INFLAMMATORY CYTOKINES LEVELS FOLLOWING VARIOUS FRACTURE PATTERNS AMONG PATIENTS MANAGED AT MOI TEACHING AND REFERRAL HOSPITAL, ELDORET, KENYA

BY

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MOI UNIVERSITY

DECLARATION

Declaration by Candidate:

This thesis is my original work and has not been presented for a degree in any other university. No part of this thesis may be reproduced without prior written permission of the author and or Moi University.

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DEDICATION

This thesis is dedicated to my lovely late mum, Dr Jennifer J. Chumo for the unwavering and unconditional support, care and most of all love.

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ABSTRACT

Background: Inflammatory cytokines are signalling molecules secreted by immune cells that mediate body's response to injury. Trauma stimulates an inflammatory response which when heightened results in undesirable outcomes like systemic inflammatory response syndrome, adult respiratory distress syndrome, multi-organ failure and death. Accurate quantification of this inflammatory response would enable better injury grading, appropriate timing of surgery, improve patient outcome and prevention of life threatening complications. The inflammatory response following various common fractures has hitherto not been quantified.

Objective: To evaluate the levels of inflammatory cytokines, vital signs and complete blood count parameters following various fracture patterns among patients managed at Moi Teaching and Referral Hospital.

Methods: A cross-sectional study conducted at MTRH between 1st April 2017 and 31st March 2019. A total of 70 adult participants comprising of 56 patients with fractures and 14 age and gender matched negative controls were studied. The patients with fractures were divided into 4 groups of 14 subjects each based on the fractured bone as follows: isolated femur fracture (F), isolated tibial fracture (T), isolated upper limb fractures (UL), and polytrauma patients (P). The control group (C) comprised of 14 age and gender matched healthy individuals without fractures. Systematic sampling was used to pick the 56 participants out of approximately 1,200 patients with fractures seen at MTRH annually. Fractures were evaluated for number, pattern, extent of soft tissue involvement and whether they were open or closed. Grading was subsequently done using the Abbreviated Injury Score (AIS) or the New Injury Severity Score (NISS). Flow-Cytometry Bead Assay was used to analyse IL-1β, IL-6, IL-8, IL-10 and TNF-α levels. In addition, patients' vital signs and complete blood count (CBC) were captured. Data was summarised as mean ± SD and presented using tables and scatter plots. One-way analysis of variance used to compare the means of the different study groups. Spearman's rank correlation tests were used to check for association between the variables.

Results: The fracture groups had higher mean IL-6 levels with the polytrauma group recording the highest levels: 181.4 ± 188.7 (P); 127.8 ± 28.6 (UL); 89.5 ± 140 (T); 106.9 ± 84.0 (F); and (12.4 ± 6.8 (C) pg/mL; $p = 0.005$). The fracture groups had higher mean IL-8 levels with the polytrauma group recording the highest levels: 126.4 ± 103.9 (P); 81.0 ± 48.8 (UL); 58.8 ± 37.3 (T); 83.1 ± 53.1 (F); (40.0 ± 30.4 (C) pg/mL; $p = 0.004$). There was positive correlation between IL-6 levels and fracture pattern severity ($r = 0.509$, $p = 0.001$), soft tissue injury ($r = 0.577$, $p = 0.001$), presence of open fractures ($r = 0.593$, $p = 0.001$), AIS ($r = 0.636$, $p = 0.001$) and NISS ($r = 0.753$, $p < 0.001$). The fracture groups had lower mean platelet counts (328.73 ± 55.4 (C); 211 ± 23 (UL); 227.15 ± 58.3 (T); 243 ± 105.4 (F); 96.2 ± 33.7 (P) X 10^9/L; $p = 0.001$). The polytrauma group (P) had statistically significant higher pulse rate, respiratory rate and shock index than the negative control group (C) ($p < 0.01$).There was inverse correlation between IL-6 and platelet count ($r = -0.252$, $p = 0.05$).

Conclusion: The IL-6 and IL-8 levels were elevated in fracture patients being highest in patients with polytrauma. There was inverse correlation between platelet count and both IL-6 and IL-8 levels. There was positive correlation between shock index and both IL-6 and IL-8.

Recommendation: Adoption of the use of inflammatory cytokines to improve trauma patients' stratification, determine best time for surgery and to refine existing trauma scores. In addition, the study recommends the use of platelet count as an alternate measure of inflammation following trauma.

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ABBREVIATIONS AND ACRONYMS

AMPATH	Academic Model Providing Access To Healthcare
ARDS	Acute Respiratory Distress Syndrome
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IREC	Institutional Research Ethics Committee
MODS	Multi-Organ Dysfunction Syndrome
MTRH	Moi Teaching and Referral Hospital
PMNs	Polymorphonuclear leukocytes
SI	Shock Index
SIRS	Systemic Inflammatory Response Syndrome
TNF – α	Tumor Necrosis Factor-α

OPERATIONAL DEFINITION OF KEY TERMS

Complete Blood Count	refers to a blood panel comprising counts and characteristics of blood cell types and the concentrations of haemoglobin.
Cytometric Bead Assay	refers to an immunologic technique used to measure physical and chemical characteristics of a population of cells or particles
Fracture	refers to a break in the continuity of bone
Inflammatory Cytokines	these are signalling molecules that are secreted by immune cells and certain other cell types that promote multi-systemic response to injury for example following trauma.
Vital Signs	these are clinical measurements, specifically pulse rate, temperature, respiration rate and blood pressure that indicate the state of an individual's essential body functions.

CHAPTER ONE: INTRODUCTION

1.1 Background

Trauma continues to be a significant cause of morbidity and mortality contributing to 20% of hospitalization and 30% of deaths for individuals aged 19-40 years old (Vos et al., 2015). Non-fatal injuries affect approximately 4% of the global population (Raina *et al*., 2015).

Sub-Saharan Africa suffers the significant proportion of trauma-associated health burden. Studies have documented up to 300,000 injuries (both trivial and significant) per 100,000 persons in a year (Nordberg *et al*., 2009). In Kenya, for instance, increased urbanization, population growth and an upsurge of motorcycle use have all resulted in a sharp rise of trauma following road traffic accidents (Saidi *et al*., 2014). Of these accident victims, three quarters are between the ages of 20-49 years-old, the most productive age group. The majority (73%) are males and the mean age of those affected is 32.4 years (Osoro *et al*., 2011). Limb fractures are seen in up to 38% of the accidents victims with head injury observed in approximately 5% (Osoro *et al*., 2011). The trends are equally disheartening with a 29% annual increase noted in the number of motorcycle injuries for the period 2004 to 2009. During this period, deaths following road traffic accidents also increased at an average annual rate of 7% (Bachani *et al*., 2012).

Mortality following trauma occurs in three main phases (Baker *et al*., 1980). Forty percent of the deaths occur immediately, at the scene of the accident due to haemorrhage and crushing injuries. Secondly, a smaller fraction die within 24 hours of hospitalization due to hypoxia, hypovolemia, or head injury. Approximately 45% of deaths occur days to weeks after hospitalization. These are mostly due to head

injury and immune mediated syndromes of acute respiratory distress syndrome (ARDS), or multi-organ dysfunction syndrome (MODS) (Baker *et al*., 1980). These immune mediated syndromes are because trauma is known to induce a rise in the level of inflammation in the body with inflammatory markers rising by between 4 and 30-fold (Giannoudis *et al*., 2004). This response is beneficial and protective as it limits further injury and initiates the reparative processes (Giannoudis *et al*., 2004). When heightened however, inflammation often results in life threatening conditions mentioned above. These are commonly seen in multiply injured patients. Quantification and characterization of the inflammatory response following common fractures would thus enable better grading injury, optimum timing of surgery, prediction of outcome and refinement of existing trauma scoring systems. Together, these will serve to improve the care of trauma patients leading to better outcomes.

1.2 Problem Statement

Heightened inflammation as shown by elevated levels of inflammatory cytokines is a common phenomenon in polytrauma patients often resulting in life threatening states of SIRS and MODS. The cumulative inflammation results from multiple isolated injuries. The levels and patterns of inflammation attributable to various common fractures are unknown. Characterization of the inflammatory load associated with various common fractures would enable more accurate assessment of trauma patients, better timed surgical intervention and improved patient outcomes.

1.3 Justification

Inflammation is an innate response to various insults including trauma. Inflammation, though initially beneficial, becomes life threatening when heightened. Surgery, the mainstay of trauma care stimulates additional inflammatory response. Evaluation of the level of inflammation is thus paramount in the care of trauma patients. The use of molecular and immunologic markers would potentially offer a more accurate assessment of the level of inflammation. Information on the level of inflammation following specific fractures would serve as a guide for the inflammatory status among patients with such fractures. This would enable better patient stratification, outcome prediction and timing for surgery. Indeed, inaccurate grading of patient's inflammatory states may result in inappropriately timed surgery and poor patient outcome.

1.4 Research Questions

What are the patterns of inflammatory cytokines, vital signs and complete blood count parameters following various fracture patterns among patients managed at Moi Teaching and Referral Hospital, Eldoret, Kenya?

1.5 Objectives

1.5.1 General objective:

To evaluate the levels of inflammatory cytokines, vital signs and complete blood count parameters following various fracture patterns among patients managed at Moi Teaching and Referral Hospital, Eldoret, Kenya.

1.5.2 Specific objectives

i. To evaluate the levels of IL-1, IL-6, IL-8 and TNF-α following various fractures in patients managed at MTRH.
ii. To evaluate the vital signs following various fractures in patients managed at MTRH.
iii. To evaluate the complete blood count parameters following various fractures in patients managed at MTRH
iv. To explore correlations between inflammatory cytokines, vital signs, complete blood count parameters and injury severity following various fractures in patients managed at MTRH.

CHAPTER TWO: LITERATURE REVIEW

2.1 Trauma Induced Inflammatory Response

2.1.1 Nature of Trauma Induced Inflammatory Response

Trauma resulting into fractures and injury to soft tissue has been shown to induce an increase in the level of inflammation in the body with inflammatory markers rising by between 4 and 30-fold (Giannoudis *et al*., 2004). This is part of the innate, non-specific immune response. The response is beneficial as it mediates vasoconstriction and platelet activation leading to haemostasis (Male, 2006). In addition, it mediates increased vascular permeability leading to in-migration of inflammatory cells as well as pro and anti-inflammatory mediators (Male, 2006).

When widespread, however, this inflammatory response turns detrimental leading to the development of systemic inflammatory reaction syndrome (SIRS), acute respiratory distress syndrome (ARDS) and multi-organ dysfunction syndrome (MODS). These are all associated with relatively high morbidity and mortality (Pape *et al*., 2007, Dinarello, 2000).

The level of the inflammatory response has been shown to be dependent on the severity of injury (Dinarello, 2000). In addition, the effect is additive meaning a patient with more than one injury would have a more heightened response. In addition, surgery causes further tissue damage and induces additional inflammatory response and may worsen the condition of severely injured patients. This is discussed further below.

2.1.2 Mechanism of Injury Induced Inflammatory Response

Following trauma a local immune mediated response is stimulated. When the trauma is severe, this response becomes systemic. The cascade of trauma induced inflammatory response is complex as demonstrated Figure 1 below. Mediators of this inflammatory response are broadly looked at as pro-inflammatory cytokines, anti-inflammatory cytokines, chemical mediators and cellular elements (Sears *et al*., 2009).

Major pro-inflammatory and anti-inflammatory cytokines include tumor necrosis factor (TNF)-α, interleukin-1, interleukin-6, interleukin -8, and interleukin-10 among others. These mediate cellular migration and activation, increased vascular permeability and vasodilation. After arriving at the site of injury, polymorphonuclear leukocytes (PMNs) generate and release numerous active substances, including proteolytic enzymes, reactive oxygen species, and vasoactive substances. These induce the microvascular endothelium to increase its permeability resulting in interstitial edema and intravascular coagulation (Giannoudis & Dinopoulos, 2004; Sears *et al*., 2009). This increased endothelial permeability, interstitial edema and intravascular occlusion all contribute to the multi-organ failure.

