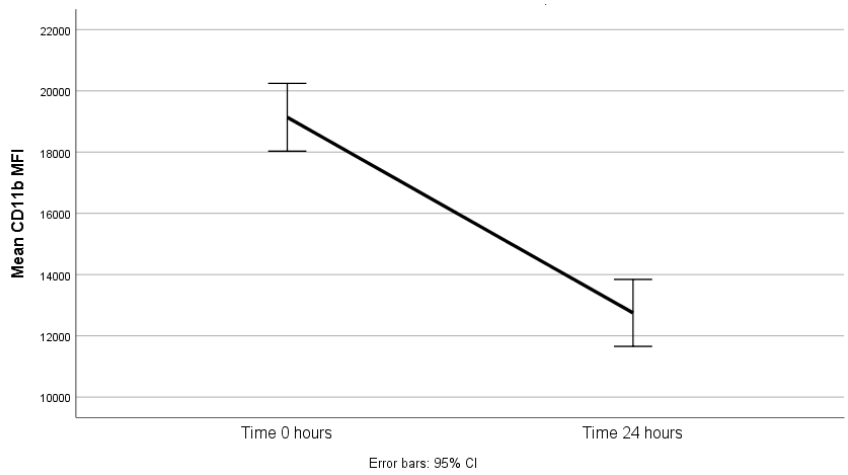
Appendix A, Table 2. ANC Coefficient of Variation for each BAM at each Preservative Type and Time Point

BAM		Time 0 Hours	Time 24 Hours
Citrate	No Preservative	5.91%	8.77%
	Streck	10.23%	12.89%
Heparin	No Preservative	11.51%	7.50%
	Streck	7.14%	2.74%
EDTA	No Preservative	5.06%	4.12%
	Streck	28.87%	2.91%

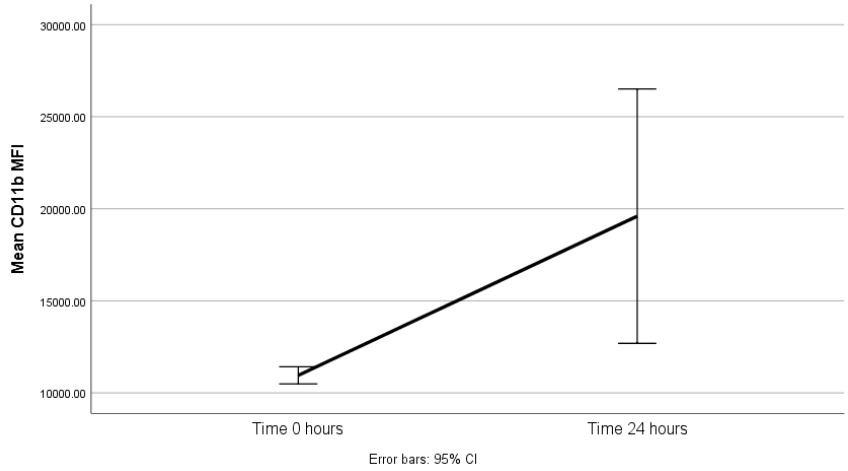
Appendix B Change in CD11b MFI over time for each BAM



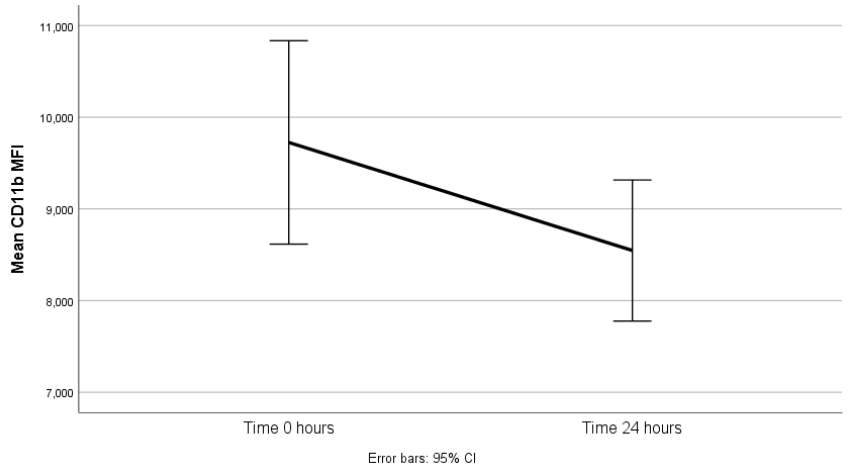
Appendix B, Figure 1. CD11b MFI in Citrate from Time 0 Hours to Time 24 Hours, with No Preservative



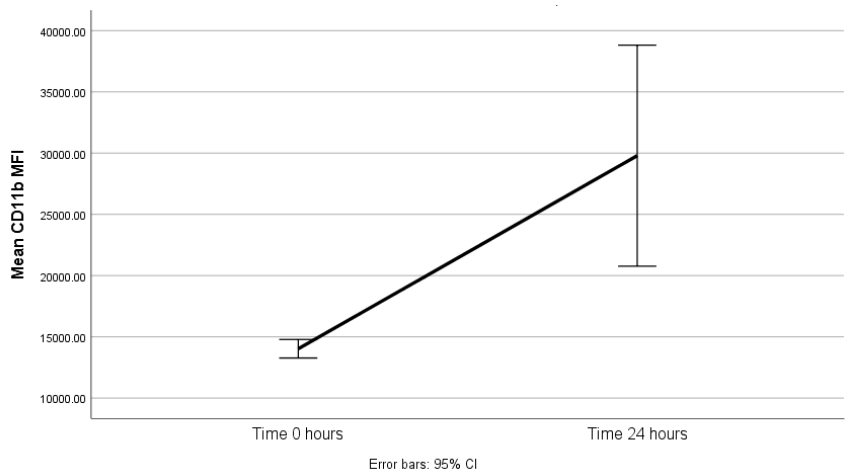
Appendix B, Figure 2. CD11b MFI in Citrate from Time 0 Hours to Time 24 Hours, with Streck Preservative