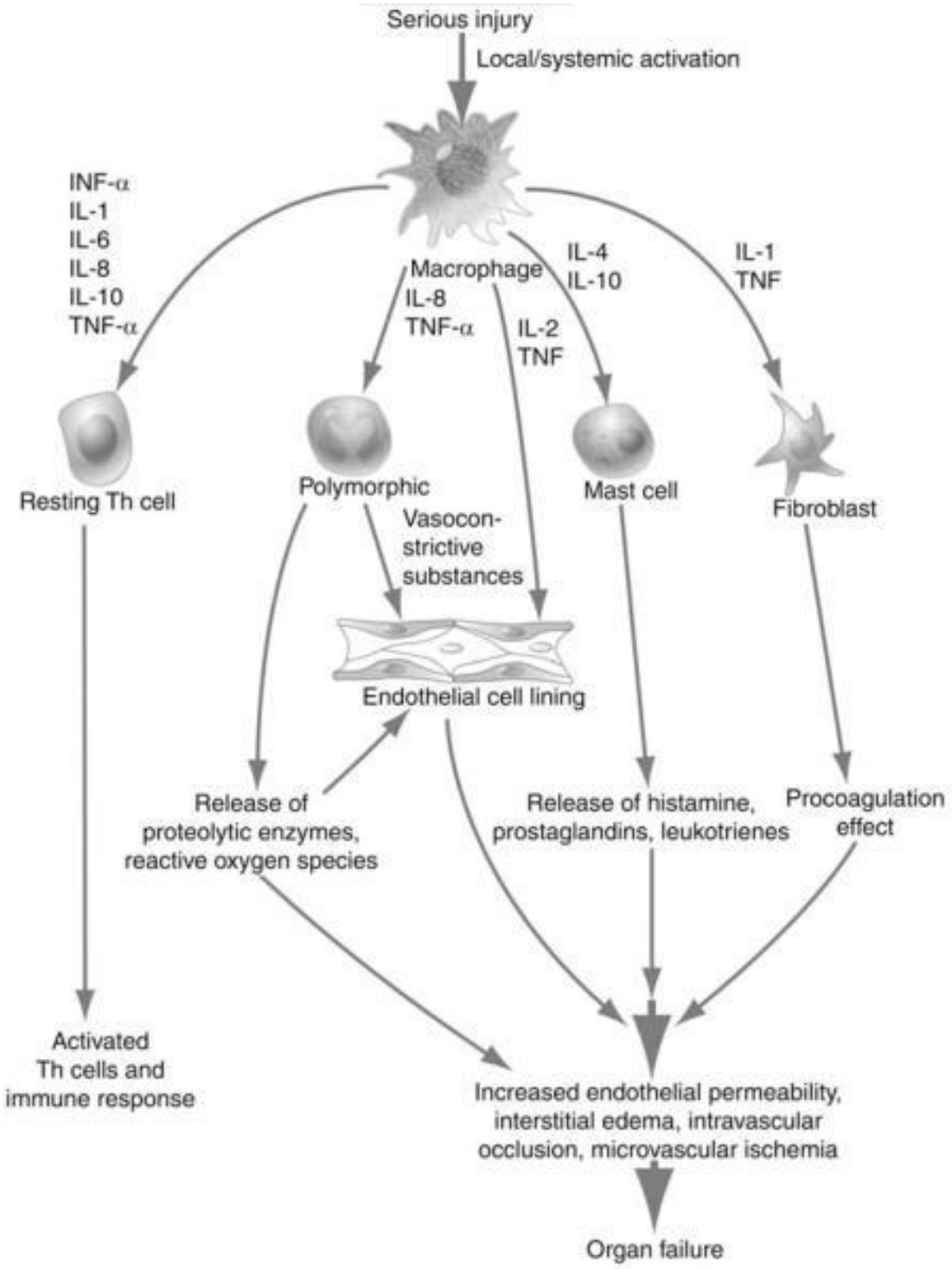


Figure 1: Systemic inflammatory response to Injury

IL = interleukin, INF = interferon, Th = T helper, TNF = tumor necrosis factor

Adopted from Sears *et al*., 2009

2.1.3 Cytokines involved in Inflammatory Response

Cytokines are a group of proteins made by the immune system that act as chemical messengers to upregulate or downregulate body's immune and inflammatory response (Lackie & O'Callaghan, 2010). They include interleukin, chemokines, interferons and lymphokines. There are tens of cytokines some that serve to increase the inflammatory response referred as pro-inflammatory cytokines, those that downregulate the response referred to us anti-inflammatory cytokines and some that are both pro and anti-inflammatory.

Pro-inflammatory cytokines include interleukin-1, tumor necrosis factor-γ, Granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-15, interleukin-16, interleukin-17 and interleukin-18. Interleukin-6, Interleukin-8 and tumor growth factor-β are both pro and anti-inflammatory (Scheller *et al.*, 2011). Anti-inflammatory cytokines include interleukin-4, interleukin-10, interleukin-11, interleukin-13 and interleukin-18.

The role of various pro-inflammatory and anti-inflammatory cytokines in the immune activity following trauma has attracted significant research interest (Giannoudis *et al*., 2004; Ozturk *et al*, 2007; Pape *et al*., 2007; Sears *et al*., 2009). The use of these biomarkers to assess the level of inflammation has also been attempted with varying success. TNF-α is produced by a variety of cells and acts to increase the permeability of endothelial cells and the expression of adhesion molecules. However, investigations into the use of TNF-α as a clinical marker of inflammation have been equivocal. IL-1 and -10 have also been studied with varying results (Volpin *et al*., 2014). Two markers, IL-6 and IL-8 have been shown to rise proportionately with the severity of injury (Volpin *et al*., 2014). In addition, IL-6 has been shown to be a

reliable marker of the magnitude of systemic inflammation. In multiply injured patients, the IL-6 levels are up to 6 times higher than the baseline levels (Giannoudis *et al*., 2008). Among children, levels of IL-8 was shown to be a sensitive predictor of post-injury mortality (Ozturk *et al*, 2007). Interleukin-6 and interleukin-8 are reviewed further below.

2.1.3.1 Interleukin-6

Interleukin-6 is a 212 amino acid cytokine coded for by genes located in chromosome 7 (Hashizume & Mihara, 2011). It possesses polymorphism owing to nucleotide changes in multiple positions. This affords the body the ability to secrete different interleukin-6 molecules (Sutherland *et al*., 2005). Interleukin-6 is secreted by different cell types including fibrobalsts, macrophages, endothelial cells, B lymphocytes, T lymphocytes, neutrophils, astrocytes, myocytes, vascular smooth muscle cells, osteoblasts and keratinocytes (Dixon, 1993) Interleukin-6 has been shown to mediate at least ten different functions in the body. These include:

- Acute phase response to trauma (Biffl, 1994),
- Stimulation of the hypothalamic-pituitary-adrenal axis resulting in:
    - Fever induction (Baumann *et al*., 1995)
    - Cortisol release (Dimopoulou *et al*., 2004)
- B and T cell maturation (Hashizume & Mihara, 2011).
- Stimulation of Immunoglobulin release from B cells
- Release of platelets from megakaryocytes leading to more hemostasis potential (Burstein *et al*., 1996),
- Red Blood Cell production (Pignatti *et al*., 2003),
- Endothelial cell activation and induction of receptor presentation (Pignatti *et al*., 2003) and

- Keratinocyte proliferation (Burstein *et al*., 1996),

Studies by Li *et al*., (2016), Perl *et al*., (2003) and Volpin *et al*., (2014) demonstrated elevation in levels of interleukin-6 following trauma. This elevation was varied based on the site and degree of injury has shown by Kleber *et al*., (2015) in a murine study. In addition, Pape *et al*., (2008) demonstrated that there is further rise in interleukin-6 levels owing to the blood loss accompanying tissue injury. Studies by Strecker, *et al*., (2003) and Giannoudis, *et al*., (2008) documented that IL-6 following trauma can accurately predict development of systemic inflammatory response syndrome, organ failure and death.

2.1.3.2 Interleukin-8

Interleukin-8 is a chemokine produced by macrophages, epithelial cells, airway smooth muscle cells and endothelial cells (Hedges *et al*.,, 2000). It is produced as a precursor molecule with 99 amino acids which is later cleaved into different isoforms commonest of which contains 72 amino acids (Brat *et al*., 2005).

Interleukin-8 is key chemotactic agent for neutrophils. In addition, it induces phagocytocis of microbes by polymorphonuclear cells (Zahn *et al*., 1997). Other documented functions include promotion of angiogenesis, histamine release by mast cells and the respiratory burst required for microbial destruction following phagocytosis.

In the setting of trauma, interleukin-8 has been shown to rise within hours of injury. This was demonstrated by Horst *et al*., (2015); Li *et al*., (2016) and Volpin *et al*., (2014). In addition, Haller *et al*., (2107) documented the rise to be dependent on the site and degree of injury. It their study, they showed higher levels of IL-8 following tibial plafond fractures than following tibial plateau fractures.

2.1.4 Surgery and Inflammatory Response

Surgery is the mainstay of definitive management of trauma patients. Surgical procedures cause further tissue injury which has been shown to stimulate additional inflammatory response. Thus, the initial trauma is often regarded as the 'primary hit' while the surgical intervention the 'secondary hit' (Giannoudis *et al*., 1998; Pape, 2008).

Approximately four decades ago, severely injured patients were offered early, aggressive and definitive surgeries which would take hours. It was noted however, that the patient would soon develop systemic inflammatory response (SIRS) and multiorgan failure dysfunction (MODS). These life threatening conditions resulted from the additive tissue insult and the accompanying inflammatory response. In a bid to improve the patient outcome, severely injured patients started being offered minimal life-saving surgery in the acute setting. This would focus on initial control of hemorrhage followed by rapid and temporary stabilization of fractures along with soft-tissue debridement. This would subsequently be followed by staged, definitive fracture fixation. This was shown to result in better patient outcome than immediate aggressive definitive surgery (Lichte *et al*., 2012; Pape *et al*., 2007). This thus served as the basis of a paradigm shift from '**early total care**' to '**damage control surgery**'. Tscherne *et al* developed a framework on the guiding principles for damage control surgery. This is initially focused on hemostasis and decompression of organ cavities. Orthopedic emergencies are subsequently addressed looking at open fractures, compartment syndrome, unstable pelvic injuries, and fractures with concomitant vascular injuries requiring stabilization. Closed fractures are stabilized next. Definitive surgery is then planned approximately 72 hours later dependent on the resolution of the inflammatory response (Tscherne *et al*. 1998).

Studies have also demonstrated that different surgical procedures induce differing levels of inflammation (Giannoudis *et al*., 1998; Husebye *et al*., 2012). The differences noted are owing to differences in soft tissue manipulation, size of bone fixed and specifics of procedures like whether reaming is done during intramedullary nail placement or not (Giannoudis *et al*., 1998).

2.1.5 Grading of Trauma Severity

Emergency trauma care, among other fields of medicine, has benefited a lot from scoring systems. These scales standardize injury/condition characterization thereby enabling better communication among clinicians as well as comparison between patients and centres. In addition, serial evaluation with these scores enable evaluation of changes in patient condition. Even more importantly, these scoring systems enable grading of the severity as well as better prediction of patient outcome.

Several factors affect the outcome of patients following trauma. One of these is the severity of injury (Boyd *et al*., 1987). This was the basis of the Injury Severity Score by Boyd *et al*. (1987) and its improvement, the New Injury Severity Score (Osler, *et al*., 1997).

2.2 Vital Signs Changes following Trauma

Vital signs are a set of important parameters that indicate the status of the body's functions necessary to sustain life. These include temperature, respiratory rate, heart rate and blood pressure.

Body temperature is measured as the core temperature with the reference range set at 36.0°C to 37.5°C. Hypothermia occurs frequently among severely injured patients and it has been described as one of the trauma triad of death (Mikhail, 1999). This results

from both the initial injury and the resuscitation maneuvers. The severity of hypothermia is graded as mild (34^{o}C – 35.9^{o}C), moderate (32^{o}C – 33.9^{o}C) and severe (<32^{o}C). The mortality rate for patients with severe hypothermia approximates 100% (Luna *et al*., 1988). Hypothermia is detrimental as it causes a rise in the peripheral vascular resistance, cardiac dysrhythmias and a left shift to the hemoglobin-oxygen dissociation curve (Frank *et al*., 1993).

Heart rate refers to the number of beats per minute. The reference range for adults is set at 60 – 100 beats per minute. Trauma leading to blood loss leads to a reduction in the blood pressure. In a bid to restore the blood pressure, the heart beats faster and harder thereby increasing the cardiac output which is a product of the heart rate and stroke volume. This increase is mediated by increased activity in the sympathetic nervous system. A rise in heart rate is the first vital sign to change following blood loss (Little *et al*., 1988). Studies by Larson *et al*., (2010) as well as by Smith & Choi (2017) demonstrated that vital signs among trauma patients could precisely predict the need for massive transfusion.

Respiratory rate is measured as the number of breaths in a minute. The reference range for an adult is 12-20 breathes per minute. In isolation, tachypnea has been shown to correctly identify severe chest injury (Yonge *et al*., 2017). When used in combination with other vital signs, respiratory rate serves to improve the triage of trauma patients (Lehmann *et al*., 2009).

Blood pressure refers to the highest and lowest recordable pressure within the major vessel during a cardiac cycle.