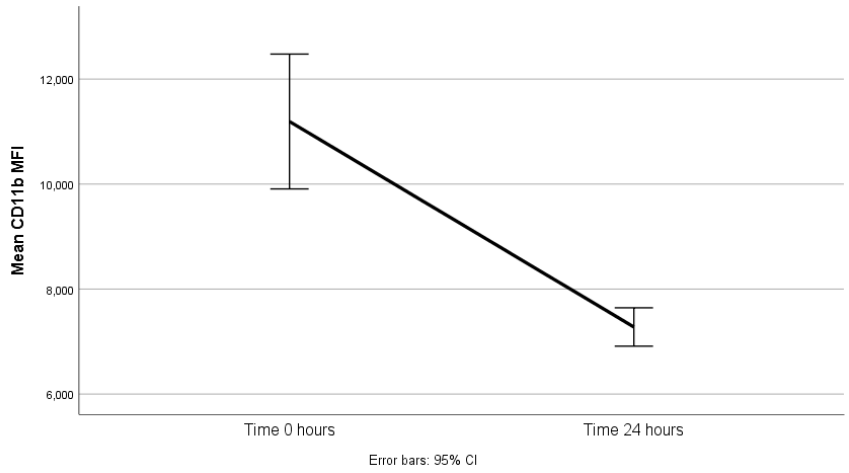
Appendix B, Figure 3. CD11b MFI in Heparin from Time 0 Hours to Time 24 Hours, with No Preservative



Appendix B, Figure 4. CD11b MFI in Heparin from Time 0 Hours to Time 24 Hours, with Streck Preservative



Appendix B, Figure 5. CD11b MFI in EDTA from Time 0 Hours to Time 24 Hours, with No Preservative



Appendix B, Figure 6. CD11b MFI in EDTA from Time 0 Hours to Time 24 Hours, with Streck Preservative

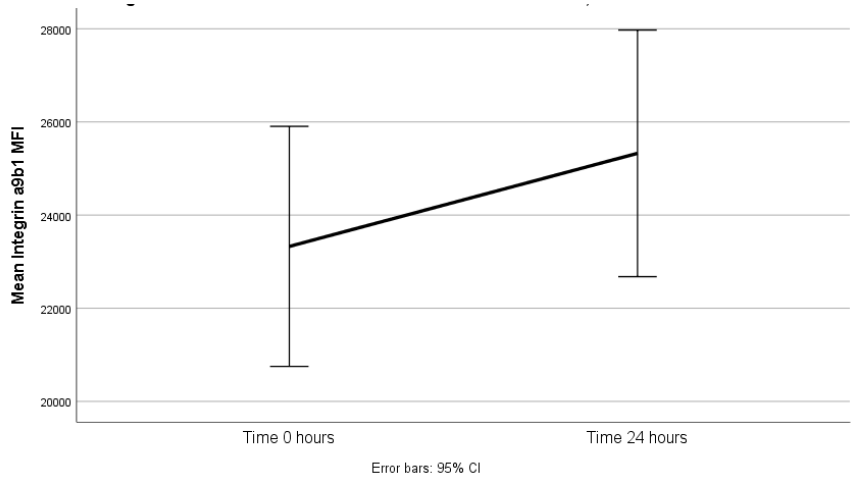
Appendix B, Table 1. Average Change in CD11b MFI over Time for each BAM

BAM		Time 0	Time 24	Total Change	Percent Change
Citrate	No Preservative	20789.33	21560.33	+771.00	3.71%
	Streck	19136.44	12748.56	-6387.88	33.38%
Heparin	No Preservative	10953.3656	19598.3226	+8644.96	78.93%
	Streck	9725.1777	8546.0456	-1179.13	12.12%
EDTA	No Preservative	14025.9233	29791.3990	+15765.48	112.40%
	Streck	11191.9934	7277.3778	-3914.62	34.98%

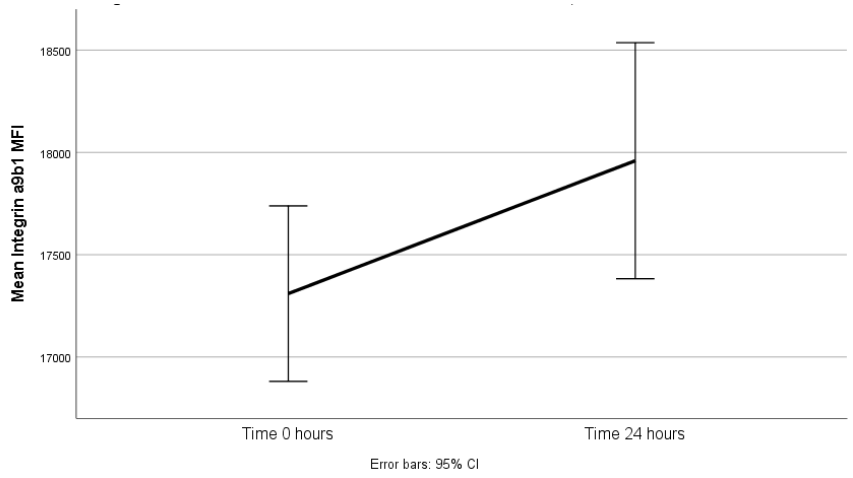
Appendix B, Table 2. CD11b MFI Coefficient of Variation for each BAM at each Preservative Type and Time Point

BAM		Time 0 Hours	Time 24 Hours
Citrate	No Preservative	5.44%	7.18%
	Streck	2.32%	1.99%
Heparin	No Preservative	3.35%	6.55%
	Streck	2.71%	1.82%
EDTA	No Preservative	2.24%	17.59%
	Streck	6.23%	3.35%

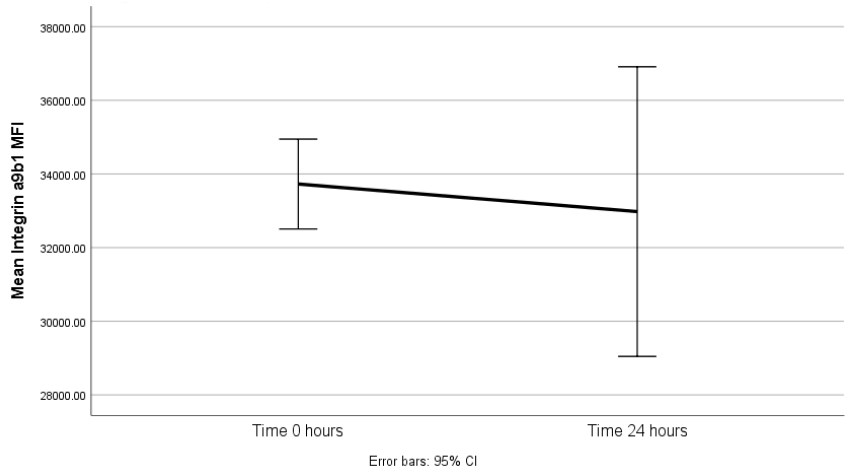
Appendix C Change in Integrin α9β1 MFI over Time for each BAM



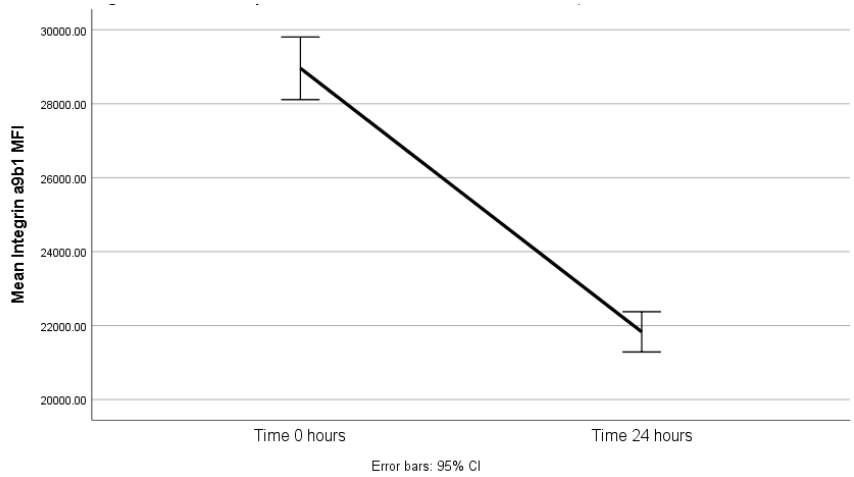
Appendix C, Figure 1. Integrin a9b1 MFI in Citrate from Time 0 Hours to Time 24 Hours, with No Preservative



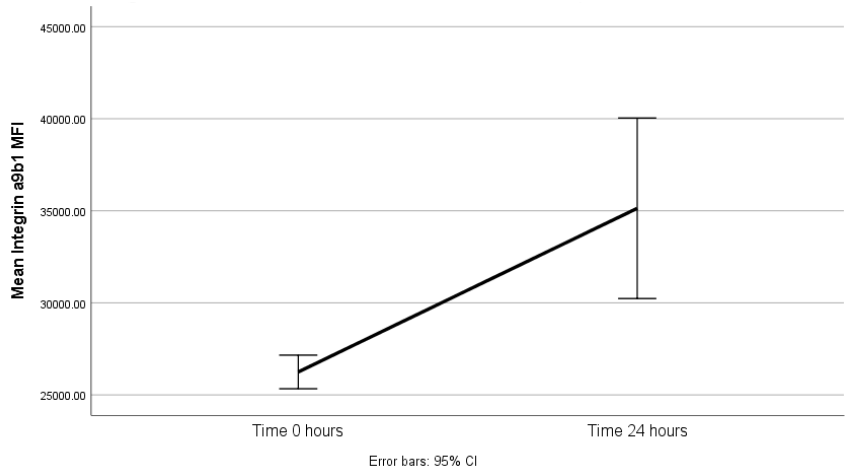
Appendix C, Figure 2. Integrin a9b1 MFI in Citrate from Time 0 Hours to Time 24 Hours, with Streck Preservative



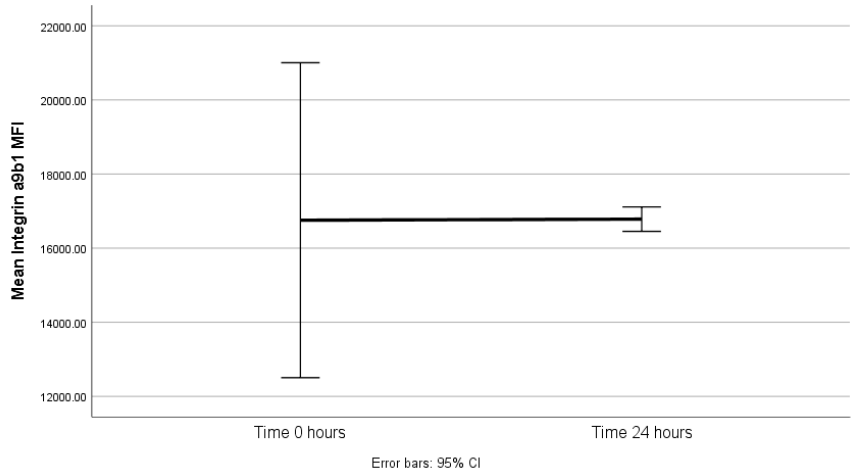
Appendix C, Figure 3. Integrin a9b1 MFI in Heparin from Time 0 Hours to Time 24 Hours, with No Preservative



Appendix C, Figure 4. Integrin a9b1 MFI in Heparin from Time 0 Hours to Time 24 Hours, with Streck Preservative



Appendix C, Figure 5. Integrin a9b1 MFI in EDTA from Time 0 Hours to Time 24 Hours, with No Preservative



Appendix C, Figure 6. Integrin a9b1 MFI in EDTA from Time 0 Hours to Time 24 Hours, with Streck Preservative