Lastly, shock index, which is defined as heart rate divided by systolic blood pressure, has been described in a bid to increase the reliability in the assessment of hypovolemic shock (Rady *et al*., 1994). Indeed, shock index has been shown to be useful in identifying patients with significant post-trauma hemorrhage requiring blood transfusion (Birkhahn *et al*., 2005) Findings by Vandromme *et al*., (2011) suggest a cut off of 1.1 better predicted need for massive transfusion as compared to a cut off of 0.9. In addition, the sensitive and specific of shock index in predicting need for blood transfusion can be increased by taking repeated measurements (Chen *et al*., 2009). Prognostically, a rise in the shock index by >0.3 has been shown to be associated by up to a five-fold increase in mortality rates. (McNab *et al*., 2012). Shock index is however prone to variations due various factors. Pain and anxiety post trauma increases the heart rate and thus the shock index. Variations in shock index are also seen due to the initial resuscitation (Hagiwara *et al*., 2010). Advancing age limits the physiological response to trauma and thus the shock index changes are stifled (Salottolo, *et al*, 2013). Despite this documentation, Kim *et al.*, (2016) demonstrated that shock index was predictive of transfusion requirement even among the geriatric population. Athletes have low baseline heart rates and thus low shock indices.

2.3 Hematological Changes following Trauma

Complete blood count (CBC) refers to a blood panel that gives information about the counts of different cell types in a patient's blood as well as the concentrations of hemoglobin. The numbers usually reflect both the need of a particular cell line as well as the capacity of the bone marrow to produce and release the same. Following trauma, there are rapid changes seen as highlighted below.

In the setting of trauma, platelet count has received significant attention with studies looking at the temporal changes as well as its utility in informing decision making and predicting patient outcome. Studies by Hobisch-Hagen *et al*., (2000) and Kutcher *et al*., (2012) demonstrated a reduction in platelet counts following trauma. This results from the consumptive coagulation that takes place in a bid to limit blood loss. In addition to hypothermia discussed above, thrombocytopenia is the second component in the triad of death in the trauma setting (Mikhail, 1999). A study by Yolcu *et al*., (2013) documented the diagnostic role of platelet count reduction in estimating injury severity. Prognostic ally, thrombocytopenia has been shown to be predictive of patient outcome including death (Jin *et al*., 2018). These findings have informed the use of platelet count in determining timing of surgery among critically ill patients (Nieto & Baroan, 2017).

White blood cells are the second cell type. Following trauma, a subtype, neutrophils are the first to rise as they are involved in the acute phase reaction. The elevation in the total white blood cell and neutrophils in particular following injury has been documented (Gürkanlar *et al*., 2009; Iyidobi *et al*., 2013; Turan *et al*., 2019). In a study by Turan *et al*. (2018), while studying patients with firearm injuries, they explored the prognostic accuracy of white blood cell count in predicting mortality.
Leucocytosis and in particular neutrophilia following trauma results from the rapid release from the bone marrow. This release is mediated by a chemicals released by damaged cells called damage-associated molecular patterns (DAMPs) (Pittman & Kubes, 2013). These DAMP molecules include DNA, histones, high mobility group protein B1, N-formyl peptides, Adenosine triphosphate, interleukin-1α and many others (Chen and Nunez 2010)

The leading cause of death among trauma patients is bleeding. This has two effects, loss of volume as well as loss of oxygen carrying capacity. Haemoglobin is the oxygen carrying molecule and packed red cell transfusion is targeted as restoring levels of this vital molecule. Unlike above two parameters, the reduction in haemoglobin level is not based on the severity of injury but on amount of blood loss. Studies have looked into strategies for managing continuing bleeding (Spahn *et al.*, 2013) as well as principles and complications of massive transfusion (Chidester *et al*., 2012; Raymer *et al*., 2012).

CHAPTER THREE: METHODOLOGY

3.1 Study Setting

The study was carried out at Moi Teaching and Referral Hospital. This is located in Eldoret town, Kenya's fifth largest urban centre and the County Headquarters of Uasin Gishu County. Eldoret is located approximately 300 kilometres west of Nairobi.

The MTRH is the second largest referral hospital in Kenya. It serves the greater western Kenya, eastern Uganda and parts of Southern Sudan, the catchment area of at least 20 million people. Approximately ten thousand patients are received at the accident and emergency department annually. Admissions to the Orthopaedics wards are approximately 1,300 annually.

Recruitment of study participant was carried out at the Accident and Emergency Department and the Orthopaedic Surgery wards. Inflammatory cytokines laboratory analysis was conducted at the AMPATH Reference Laboratories whereas the complete blood count analysis was carried out at the Moi Teaching and Referral Hospital Main Laboratory.

3.2 Study Design

The study employed a cross-sectional study design as illustrated in Figure 2.

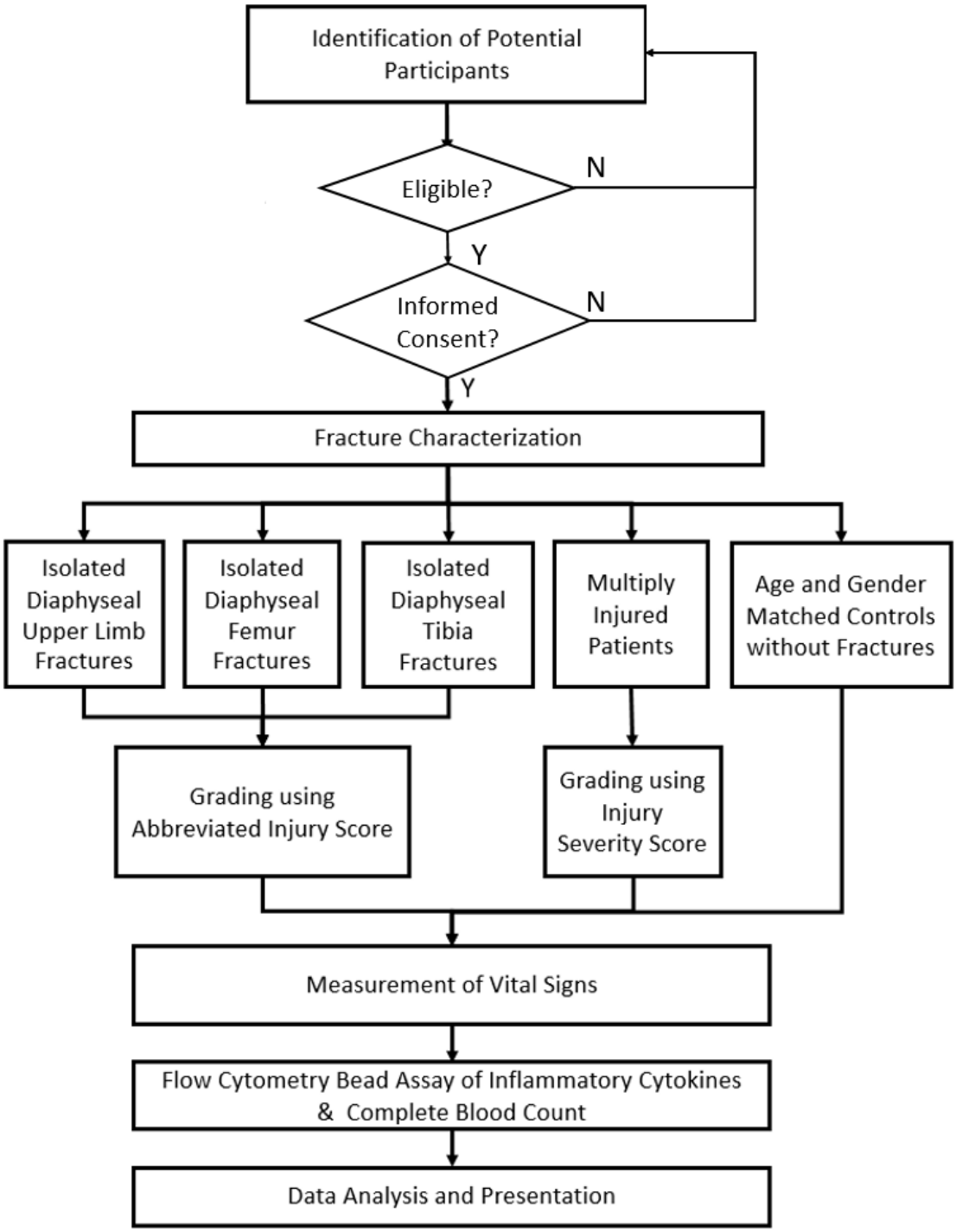


Figure 2: Study Flowchart

3.3 Study Population

The study population comprised of patients who presented to Moi Teaching and Referral Hospital with fractures of various long bones in the upper and lower limbs. The participants were assigned to different study groups based on the bone involved as highlighted below:

i. The 'Femur' Group – this group comprised patients presenting with isolated fractures of the diaphysis of the femur as the only major injury.
ii. The 'Tibia' Group – this group comprised patients presenting with isolated fractures of the diaphysis of the tibia as the only major injury.
iii. The 'Upper Limb' Group – this group comprised patients presenting with isolated upper limb long bone diaphyseal fracture as the only major injury.
iv. The 'Polytrauma' Group – this group comprised of patients presenting with significant injuries involving at least three body systems. This group served as the positive control group.
v. The 'Negative Control' Group – this group comprised age and gender matched individuals without fractures.

3.3.1 Inclusion Criteria

The study included participants meeting the following criteria:

- Fracture patients presenting within 36 hours of injury
- Fracture patients aged between 18 and 60 years.

3.3.2 Exclusion criteria

The study excluded:

- Patients who had already undergone surgical manipulation. This is because surgery induces additional inflammatory response over and above that caused by the initial trauma.
- Patients known to have active infection, inflammation or autoimmune disease prior to the injury. These were identified using a set of questions based on common symptoms. These patients were excluded because these conditions induce an elevation in the inflammatory markers just as trauma does.

3.3.3 Sample Size

The study comprised five comparison groups. The sample size was determined using power analysis for one-way ANOVA. Results for IL-6 and IL-8 from previous studies were used in the determination of total sample size.

Calculations for IL-6 yielded a higher sample size per group. Power was set at 0.8 and α value at 0.05. To determine the effect size, the standard deviation from previous studies of 80 was used together with the difference of the means between the lowest (negative control) group and the highest (positive control) group. This gave a treatment effect size (ω^2) of 0.4390.

For α value of 0.05, β value of 0.8 and effect size of 0.4390, the total sample size was determined using the formulae below by Kim (2016):

$$n = \lambda^2 \times \frac{(1-\omega^2)}{/\ \omega^2}$$

Where ‘n’ is total sample size, ‘λ’ is the non-centrality parameter and ω is the calculated effect size.

This generated a total sample size of 70 for the four groups. Thus, the sample size per group was set at 14 (Figure 3). No drop-out was expected as there was no follow-up of patients.

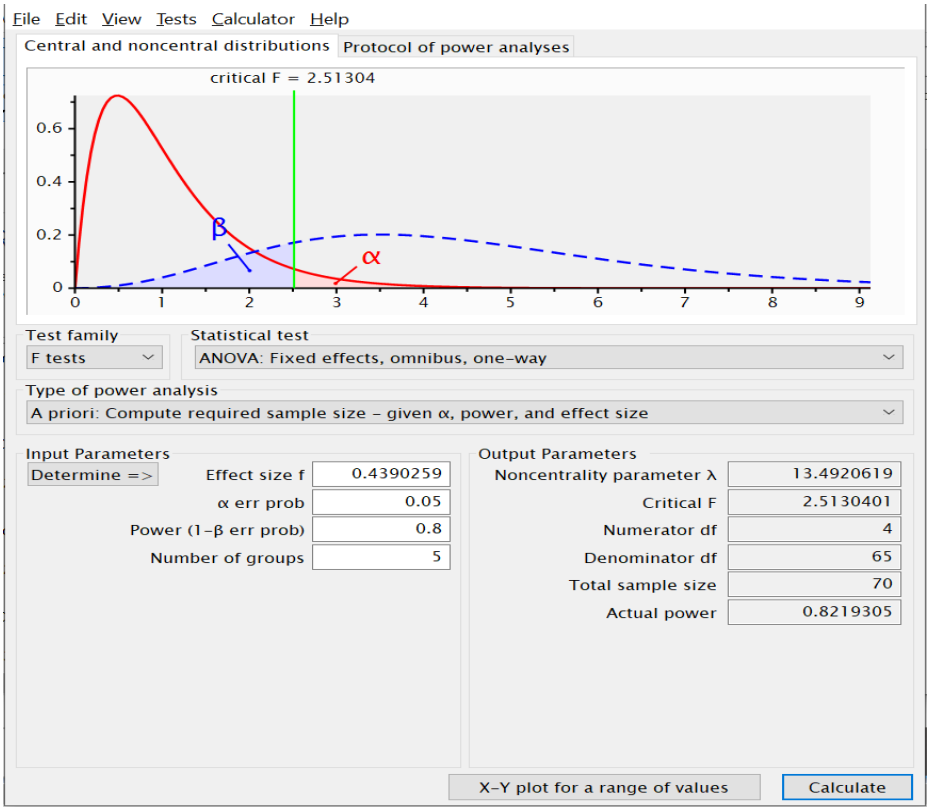


Figure 3: Output window for Sample Size Determination

3.3.4 Sampling Technique

Systematic sampling method was used to identify study participants. From the preceding year, 2016, there had been 153 patients with femur shaft fractures, 188 patients with tibia shaft fractures, 72 with upper limb fractures and 58 multiply injured patients. It was expected that approximately 50% of the presenting patients would be excluded due to delayed presentation or associated injuries. Based on the calculated sample size of 14 per group, the researcher recruited every 4th patient with isolated femur fracture, every 5th patient with isolated tibia fracture and every 2nd patient with isolated upper limb fracture and every 2nd multiply injured patient.

3.4 Study Procedures

3.4.1 Consenting Process

After adequate resuscitation of patients presenting with fractures at MTRH, a good rapport was established. Patient eligibility was subsequently evaluated and for those meeting the inclusion criteria, a brief introduction on the objectives and the scope of the study was shared. The participants were given a chance to ask any questions about the study. The participants were also informed that their participation in the study was entirely voluntary and that the care they would receive would not in any way be affected by their acceptance or refusal to participate in the study.

It was explained that participation in the study would entail clinical and radiological evaluation of patients. In addition, 6 millilitres of venous blood would then be drawn using an aseptic technique. The blood would be used for complete blood count and inflammatory cytokine evaluation only.

The participants were also informed that although they might not benefit directly from the results of the study, the information gathered would help guide the treatment of other patients within the hospital and elsewhere.

Subsequent to answering all the queries raised, consent was sought from potential participants. There was no coercion. The participants were given the researcher's contacts as well as those of the Moi University/MTRH Institutional Research Ethics Committee (IREC).

This consenting process was conducted in either English or Kiswahili languages. The Consent Form (Appendix II) and introductory letter (Appendix II) were used.

3.4.2 Recruitment of Controls

The control subjects for the study were age and gender matched healthy individuals without fractures. A brief introduction on the objectives and the scope of the study was shared. These participants were informed that they would serve as control subjects for comparison. The control subjects were free to ask any questions about the study. They were also informed that their participation was entirely voluntary and that the care of their loved ones would not in any way be affected by their acceptance or refusal to participate.

This consenting process was done in either English or Kiswahili languages. The Consent Form (Appendix II) and Introductory Letter (Appendix III) were used.

3.4.3 Sample Collection and Processing

Using an aseptic procedure, six millilitres of venous blood were drawn into Ethylenediaminetetraacetic acid (EDTA) vacutainers between 18 and 36 hours after injury. The antecubital vein was preferably used. Plasma separation by centrifugation was done within 4 hours and subsequently stored at -80^{o}C.

Batch evaluation of inflammatory cytokines as carried out by Flow-Cytometry Bead Assay at the AMPATH Reference Laboratories using BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit for IL-1, IL-6, IL-8 and TNF-α. The procedure outlined by the manufacturer, BD BioScience was followed (Appendix VII).

3.5 Data Management

3.5.1 Data Collection, Cleaning and Entry

Data on injury severity was collected using data collection sheet (Appendix III). Cytokine levels generated by the flow-cytometry machine was recorded in data

sheets. Data was checked for completeness and consistency. Data entry into SPSS was subsequently done.

3.5.2 Data Protection and Security

All paper records were kept under lock and key. Computers used for data entry and analysis were password protected.

3.5.3 Statistical Analysis

Data analysis was done using SPSS statistical package version 20.0. The results were expressed as either mean ± standard deviation or median with corresponding interquartile range. To compare the means between the groups, analysis of variance (ANOVA) was used. For comparisons demonstrating statistical difference, Tukey's post hoc test was subsequently conducted to establish the differences between respective groups. Spearman's correlation test was used to test for association between the trauma scores and the levels of inflammatory cytokines, vital signs and complete bold count parameters. Alpha (α) value was set at 0.05.

3.6 Ethical Consideration:

Ethical approval was granted by the Moi University/MTRH Research and Ethics Committee, IREC number IREC/2016/98. Permission to conduct the study at MTRH was subsequently sought from the hospital administration.

Informed consent was sought from the participants. There was no coercion. The blood samples collected were used for the purposes of the study only. No direct identifiers were used and participants' confidentiality were maintained at all times. The data in soft copy was password protected while the hard copy form were kept under lock and key. Lastly, the study participants had the freedom to terminate their participation in the study at any time.

Dissemination of the findings of the current study are through an oral defence of this thesis, presentation at relevant conferences, publication in peer reviewed scientific journals and through a printed thesis.

3.7 Study Limitations

Individuals with either autoimmune disease, active infection or active inflammation have elevated levels of inflammatory cytokines. The current study sought to exclude them so as to eliminate any potential bias. Identification of these individuals was done clinically. This identification would ideally have been done using laboratory cytokine measurement as this would be more objective. To mitigate this limitation, a standard set of signs and symptoms was used for case identify and exclusion.

The measurement of vital signs and drawing of venous blood samples for haematological measurement and inflammatory cytokines analysis was carried out after initial resuscitation of patients. This is the ethical practice to enable best patient outcome. This potentially had the findings reflecting the effects of both the injury and the initial resuscitation. This impact on the results was however minimised by the measurements and sample collection being done soon after before the full effects of the resuscitation.

CHAPTER FOUR: RESULTS

4.1 Age

The five study groups demonstrated no statistically significant difference in age (31.7 ± 8.4 (negative control); 36.5 ± 9.2 (upper limb); 30.3 ± 12.1 (tibia); 40.3 ± 18.2 (femur); 27.6 ± 4.4 (polytrauma); p= 0.208). The data are illustrated in Table 1.

Table 1: Age Distribution

STUDY GROUPS	AGE (years)
Control Group	31.7 ± 8.4*
Upper Limb Group	36.5 ± 9.2*
Tibia Group	30.3 ± 12.1*
Femur Group	40.3 ± 18.2*
Polytrauma Group	27.6 ± 4.4*

Values are Mean ± SD
*p = 0.208

4.2 Inflammatory Cytokines

Interleukin-6

The mean interleukin 6 (IL-6) levels were (0.78 ± 0.1 (negative control); 38.5 ± 17.1 (upper limb); 89.5 ± 140 (tibia); 106.9 ± 84.2 (femur); 181.4 ± 188 (polytrauma); p= 0.001). The polytrauma group had statistically significant higher levels as compared to the negative control (p = 0.005). The upper limb, tibia and femur groups recorded higher mean IL-6 levels than the negative control group although these did not achieve statistical significance (p = 0.42, p = 0.29 and p = 0.28 respectively). This is illustrated in Figure 4.

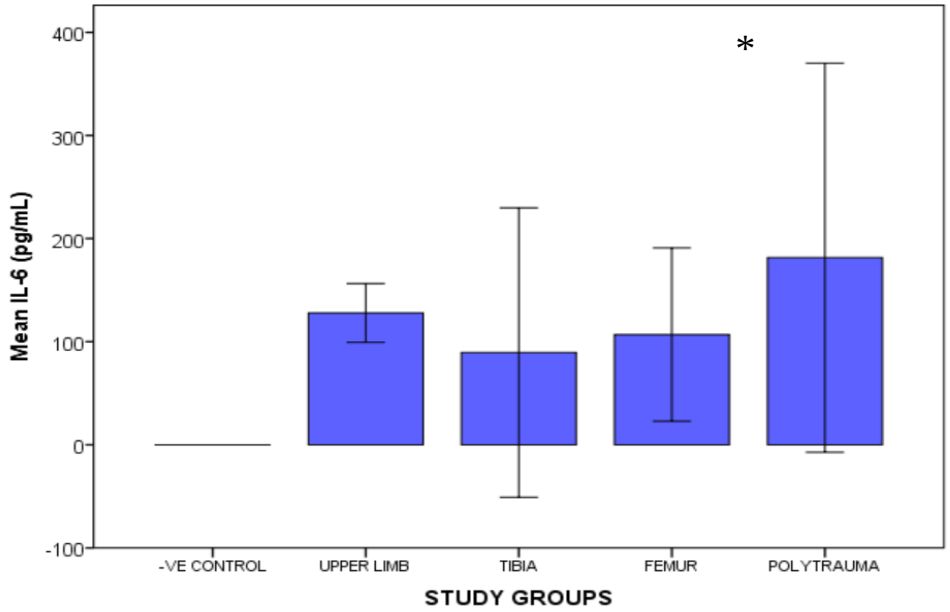


Figure 4: Bar Graph of Mean IL-6 following Various Fractures

Bars represent Mean ± SD
*p = 0.001

Interleukin-8

The mean interleukin 8 (IL-8) levels were (40.03 ± 30.4 (negative control); 80.97 ± 48.80 (upper limb); 58.75 ± 37.3 (tibia); 83.09 ± 53.14 (femur); 126.44 ± 103.94 (polytrauma); p= 0.004). The polytrauma group had statistically significant higher levels as compared to the negative control (p = 0.003). The upper limb, isolated tibia and isolated femur groups recorded higher IL-8 levels than the negative control group although these did not achieve statistical significance (p = 0.80, p = 0.92 and p = 0.50 respectively). This is illustrated in Figure 5.

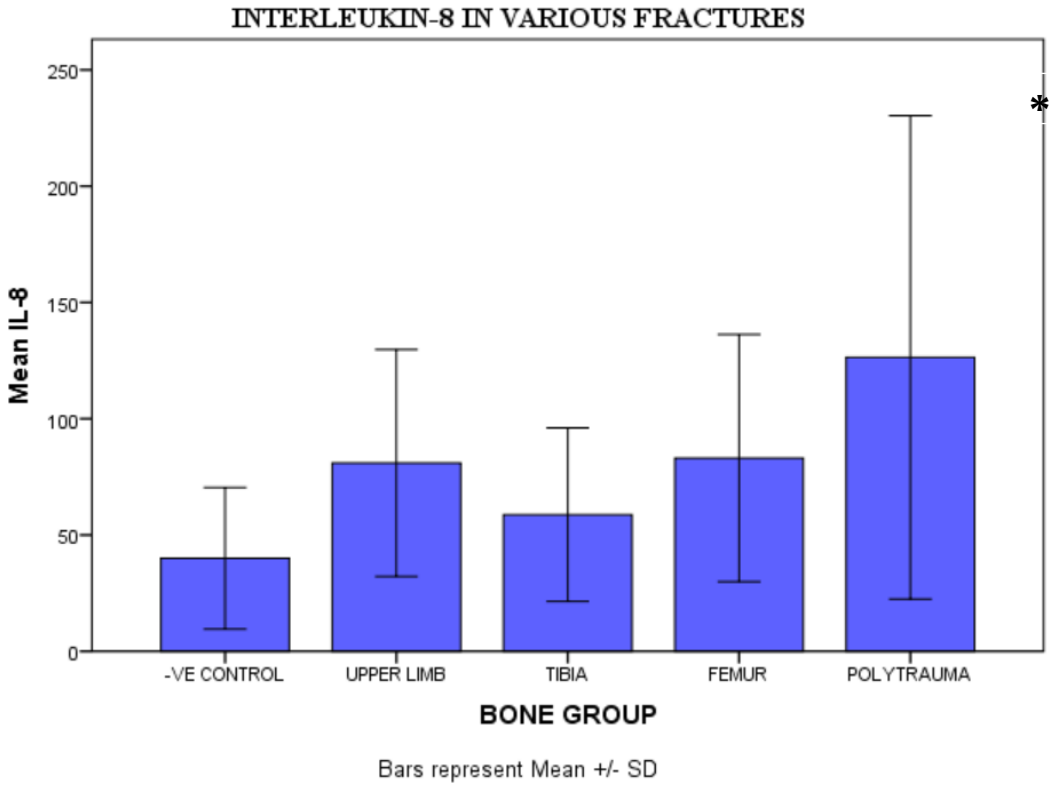


Figure 5: Bar Graph of Mean I **Study Groups** **es**

Bars represent Mean ± SD
*p = 0.003

Interleukin-10

The mean interleukin 10 (IL-10) levels were (0.51 ± 0.46 (negative control); 3.66 ± 4.00 (upper limb); 4.54 ± 6.70 (tibia); 4.01 ± 6.50 (femur); 37.6 ± 98.71 (polytrauma); p= 0.232). The polytrauma group had higher mean IL-10 levels than the negative control group although this did not achieve statistical significance (p = 0.272). This is illustrated in Figure 6.