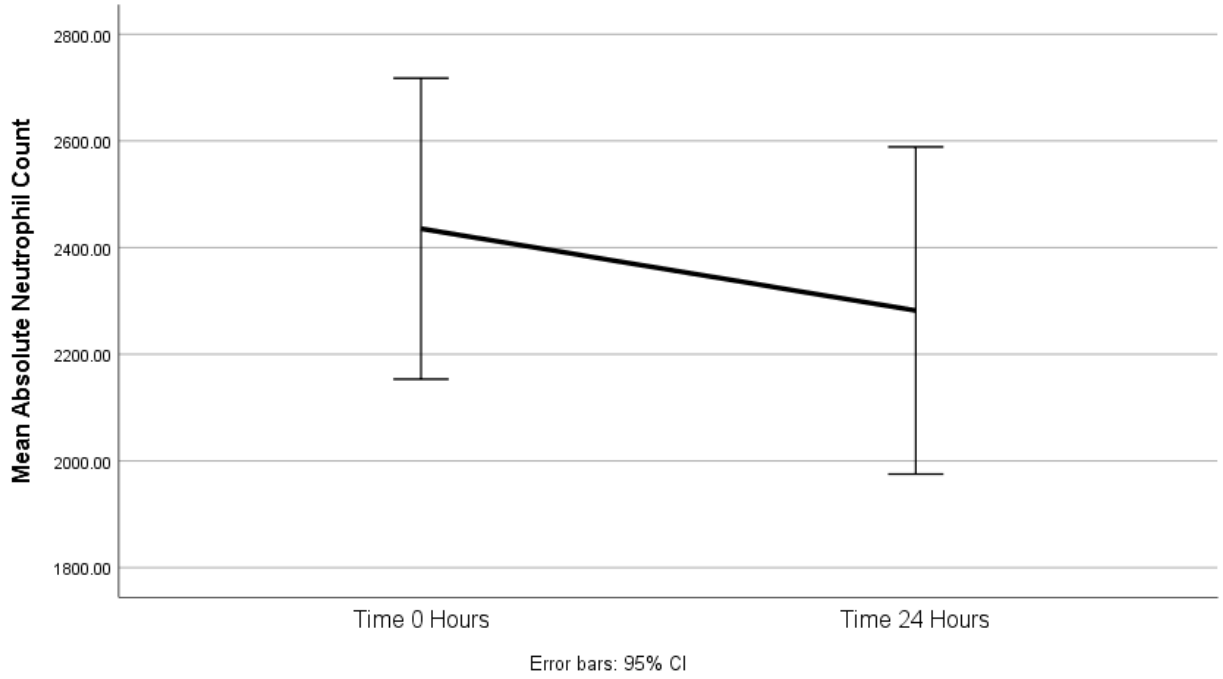
Appendix C, Table 1. Average Change in Integrin α9β1 MFI over Time for each BAM

BAM		Time 0	Time 24	Total Change	Percent Change
Citrate	No Preservative	23328.00	25325.39	+1997.39	8.56%
	Streck	17309.78	17959.44	+649.66	3.75%
Heparin	No Preservative	33726.6224	32978.3854	-748.237	2.22%
	Streck	28957.8466	21830.2912	-7127.56	24.61%
EDTA	No Preservative	26247.0866	35134.3676	+8887.28	33.86%
	Streck	16753.9535	16782.5275	+28.57	0.17%

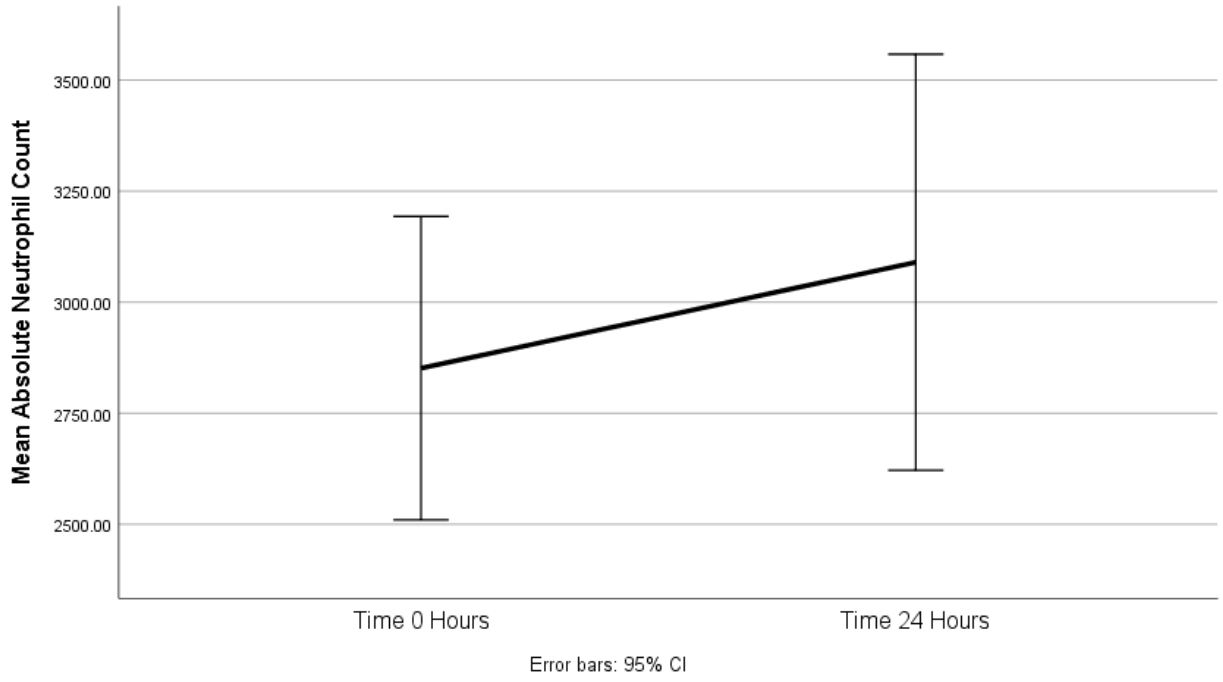
Appendix C, Table 2. Integrin α9β1 MFI Coefficient of Variation for each BAM at each Preservative Type and Time Point

BAM		Time 0 Hours	Time 24 Hours
Citrate	No Preservative	4.48%	7.92%
	Streck	3.52%	3.37%
Heparin	No Preservative	2.98%	4.57%
	Streck	2.72%	2.73%
EDTA	No Preservative	3.06%	1.59%
	Streck	29.02%	1.62%