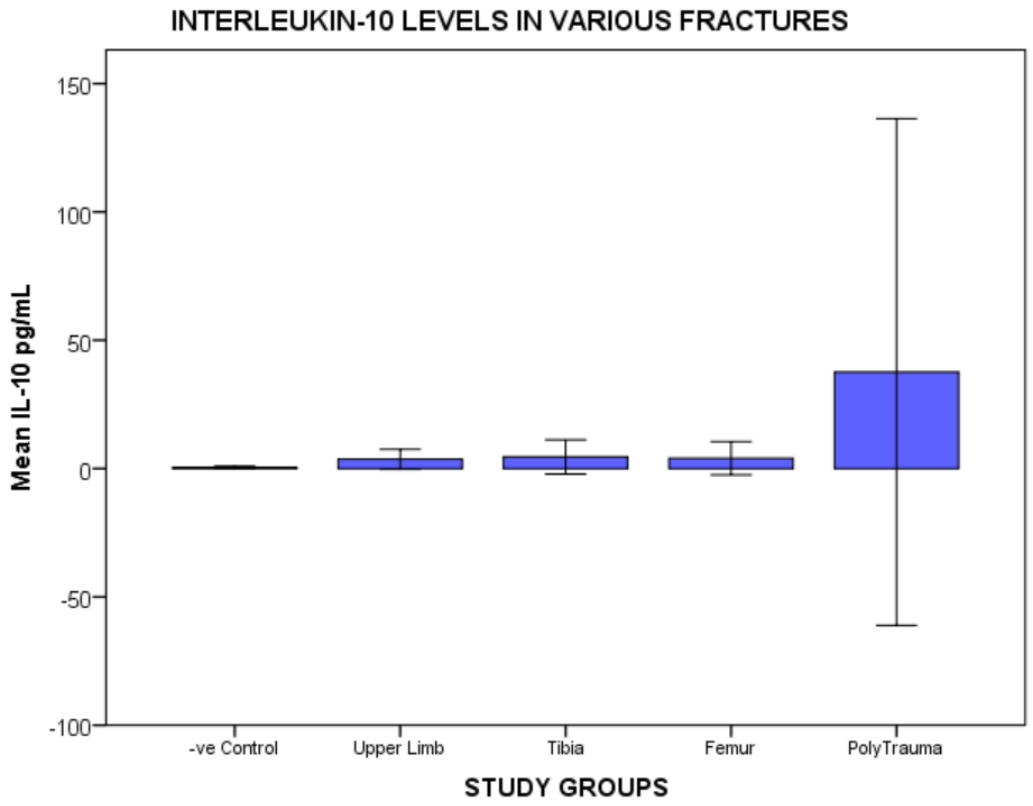


Figure 6: Bar Graph of Mean IL-10 following Various Fractures

Bars represent Mean ± SD

Tumor Necrosis Factor-α (TNF-α)

The levels of TNF-α for the five study groups were undetectably low based on the technique and dilution used. This precluded assessment and comparison between groups.

Interleukin-12 (IL-12)

The levels of Interleukin-12 (IL-12) for the five study groups were undetectably low based on the technique and dilution used. This precluded assessment and comparison between groups.

Interleukin-1β (IL-1β)

The mean levels of Interleukin-1β (IL-1β) in the negative control group was 36.8 ± 110. The levels of IL-1β for the four fracture groups were undetectably low based on the technique and dilution used. This precluded assessment and comparison between groups.

4.3 Vital Signs

The mean pulse rate for the negative control, upper limb, tibia, femur and polytrauma groups were 73.33 ± 4.76, 61.00 ± 1.16, 85.11 ± 12.88, 83.00 ± 17.90 and 98.74 ± 21.57 beats/minute respectively. The polytrauma study groups had statistically significant higher pulse rate than the negative control group (p = 0.001). This is illustrated in Table 2.

Table 2: Table of Vital Signs following Various Fractures

Groups	Pulse Rate (beats/ minute)	Respiratory Rate (beats/ minute)	Shock Index	Systolic Blood Pressure (mmHg)	Diastolic Blood Pressure (mmHg)
Control Group	**73.33 ± 4.76***	**17.33 ± 1.40***	**.64 ± .063***	114.80 ± 5.94	72.53 ± 6.64
Upper Limb Group	61.00 ± 1.16	**21.00 ± 1.16***	.50 ± .023	123.00 ± 3.46	72.50 ± 5.20
Tibia Group	85.11 ± 12.88	**19.00 ± 1.41***	.73 ± .13	117.37 ± 14.92	74.42 ± 13.87
Femur Group	83.00 ± 17.90	19.00 ± 1.05	.70 ± .15	123.70 ± 34.49	74.40 ± 21.23
Polytrauma Group	**98.74 ± 21.57***	**20.26 ± 2.078***	**.89 ± .29***	114.11 ± 14.48	65.79 ± 14.02

Values are Mean ± SD
* $p < 0.05$

The mean respiratory rate for the negative control, upper limb, tibia, femur and polytrauma groups were 17.33 ± 1.40, 21.00 ± 1.16, 19.00 ± 1.41, 19.00 ± 1.05 and 20.26 ± 2.078 breathes/minute respectively. The polytrauma study groups had statistically significant higher respiratory rate than the negative control group (p = 0.001). In addition the upper limb fracture and isolated tibia fracture groups recorded a statistically significant higher respiratory rate as compared to the negative control (p = 0.001). This is illustrated in Table 2.

The mean shock index for the negative control, upper limb, tibia, femur and polytrauma groups were .64 ± .063, .50 ± .023, .73 ± .13, .70 ± .15 and .89 ± .29 respectively. The polytrauma study groups had statistically significant higher shock index than the negative control group (p = 0.002). This is illustrated in Table 2.

The mean systolic blood pressure for the negative control, upper limb, tibia, femur and polytrauma groups were 114.80 ± 5.94, 123.00 ± 3.46, 117.37 ± 14.92, 123.70 ± 34.49 and 114.11 ± 14.48 respectively demonstrating no statistical significant difference between the groups (p = 0.613). This is illustrated in Table 2.

The mean diastolic blood pressure for the negative control, upper limb, tibia, femur and polytrauma groups were 72.53 ± 6.64, 72.50 ± 5.20, 74.42 ± 13.87, 74.40 ± 21.23 and 65.79 ± 14.02 respectively demonstrating no statistical significant difference between the groups (p = 0.334). This is illustrated in Table 2.

4.4 Hematological Parameters

The mean platelet counts were statistically lower in all the fracture groups as compared to the negative control group (328.73 ± 55.4 (negative control); 211 ± 23 (upper limb); 227.15 ± 58.3 (tibia); 243 ± 105.4 (femur); 96.2 ± 33.7 (polytrauma) X 109/L; p= 0.001). The data are illustrated in Table 3

Table 3: Table of Complete Blood Count Parameters following Various Fractures

Groups	Haemoglobin (mg/dL)	White Blood Cell Count (X 10^9/L)	Neutrophil Count (X 10^9/L)	Neutrophil Percentage (%)	Platelet Count (X 10^9/L)
Control Group	**13.713 ± 0.95¥**	**6.04 ± 0.75*¥**	**3.37 ± 0.6*¥**	**56 ± 8.4*¥**	**328.73 ± 55.37¥**
Upper Limb Group	13.000 ± 0.01	7.53 ± 1.14	5.49 ± 1.23	**73 ± 9.3***	**210.25 ± 35.02¥**
Tibia Group	13.754 ± 1.58	**10.69 ± 3.67***	**8.07 ± 2.27***	**76.42 ± 11.02¥**	**227.15 ± 58.35¥**
Femur Group	13.970 ± 1.46	**13.04 ± 3.24***	**11.35 ± 3.44¥**	**85 ± 8.8¥**	**243.00 ± 105.42¥**
Polytrauma Group	**10.958 ± 1.14¥**	**20.11 ±7.55¥**	**16.19 ± 5.64¥**	**81.12 ± 10.3¥**	**96.21 ± 33.75¥**

Values are Mean ± SD
¥ $p = 0.001$
* $p < 0.05$

The mean haemoglobin levels for the negative control, upper limb, tibia, femur and polytrauma groups were 13.713 ± 0.95, 13.000 ± 0.01, 13.754 ± 1.58, 13.970 ± 1.46 and 10.958 ± 1.14 (mg/dL) respectively. The polytrauma study group had statistically significant lower haemoglobin levels than the negative control group ($p = 0.001$). The data are illustrated in Table 3.

The mean total white blood cell counts for the negative control, upper limb, tibia, femur and polytrauma groups were 6.04 ± 0.75, 7.53 ± 1.14, 10.69 ± 3.67, 13.04 ± 3.24 and 20.11 ±7.55 (X 109/L) respectively. The polytrauma, isolated tibia fracture and isolated femur fracture groups recorded a statistically significant higher total white blood cell counts as compared to the negative control ($p = 0.001$,$p = 0.04$ and $p = 0.006$ respectively). The data are illustrated in Table 3.

The mean neutrophil counts for the negative control, upper limb, tibia, femur and polytrauma groups were 3.37 ± 0.6, 5.49 ± 1.23, 8.07 ± 2.27, 11.35 ± 3.44 and 16.19 ± 5.64 (X 109/L) respectively. The polytrauma, isolated tibia fracture and isolated femur fracture groups recorded a statistically significant higher mean neutrophil counts as compared to the negative control ($p = 0.001$, $p = 0.013$ and $p = 0.001$ respectively). The data are illustrated in Table 3.

The mean neutrophil percentage for the negative control, upper limb, tibia, femur and polytrauma groups were 56 ± 8.4, 73 ± 9.3, 76.42 ± 11.02, 85 ± 8.8 and 81.12 ± 10.3

The polytrauma, isolated femur fracture, isolated tibia fracture and isolated upper limb fracture groups recorded a statistically significant higher mean neutrophil percentage as compared to the negative control ($p = 0.001$, $p = 0.001$, $p = 0.001$ and $p = 0.026$ respectively). The data are illustrated in Table 3

4.5 Trauma Scores

4.5.1 Abbreviated Injury Score for Isolated Injury Groups

The mean Abbreviated Injury Score for the isolated upper limb fracture, isolated tibia fracture and isolated femur fracture groups were 1.5 ± 0.577, 3.0 ± 0.485 and 3 ± 0.625. The isolated femur fracture and isolated tibia fracture groups recorded a statistically significant higher mean Abbreviated Injury Scores as compared to the isolated upper limb fracture group ($p < 0.001$). This data is illustrated in Table 4.

Table 4: Table of Abbreviated Injury Score and Injury Severity Score Results

Group	Abbreviated Injury Score	Injury Severity Score
Isolated Upper Limb Fracture	1.5 ± 0.577*	
Isolated Tibia Fracture	3.0 ± 0.485*	
Isolated Femur Fracture	3.0 ± 0.625*	
Polytrauma		32.87 ± 13.64

Values are Mean ± SD
* $p < 0.001$

4.5.2 Injury Severity Score for Polytrauma Group

The mean Injury Severity Score for the polytrauma group was 32.87 ± 13.64. This data is illustrated in Table 4 above.

4.6 Correlations

4.6.1 Correlation of Interleukin-6 and Interleukin-8

The levels of interleukins-6 were positively correlated with the levels of interleukin-8 (r = 0.522, p = 0.001) as shown in Figure 7.

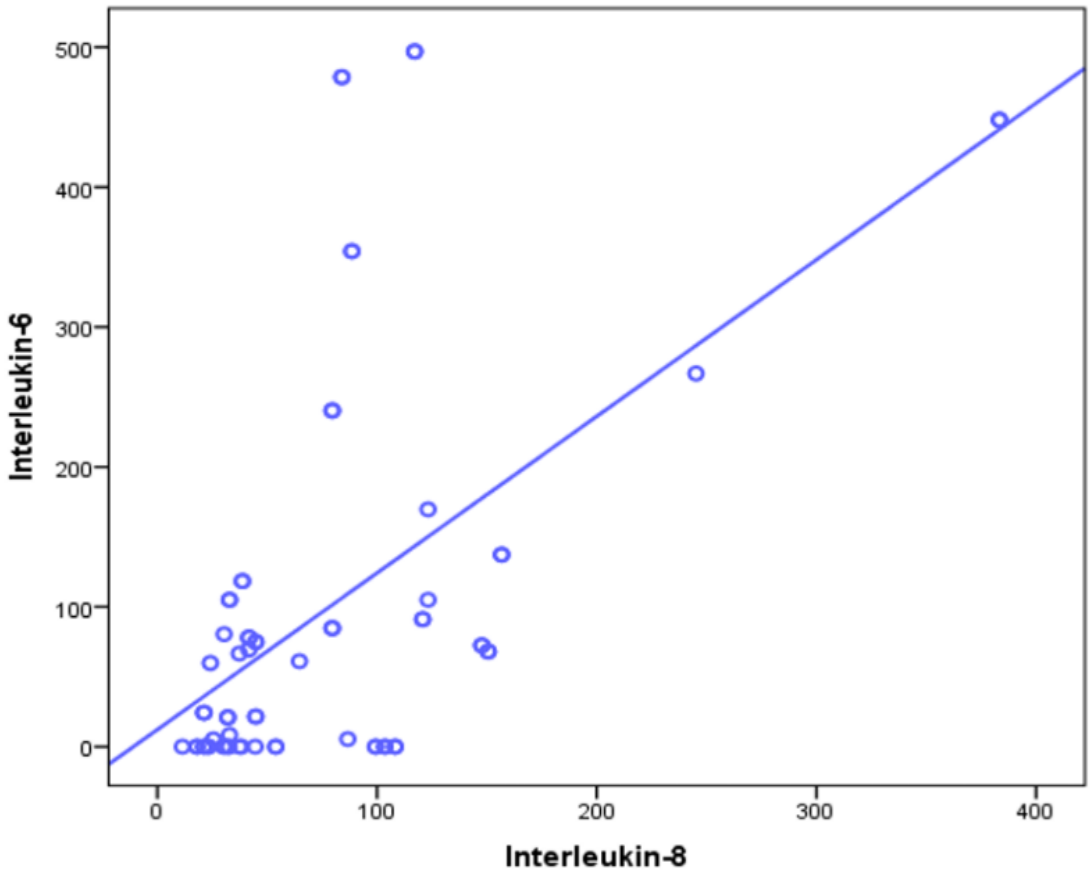


Figure 7: A Scatter Plot of Interleukin-6 and Interleukin-8 following Various Fractures

r = 0.522
p = 0.001

4.6.2 Correlation of Platelet Count and both Interleukin-6 and Interleukin-8

The platelet levels were negatively correlated with interleukin-6 (r = -0.252, p = 0.05) and interleukin-8 (r = -0.389, p = 0.002) as illustrated in Figure 8.