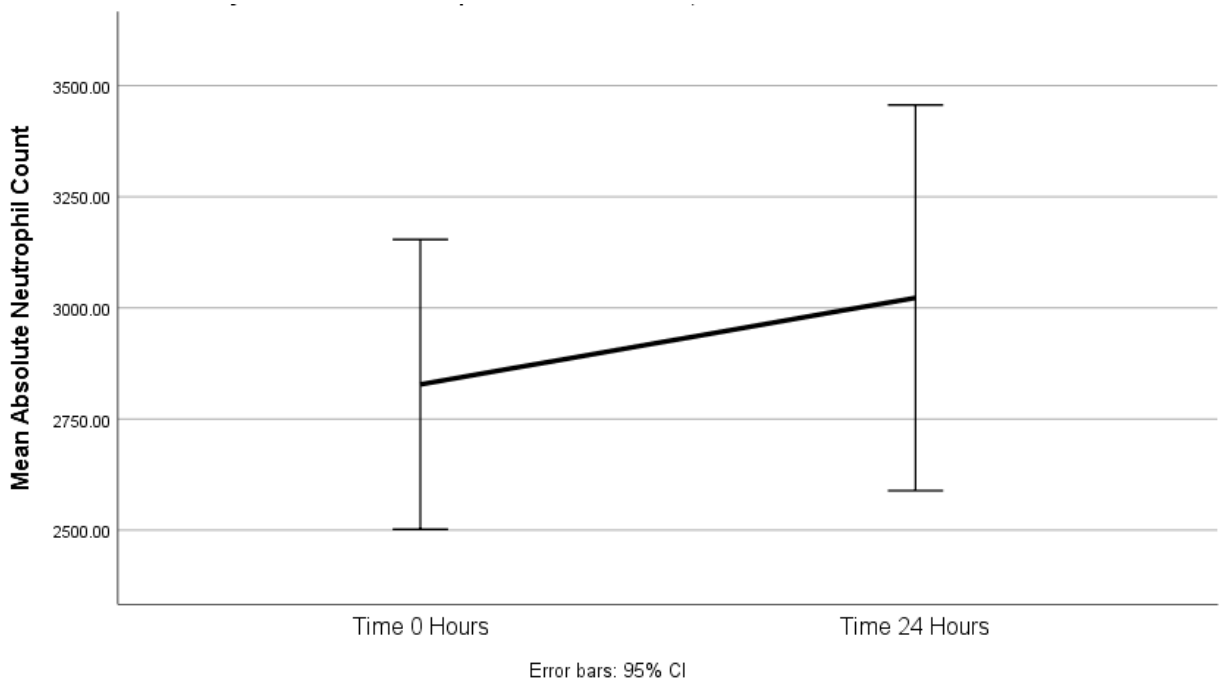
Appendix D Variability in Absolute Neutrophil Count over Time at each Preservative Type



Appendix D, Figure 1. Variability of Absolute Neutrophil Count over Time, with No Preservative



Appendix D, Figure 2. Variability of Absolute Neutrophil Count over Time, with Streck Preservative Solution

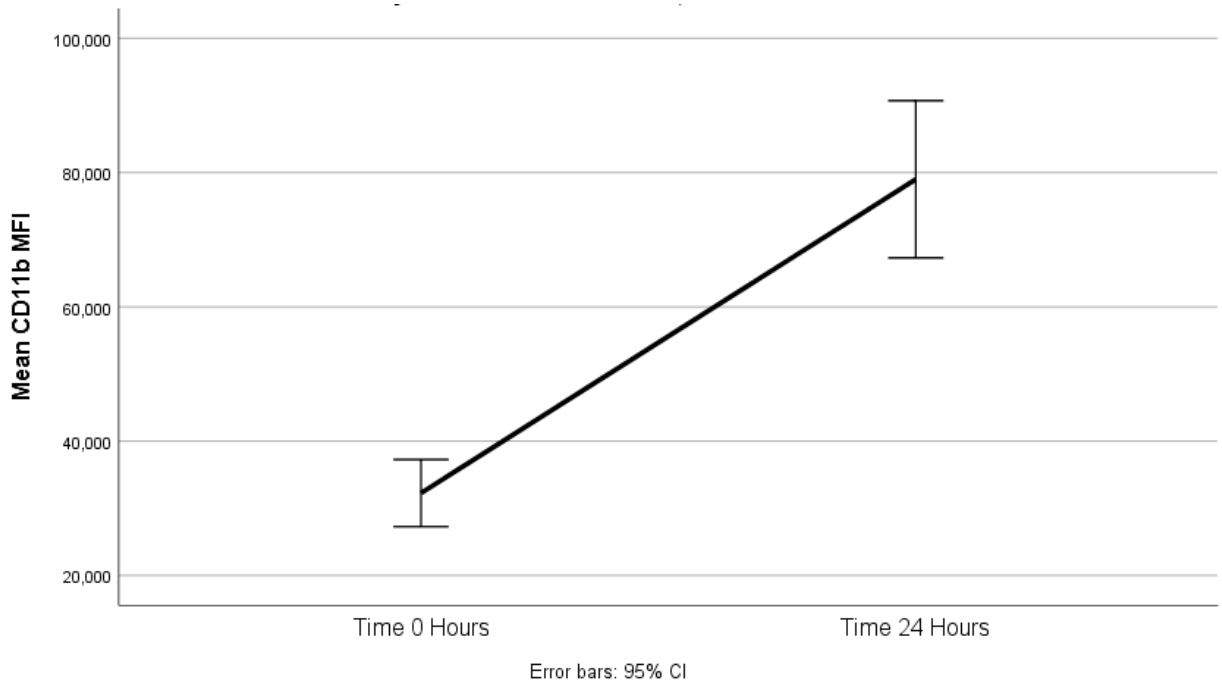


Appendix D, Figure 3. Variability of Absolute Neutrophil Count over Time, with Streck Blood Collection Tube