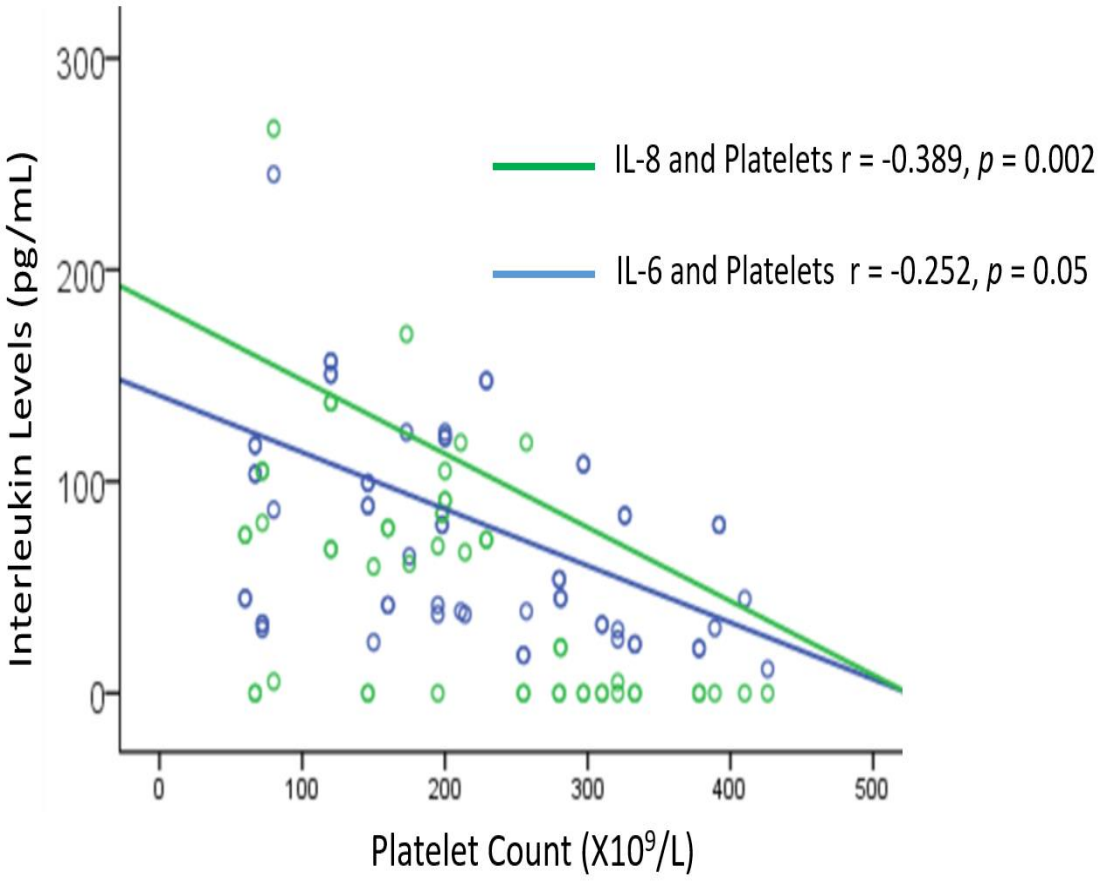


Figure 8: An Overlay Scatter Plot of Platelet Count and both Interleukin-6 and Interleukin-8

4.6.3 Correlation of Interleukin-6 and Fracture Properties

Interleukin-6 levels were positively correlated with increased fracture pattern severity (r = 0.509, p = 0.001), increased soft tissue injury (r = 0.577, p = 0.001) and presence of open fractures (r = 0.593, p = 0.001) as illustrated in Figure 9.

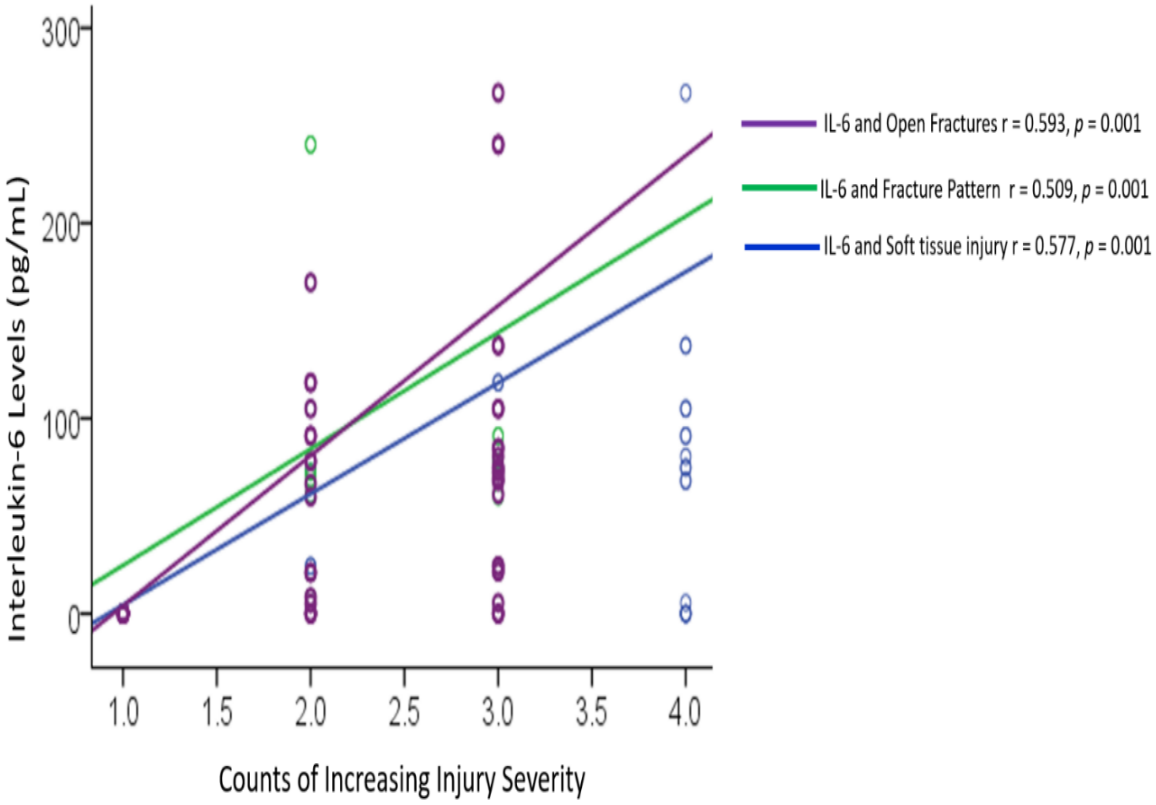


Figure 9: An Overlay Scatter Plot of Interleukin-6 and Severity of Fracture Pattern, Soft Tissue Injury and Open Fractures

4.6.4 Correlation of Platelet Count and Shock Index

The shock index among the patients was negatively correlated with the platelet count (r = 0.566, p = 0.001) as illustrated in Figure 10.

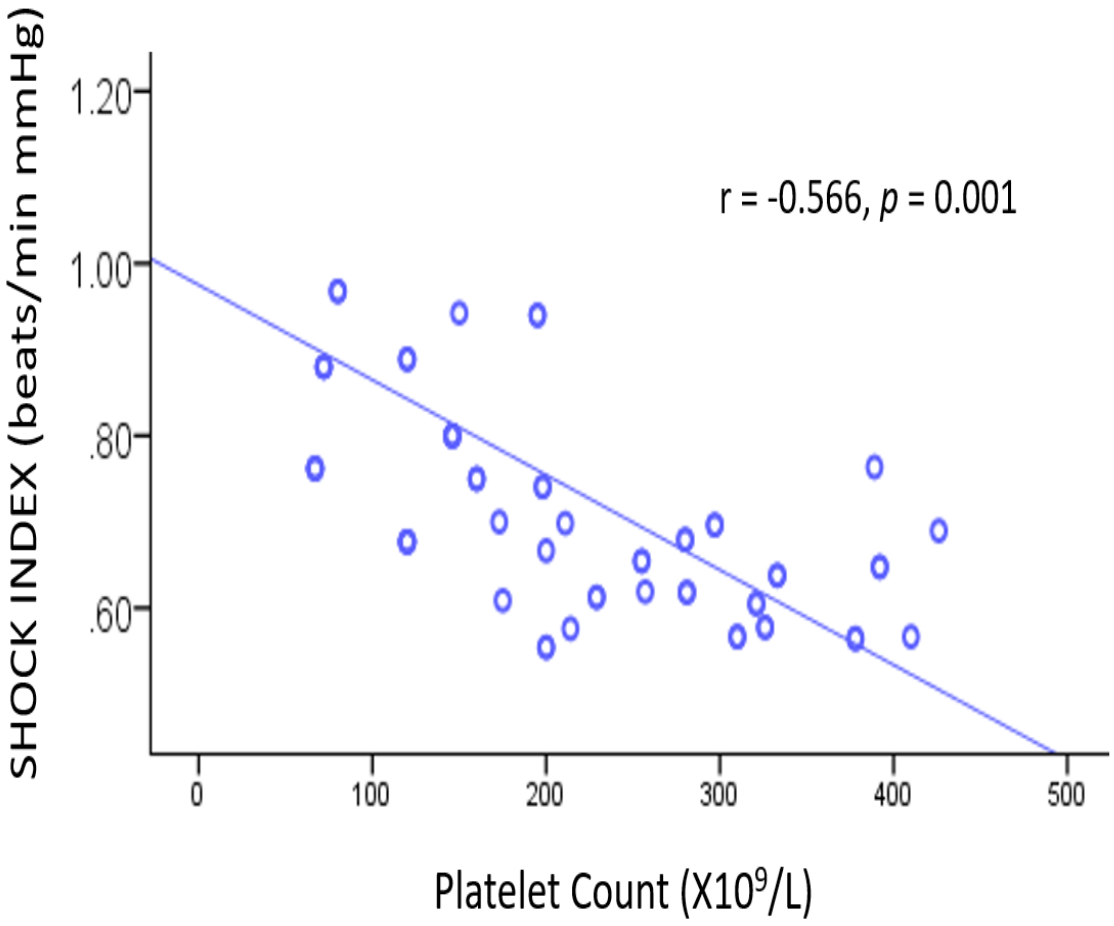


Figure 10: A Scatter Plot of Shock Index and Platelet Count

4.6.5 Correlation of Platelet Count and Fracture Properties

Platelet count was inversely correlated with increased fracture pattern severity (r = -0.802, p = 0.001), increased soft tissue injury (r = -0.808, p = 0.001) and presence of open fractures (r =- 0.586, p = 0.001) as illustrated in Figure 11.