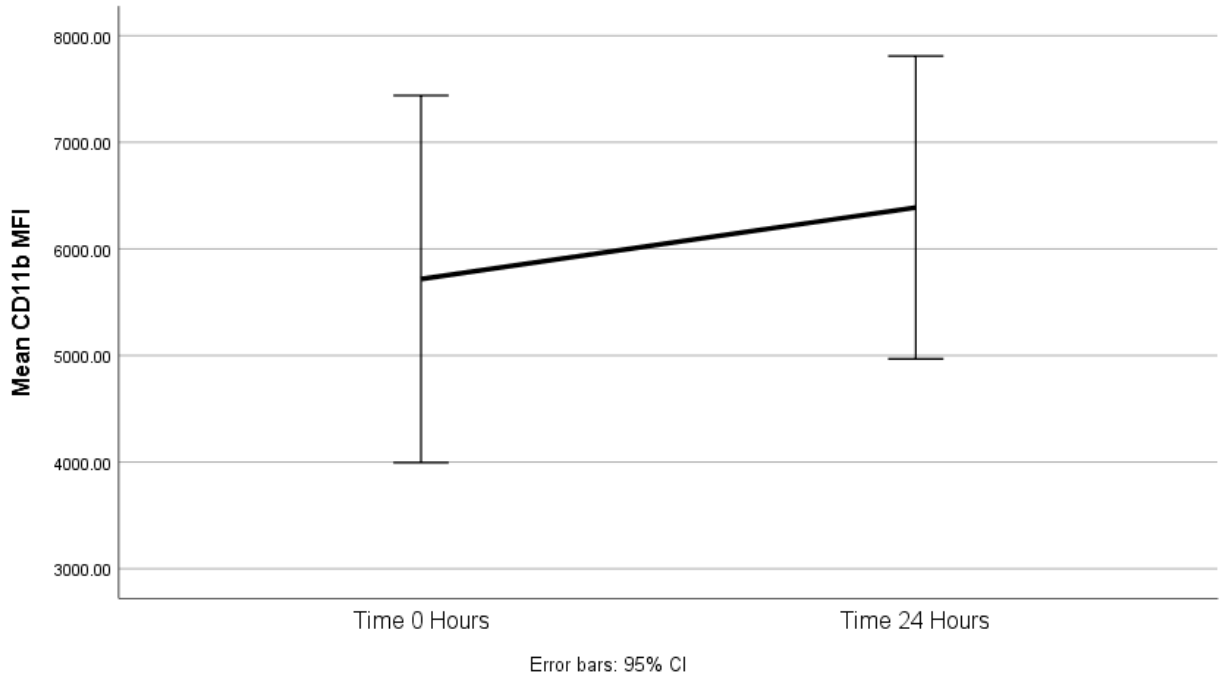
Appendix D, Table 1. Average Change in ANC over Time for each Preservative Type

Preservative	Time 0	Time 24	Total Change	Percent Change
No Preservative	2435.382	2282.038	- 153.344	6.30%
Streck	2851.431	3089.891	+ 238.460	8.36%
BCT	2827.927	3022.725	+ 194.798	6.89%

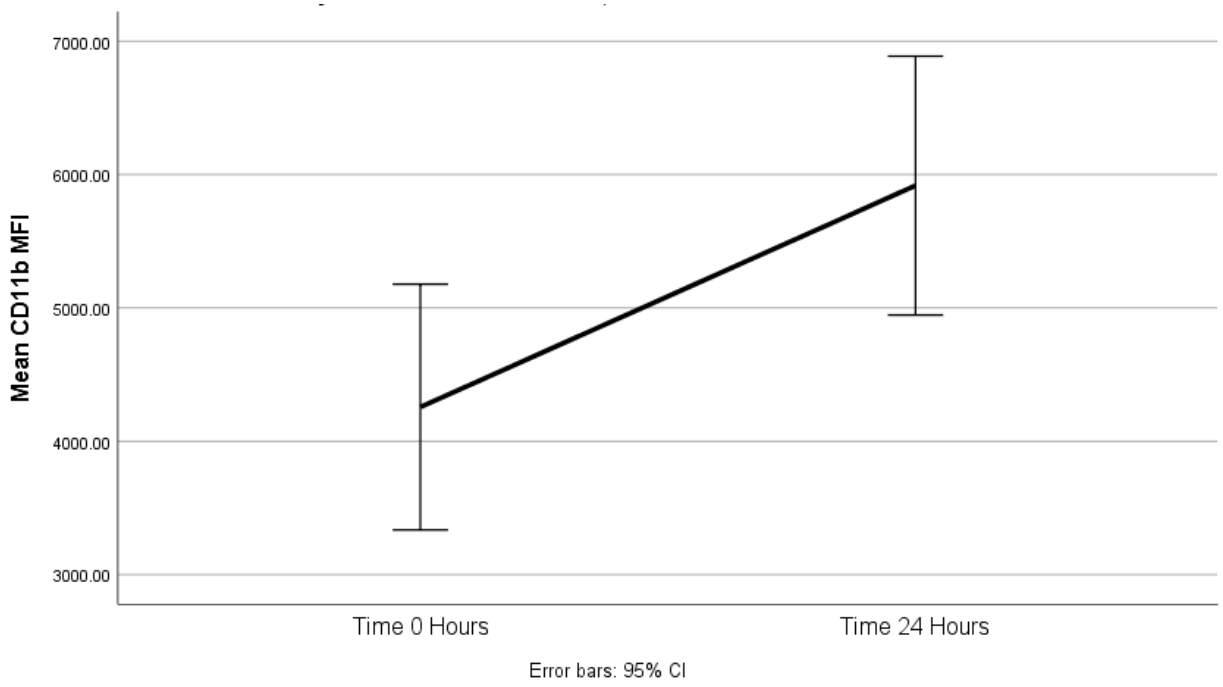
Appendix E Variability in CD11b MFI over Time at each Preservative Type



Appendix E, Figure 1. Variability of CD11b MFI over Time, with No Preservative



Appendix E, Figure 2. Variability of CD11b MFI over Time, with Streck Preservative Solution

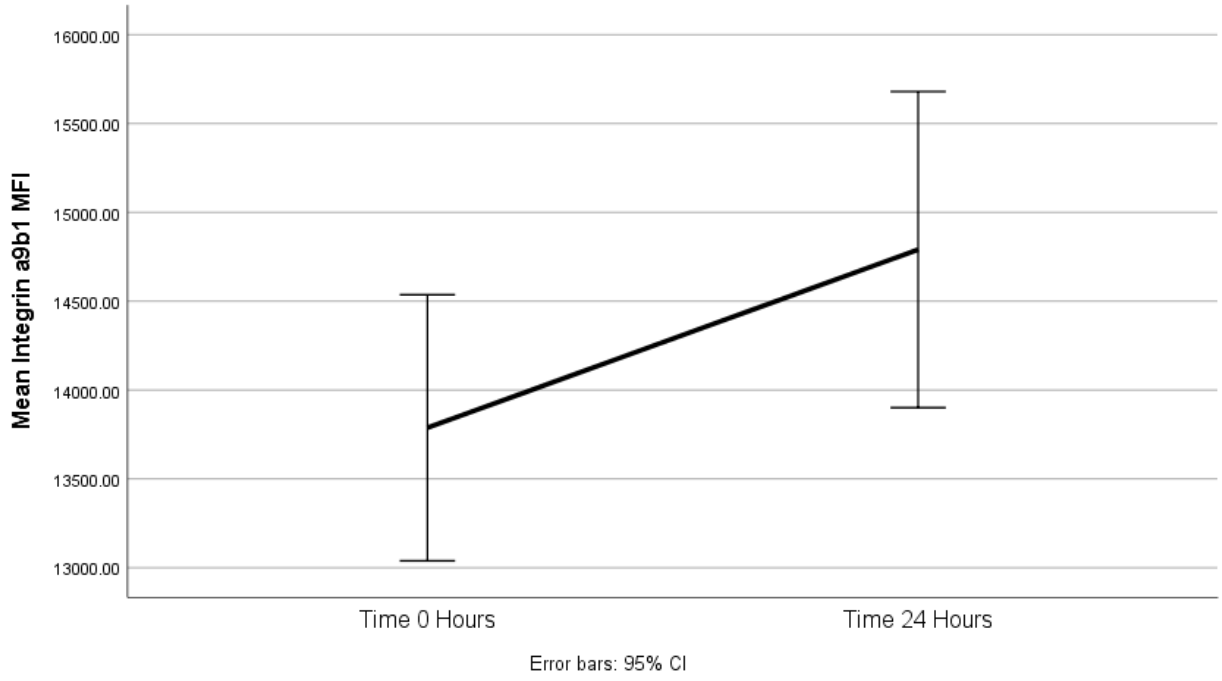


Appendix E, Figure 3. Variability of CD11b MFI over Time, with Streck Blood Collection Tube

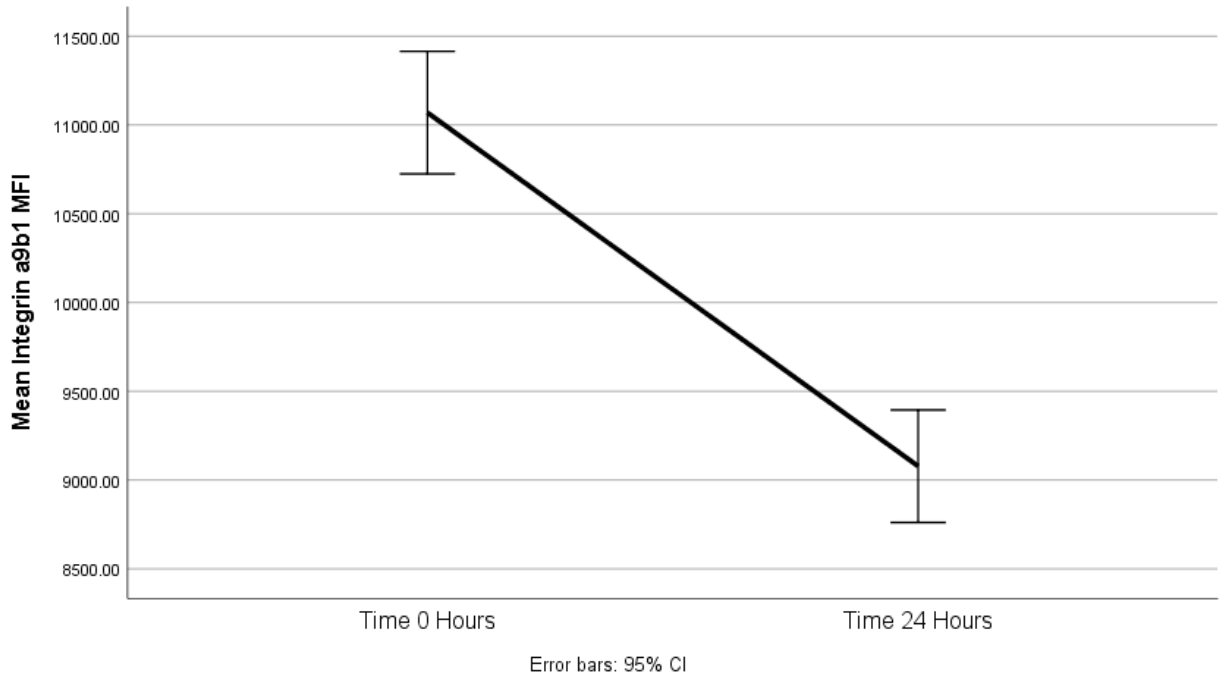
Appendix E, Table 1. Average Change in CD11b MFI over Time for each Preservative Type

Preservative	Time 0	Time 24	Total Change	Percent Change
No Preservative	32269.100	78993.940	+ 46724.840	144.80%
Streck	5717.239	6388.348	+ 671.109	11.74%
BCT	4256.392	5916.869	+ 1660.477	39.01%

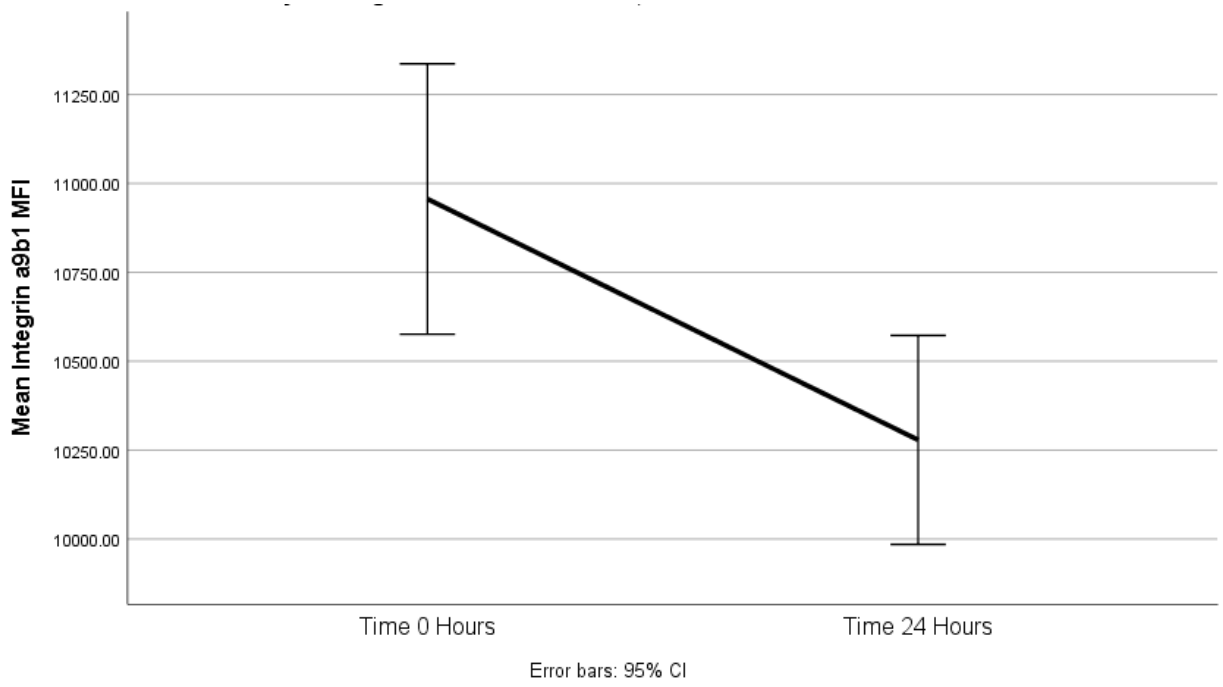
Appendix F Variability in Integrin α9β1 MFI over Time at each Preservative Type