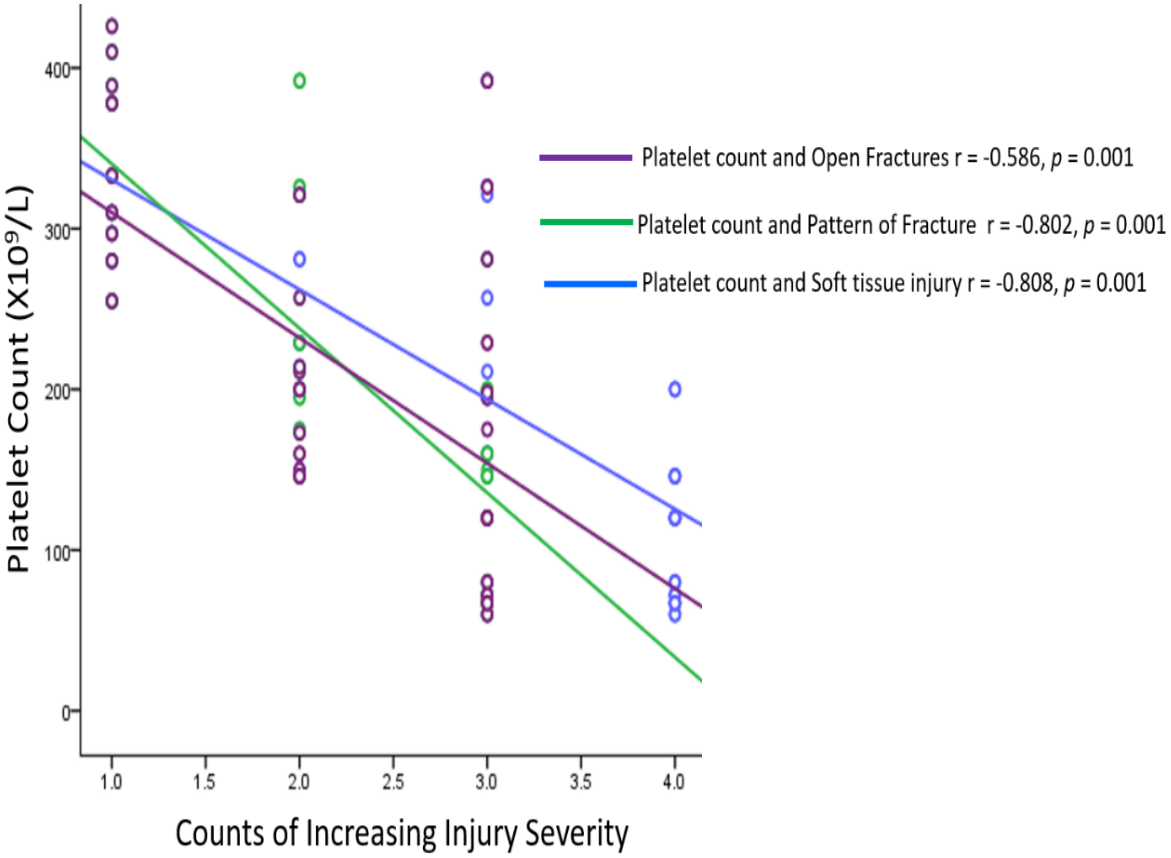


Figure 11: An Overlay Scatter Plot of Platelet Count and Severity of Fracture Pattern, Soft Tissue Injury and Open Fractures

CHAPTER FIVE: DISCUSSION

5.1 Levels of Inflammatory Cytokines following Various Fractures

5.1.1 Interleukin-6

The current study demonstrated an elevation in the level of IL-6 among all fracture groups. The levels were highest among the multiply injured patients. Patients with single bone involvement had moderately elevated levels. This finding is in agreement with work done by Li *et al*., (2016); Perl *et al*., (2003) and Volpin *et al*., (2014).

The upper limb group had levels higher than both the isolated tibia and isolated femur fracture groups. Variation in the level of inflammation following injury to different body regions has previously been documented by Kleber *et al*., (2015) in a murine study.

In addition to variation due to extent and site of injury, Pape *et al*., (2008) demonstrated that post traumatic and perioperative blood loss independently caused IL-6 elevation. Giannoudis, *et al*., (2008) and Strecker, *et al*., (2003) documented that IL-6 following trauma can accurately predict development of systemic inflammatory response syndrome, organ failure and death.

Immunologically, this elevation of IL-6 is expected in the setting of trauma as it serves to stimulate further release of acute phase proteins, leads to release of neutrophils and down regulates levels of regulatory T cells. Further rise is expected due to blood loss and entry of microbial which both occur with fractures.

5.1.2 Interleukin-8

The current study demonstrated an elevation in the level of IL-8 among fracture patients. This levels were highest among the multiply injured patients. Patients with single bone involvement had moderately elevated levels. These findings concur with work done by Horst *et al*., (2015); Volpin *et al*., (2014) and Li *et al*., (2016). The

upper limb group had levels higher than both the isolated tibia and isolated femur fracture groups. Haller *et al*., (2107) documented higher levels of IL-8 following tibial plafond fractures than tibial plateau fractures. Similar to IL-6, it is expected that IL-8 would be elevated in the setting of acute trauma as it is a chemokine key in cascade leading to acute inflammation. It does this by acting as a chemo-attractant for neutrophils, fostering phagocytosis and inducing respiratory burst key to neutrophil function.

5.1.2 Interleukin-10

The current study demonstrated no statistical significant difference in the levels of IL-10 in various study groups. The findings of this study agree with prior studies that did not demonstrate elevation of IL-10 among trauma patients (Yousefzadeh, *et al*., 2015). This is likely because IL-10 is a purely anti-inflammatory cytokine and its levels are expected to rise when the inflammatory response is subsiding (Giannoudis *et al*., 2004).

5.2 Vital Signs Following Various Fractures

The current study demonstrated an elevation in the pulse rate among the fracture patients. The mean pulse rate was highest among the multiply injured patients. In addition the fracture patients had elevated respiratory rates. These changes in the vital signs following trauma agree with those demonstrated by other studies (Larson *et al*., 2010; Smith & Choi, 2017). The findings of this study contrast those by Yonge *et al*., (2017) who documented respiratory rate when used in isolation was only predictive with regard to chest trauma. It is only when used in combination with other vital signs, that respiratory rate serves to improve the triage of trauma patients (Lehmann, *et al*., 2009).

These changes reflect the normal physiological response that is aimed at maintaining normal blood pressure to enable perfusion of vital organs. The blood pressure is a product of heart rate, ejection fraction and resistance within the vascular system. The rise in heart rate in trauma patients thus serves to restore the blood pressure.

The shock index was also statistically significant higher among the fracture patients. This finding is in agreement with prior studies that documented shock index is useful in identifying patients with significant hemorrhage following trauma (Birkhahn, *et al*., 2005; Rady, *et al*., 1994). Kim *et al.*, (2016) demonstrated that shock index was predictive even among the geriatric population. The shock index is the quotient when the heart rate is divided by the systolic blood pressure. A high shock index indicates a reduction in the systolic blood pressure and a concomitant compensatory elevation of the heart rate. This is the haemodynamic picture seen among trauma patients due to hypovolemia.

The rise in the respiratory rate serves to supply additional oxygen required by the vital organs as well as the oxidative process needed by granulocytes to fight microbial infiltration.

5.3 Complete Blood Count Parameters following Various Fractures

5.3.1 Platelet Count

The current study demonstrated a reduction in the mean platelet count among all the fracture groups. The reduction was greatest among the multiply injured patients. The patients with single bone isolated fractures had lowering of the platelet count but not as much as the multiply injured patients. These findings are similar to those documented by Hobisch-Hagen *et al*., (2000) and Kutcher *et al*., (2012). In addition, platelet count has been shown to be predictive of patient outcome including death (Jin

et al., 2018; Yolcu *et al*., 2013). This has led to the adoption of platelet count in decision making and timing of surgery (Nieto & Baroan, 2017; Thiele, *et al*., 2013).

The reduction in platelet is indicative of a consumptive coagulopathy whereby platelets in circulation get used up in the formation of the platelet plug thereby limiting further blood loss prior to the formation of a definitive clot by the intrinsic and extrinsic clotting pathways. More injury would thus be expected to have more platelet being used up and thus the much lower levels of platelet seen in multiply injured patients.

5.3.2 White Blood Cell Count

The current study demonstrated that the patients with fractures had statistically significant higher white blood cell counts. In addition, the neutrophil percentage was statistically higher indicating the leucocytosis was largely attributable to the neutrophilia. These findings are in agreement with prior studies looking at leucocyte count following trauma (Gürkanlar, *et al*., 2009; Iyidobi, *et al*., 2013; Turan, *et al*., 2019). In addition, Turan *et al*. (2018) while studying patients with firearm injuries demonstrated that the white blood cell count at presentation accurately predicted mortality.

Physiologically, leucocytosis and in particular neutrophilia following trauma results from the rapid release from the bone marrow. This release is mediated by a chemicals released by damaged cells called damage-associated molecular patterns (DAMPs) (Pittman & Kubes, 2013). These DAMP molecules include DNA, histones, high mobility group protein B1, N-formyl peptides, Adenosine triphosphate, interleukin-1α and many others (Chen and Nunez 2010)

5.4 Correlations

5.4.1 Correlation between Interleukin-6 and Interleukin-8

The current study demonstrated a positive correlation between IL-6 and IL-8 among the fracture patients. This is in agreement with findings by Mimasaka *et al*., (2007) and Reikerås, (2010). This is expected as both IL-6 and IL-8 are involved in the cascade of inflammation following trauma as chemo-attractants and concordance in the elevation indicates good coordination of the inflammatory cascade.

5.4.2 Correlation between Platelet Count and both IL-6 and IL-8

In the current study, a negative correlation between Platelet count and both IL-6 and IL-8 was found. At the time of writing this thesis literature search did not yield documentation of similar findings. This inverse correlation is however expected as the trauma induced inflammatory response stimulates and elevation of pro-inflammatory cytokines IL-6 and IL-8 and at the same time the concomitant consumptive coagulation causes a reduction in the platelet count.

5.4.3. Correlation between IL-6 and Injury Properties

The levels of interleukin-6 were positively correlated to the severity of fracture pattern, extent of soft tissue involvement as well as the presence of an open fracture. Similar correlation was documented by Perl *et al*., (2003) and Strecker *et al*., (2003). This was an expected correlation as the cumulative extent of injury is a summation of the injury to bone and soft tissues. In addition, open fractures would be associated with higher energy trauma and thus more tissue damage and higher levels of inflammatory cytokines.

5.4.4 Correlation of Platelet Count and Shock Index

The current study demonstrated an inverse correlation between platelet count and shock index. Despite absence of prior publication of this correlation, this correlation was expected as increased trauma induced haemorrhage would lower the systolic blood pressure inducing a compensatory rise in the heart rate. This would result in elevation of the shock index. The blood loss would also consume platelets in a bid to caused haemostasis.

5.4.5 Correlation between Platelet Count and Injury Properties

Lastly, an inverse correlation was demonstrated between Platelet Count and severity of fracture pattern, extent of soft tissue involvement and presence of an open fracture. This is the index documentation of this phenomenon. This inverse correlation is expected as increasing severity of injury induces higher clot formation and thus lower platelet counts.

CHAPTER SIX: CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The current study demonstrated that the level of trauma induced inflammatory response is dependent on the number and pattern of fractures as well as extent of soft tissue injury.

In addition, the vital signs that closely rise in tandem with level of post-trauma inflammation are shock index and respiratory rate.

Among the haematological parameters, the platelet count was shown to decrease with increasing level of post-trauma inflammation as indicated by levels of IL-6 and IL-8.

Lastly, the level of inflammation as indicated by inflammatory markers was fairly well correlated with trauma severity as scored using either the Abbreviated Injury Score or the Injury Severity Score.

6.2 Recommendation

The current study recommends the development of techniques to enable rapid evaluation of inflammatory cytokines to enable individualized trauma care. In addition, the researcher recommends the use of shock index, respiratory rate and platelet count as surrogate markers for level of inflammation following trauma.

REFERENCES

Abdul-Ahad, A., Baumann, H., Elias, J. A., Fey, G., Gordon, M. S., Heinrich, P. C., ... & Revel, M. (1995). Interleukin-6-type cytokines in diagnostics and therapeutics: roundtable discussion. *Annals of the New York Academy of Sciences-Paper Edition*, *762*, 375-387.

Ärnlöv, J., & Larsson, A. (2015). Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *The Lancet*, *386*(9995), 743-800. https://doi.org/10.1016/s0140-6736(15)60692-4

Bachani, A. M., Koradia, P., Herbert, H. K., Mogere, S., Akungah, D., Nyamari, J., ... & Stevens, K. A. (2012). Road traffic injuries in Kenya: the health burden and risk factors in two districts. *Traffic injury prevention*, *13*(sup1), 24-30.

Baker, C. C., Oppenheimer, L., Stephens, B., Lewis, F. R., & Trunkey, D. D. (1980). Epidemiology of trauma deaths. *The American Journal of Surgery*, *140*(1), 144-150.

Birkhahn, R. H., Gaeta, T. J., Terry, D., Bove, J. J., & Tloczkowski, J. (2005). Shock index in diagnosing early acute hypovolemia. *The American journal of emergency medicine*, *23*(3), 323-326.

Brat, D. J., Bellail, A. C., & Van Meir, E. G. (2005). The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro-oncology*, *7*(2), 122-133.

Burstein, S. A., Peng, J., Friese, P., Wolf, R. F., Harrison, P., Downs, T., ... & Dale, G. L. (1996). Cytokine–Induced Alteration of Platelet and Hemostatic Function. *Stem cells*, *14*(S1), 154-162.

Chen, G. Y., & Nuñez, G. (2010). Sterile inflammation: sensing and reacting to damage. *Nature Reviews Immunology*, *10*(12), 826-837.

Chen, L., Reisner, A. T., Gribok, A., & Reifman, J. (2009). Exploration of prehospital vital sign trends for the prediction of trauma outcomes. *Prehospital Emergency Care*, *13*(3), 286-294.

Chidester, S. J., Williams, N., Wang, W., & Groner, J. I. (2012). A pediatric massive transfusion protocol. *The journal of trauma and acute care surgery*, *73*(5).

Dimopoulou, I., Tsagarakis, S., Kouyialis, A. T., Roussou, P., Assithianakis, G., Christoforaki, M., ... & Roussos, C. (2004). Hypothalamic-pituitary-adrenal axis dysfunction in critically ill patients with traumatic brain injury: incidence, pathophysiology, and relationship to vasopressor dependence and peripheral interleukin-6 levels. *Critical care medicine*, *32*(2), 404-408.