Appendix F, Figure 1. Variability of Integrin a9b1 MFI over Time, with No Preservative



Appendix F, Figure 2. Variability of Integrin a9b1 MFI over Time, with Streck Preservative Solution

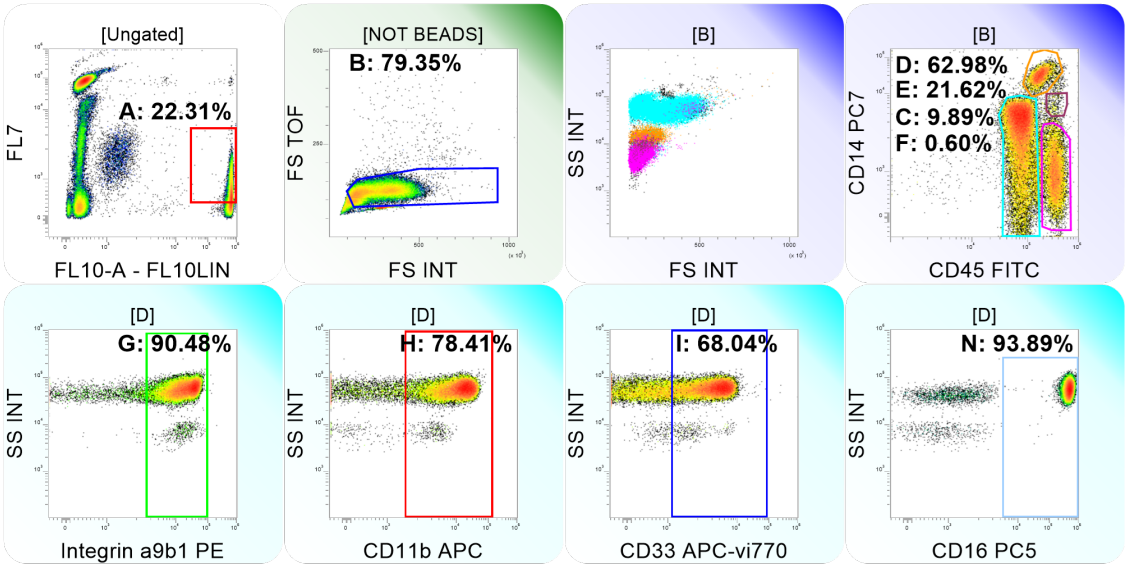


Appendix F, Figure 3. Variability of Integrin a9b1 MFI over Time, with Streck Blood Collection Tube

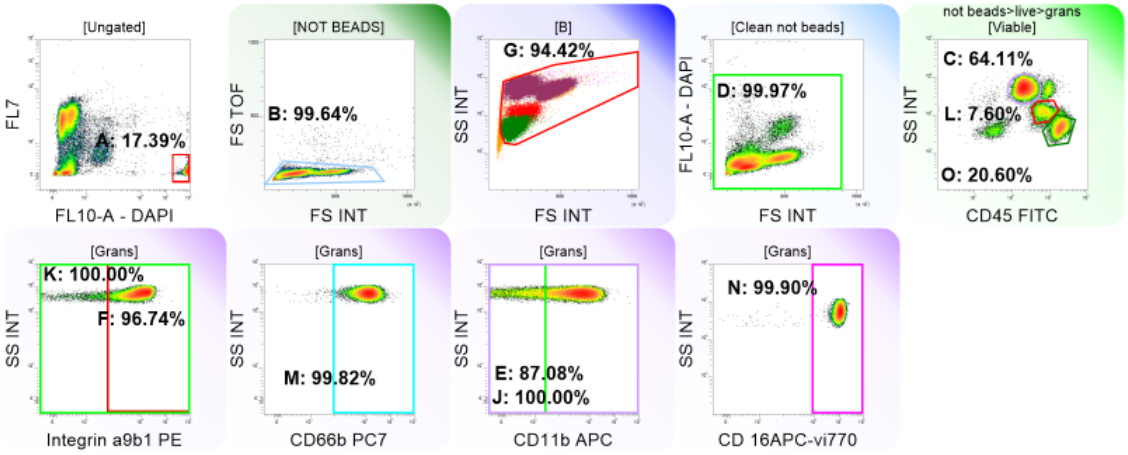
Appendix F, Table 1. Average Change in Integrin α9β1 MFI over Time for each Preservative Type

Preservative	Time 0	Time 24	Total Change	Percent Change
No Preservative	13787.530	14790.340	+ 1002.810	7.27%
Streck	11069.430	9077.850	- 1991.580	17.99%
BCT	10956.140	10278.630	- 677.510	6.18%

Appendix G . Gating Schemes



Appendix G, Figure 1. Representative Gating Scheme for BAM Analyses
Gated through B (not beads): D – Neutrophils, E – Monocytes, C – Lymphocytes, F – Eosinophils



Appendix G, Figure 2. Representative Gating Scheme for Preservative Analyses
Gated through B (not beads) and D (all cells): C – Neutrophils, O – Monocytes, L – Lymphocytes

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