Dinarello, C. A. (2000). Proinflammatory cytokines. *Chest*, *118*(2), 503-508.

Dixon, F. (1993). Advances in immunology. Academic Press.

Fenger-Eriksen, C., Anker-Møller, E., Heslop, J., Ingerslev, J., & Sørensen, B. (2005). Thrombelastographic whole blood clot formation after ex vivo addition of plasma substitutes: improvements of the induced coagulopathy with fibrinogen concentrate. *British Journal of Anaesthesia*, *94*(3), 324-329.

Frank, S. M., Beattie, C., Christopherson, R., Norris, E. J., Perler, B. A., Williams, G. M., & Gottlieb, S. O. (1993). Unintentional hypothermia is associated with postoperative myocardial ischemia. The Perioperative Ischemia Randomized Anesthesia Trial Study Group. *Anesthesiology*, *78*(3), 468-476.

Giannoudis, P. V., Smith, R. M., Bellamy, M. C., Morrison, J. F., Dickson, R. A., & Guillou, P. J. (1999). Stimulation of the inflammatory system by reamed and unreamed nailing of femoral fractures: an analysis of the second hit. *The Journal of bone and joint surgery. British volume*, *81*(2), 356-361.

Giannoudis, P. V., & Dinopoulos, H. (2004). Current concepts of the inflammatory response after major trauma: an update. *Injury-international journal of the care of the injured*, *35*(8), 836-837.

Giannoudis, P. V., Hildebrand, F., & Pape, H. C. (2004). Inflammatory serum markers in patients with multiple trauma: Can they predict outcome?. *The Journal of bone and joint surgery. British volume*, *86*(3), 313-323.

Giannoudis, P. V., Harwood, P. J., Loughenbury, P., Van Griensven, M., Krettek, C., & Pape, H. C. (2008). Correlation between IL-6 levels and the systemic inflammatory response score: can an IL-6 cutoff predict a SIRS state?. *Journal of Trauma and Acute Care Surgery*, *65*(3), 646-652.

Gürkanlar, D., Lakadamyali, H., Ergun, T., Yilmaz, C., Yücel, E., & Altinörs, N. (2009). Predictive value of leucocytosis in head trauma. *Turkish Neurosurgery*, *19*(3), 211.

Hagiwara, A., Kimura, A., Kato, H., Mizushima, Y., Matsuoka, T., Takeda, M., ... & Sasaki, J. (2010). Hemodynamic reactions in patients with hemorrhagic shock from blunt trauma after initial fluid therapy. *Journal of Trauma and Acute Care Surgery*, *69*(5), 1161-1168.

Hashizume, M., & Mihara, M. (2011). The Roles of Interleukin-6 in the Pathogenesis of Rheumatoid Arthritis. *Arthritis*, 2011, 1-8.

Hedges, J. C., Singer, C. A., & Gerthoffer, W. T. (2000). Mitogen-activated protein kinases regulate cytokine gene expression in human airway myocytes. *American journal of respiratory cell and molecular biology*, *23*(1), 86-94.

Hobisch-Hagen, P., Jelkmann, W., Mayr, A., Wiedermann, F., Fries, D., Herold, M., ... & Schobersberger, W. (2000). Low platelet count and elevated serum thrombopoietin after severe trauma. *European journal of haematology*, *64*(3), 157-163.

Husebye, E. E., Lyberg, T., Opdahl, H., Aspelin, T., Støen, R. Ø., Madsen, J. E., & Røise, O. (2012). Intramedullary nailing of femoral shaft fractures in polytraumatized patients. A longitudinal, prospective and observational study of the procedure-related impact on cardiopulmonary-and inflammatory responses. *Scandinavian journal of trauma, resuscitation and emergency medicine*, *20*(1), 2.

JC, I., & EE, I. (2013). Leukocyte Profile of Adult Nigerian Subjects with Acute Musculoskeletal Trauma. *Nigerian Journal of Medicine*, *22*(1).

Jin, W. Y. Y., Jeong, J. H., Kim, D. H., Kim, T. Y., Kang, C., Lee, S. H., ... & Lim, D. (2018). Factors predicting the early mortality of trauma patients. *Ulus Travma Acil Cerrahi Derg*, *24*(6), 532-538.

Kim, H. Y. (2016). Statistical notes for clinical researchers: Sample size calculation 3. Comparison of several means using one-way ANOVA. *Restorative dentistry & endodontics*, *41*(3), 231-234.

Kim, S. Y., Hong, K. J., Shin, S. D., Ro, Y. S., Ahn, K. O., Kim, Y. J., & Lee, E. J. (2016). Validation of the shock index, modified shock index, and age shock index for predicting mortality of geriatric trauma patients in emergency departments. *Journal of Korean medical science*, *31*(12), 2026-2032.

Kutcher, M. E., Redick, B. J., McCreery, R. C., Crane, I. M., Greenberg, M. D., Cachola, L. M., ... & Cohen, M. J. (2012). Characterization of platelet dysfunction after trauma. *The journal of trauma and acute care surgery*, *73*(1), 13.

Lackie, J. (2010). *A dictionary of biomedicine*. Oxford University Press.

Larson, C. R., White, C. E., Spinella, P. C., Jones, J. A., Holcomb, J. B., Blackbourne, L. H., & Wade, C. E. (2010). Association of shock, coagulopathy, and initial vital signs with massive transfusion in combat casualties. *Journal of Trauma and Acute Care Surgery*, *69*(1), S26-S32.

Lehmann, R., Brounts, L., Lesperance, K., Eckert, M., Casey, L., Beekley, A., & Martin, M. (2009). A simplified set of trauma triage criteria to safely reduce overtriage: a prospective study. *Archives of surgery*, *144*(9), 853-858.

Li, H., Liu, J., Yao, J., Zhong, J., Guo, L., & Sun, T. (2016). Fracture initiates systemic inflammatory response syndrome through recruiting polymorphonuclear leucocytes. *Immunologic research*, *64*(4), 1053-1059.

Li, M. (2015). *The detection of potentially harmful hand postures in pianists using Kinect depth images* (Doctoral dissertation, University of Missouri--Columbia).

Little, R. A., Jones, R. O., & Eltraifi, A. E. (1988). Cardiovascular reflex function after injury. *Progress in clinical and biological research*, *264*, 191.

Luna, G., Maier, R., & Pavlin, E. (1988). Incidence and effect of hypothermia in seriously injured patients. *Journal Of Critical Care*, *3*(2), 159.

Male, D., Brostoff, J., Roth, D. B., & Roitt, I. (2006). Immunology, 7. izdaja. *Mosby Elsevier, Edinburgh*, 59-86.

McNab, A., Burns, B., Bhullar, I., Chesire, D., & Kerwin, A. (2012). A prehospital shock index for trauma correlates with measures of hospital resource use and mortality. *Surgery*, *152*(3), 473-476.

Mimasaka, S., Funayama, M., Hashiyada, M., Nata, M., & Tsunenari, S. (2007). Significance of levels of IL-6 and IL-8 after trauma: a study of 11 cytokines post-mortem using multiplex immunoassay. *Injury*, *38*(9), 1047-1051.

Nieto, H., & Baroan, C. (2017). Limits of internal fixation in long-bone fracture. *Orthopaedics & Traumatology: Surgery & Research*, *103*(1), S61-S66.

Nordberg, E., Kimani, V., & Diwan, V. (2000). Household survey of injuries in a Kenyan district. *East African medical journal*, *77*(5).

Osler, T., Baker, S. P., & Long, W. (1997). A modification of the injury severity score that both improves accuracy and simplifies scoring. *Journal of Trauma and Acute Care Surgery*, *43*(6), 922-926.

Osoro, M. E., Ng, Z., Oundo, J., Omolo, J., & Luman, E. (2011). Factors associated with severity of road traffic injuries, Thika, Kenya. *Pan African medical journal*, *8*(1).

Ozturk, H., Yagmur, Y., & Ozturk, H. (2008). The prognostic importance of serum IL-1β, IL-6, IL-8 and TNF-α levels compared to trauma scoring systems for early mortality in children with blunt trauma. *Pediatric surgery international*, *24*(2), 235-239.

Pape, H. C., Rixen, D., Morley, J., Husebye, E. E., Mueller, M., Dumont, C., ... & Pardini, D. (2007). Impact of the method of initial stabilization for femoral shaft fractures in patients with multiple injuries at risk for complications (borderline patients). *Annals of surgery*, *246*(3), 491.

Pape, H. C. (2008). Effects of changing strategies of fracture fixation on immunologic changes and systemic complications after multiple trauma: damage control orthopedic surgery. *Journal of Orthopaedic Research*, *26*(11), 1478-1484.

Perl, M., Gebhard, F., Knöferl, M. W., Bachem, M., Gross, H. J., Kinzl, L., & Strecker, W. (2003). The pattern of preformed cytokines in tissues frequently affected by blunt trauma. *Shock*, *19*(4), 299-304.

Pignatti, P., Ciapponi, L., Galle, P., Hansen, M. B., Massa, M., Meazza, C., ... & De Benedetti, F. (2003). High circulating levels of biologically inactive IL-6/SIL-6 receptor complexes in systemic juvenile idiopathic arthritis: evidence for serum factors interfering with the binding to gp130. *Clinical & Experimental Immunology*, *131*(2), 355-363.

Pittman, K., & Kubes, P. (2013). Damage-associated molecular patterns control neutrophil recruitment. *Journal of innate immunity*, *5*(4), 315-323.

Rady, M. Y., Smithline, H. A., Blake, H., Nowak, R., & Rivers, E. (1994). A comparison of the shock index and conventional vital signs to identify acute, critical illness in the emergency department. *Annals of emergency medicine*, *24*(4), 685-690.

Raina, P., Sohel, N., Oremus, M., Shannon, H., Mony, P., Kumar, R., ... & Yusuf, R. (2016). Assessing global risk factors for non-fatal injuries from road traffic accidents and falls in adults aged 35–70 years in 17 countries: a cross-sectional analysis of the Prospective Urban Rural Epidemiological (PURE) study. *Injury prevention*, *22*(2), 92-98.

Raymer, J. M., Flynn, L. M., & Martin, R. F. (2012). Massive transfusion of blood in the surgical patient. *Surgical Clinics*, *92*(2), 221-234..

Reikerås, O. (2010). Immune depression in musculoskeletal trauma. *Inflammation research*, *59*(6), 409-414.

Saidi, H., Mutiso, B. K., & Ogengo, J. (2014). Mortality after road traffic crashes in a system with limited trauma data capability. *Journal of trauma management & outcomes*, *8*(1), 4.

Salottolo, K. M., Mains, C. W., Offner, P. J., Bourg, P. W., & Bar-Or, D. (2013). A retrospective analysis of geriatric trauma patients: venous lactate is a better predictor of mortality than traditional vital signs. *Scandinavian journal of trauma, resuscitation and emergency medicine*, *21*(1), 7.

Scheller, J., Chalaris, A., Schmidt-Arras, D., & Rose-John, S. (2011). The pro-and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1813*(5), 878-888.

Sears, B. W., Stover, M. D., & Callaci, J. (2009). Pathoanatomy and clinical correlates of the immunoinflammatory response following orthopaedic trauma. *The Journal of the American Academy of Orthopaedic Surgeons*, *17*(4), 255.

Smith, A. F., & Choi, S. W. (2017). Major trauma and the need for massive transfusion. *Anaesthesia*, *72*(11), 1299-1301.

Spahn, D. R., Bouillon, B., Cerny, V., Coats, T. J., Duranteau, J., Fernández-Mondéjar, E., ... & Neugebauer, E. (2013). Management of bleeding and coagulopathy following major trauma: an updated European guideline. *Critical care*, *17*(2), R76.

Strecker, W., Gebhard, F., Perl, M., Rager, J., Buttenschön, K., Kinzl, L., & Beck, A. (2003). Biochemical characterization of individual injury pattern and injury severity. *Injury*, *34*(12), 879-887.

Sutherland, A. M., Walley, K. R., Manocha, S., & Russell, J. A. (2005). The association of interleukin 6 haplotype clades with mortality in critically ill adults. *Archives of internal medicine*, *165*(1), 75-82.

Tscherne, H., Regel, G., Pape, H. C., Pohlemann, T., & Krettek, C. (1998). Internal fixation of multiple fractures in patients with polytrauma. *Clinical orthopaedics and related research*, (347), 62-78.

Vandromme, M. J., Griffin, R. L., Kerby, J. D., McGwin Jr, G., Rue III, L. W., & Weinberg, J. A. (2011). Identifying risk for massive transfusion in the relatively normotensive patient: utility of the prehospital shock index. *Journal of Trauma and Acute Care Surgery*, *70*(2), 384-390.

Volpin, G., Cohen, M., Assaf, M., Meir, T., Katz, R., & Pollack, S. (2014). Cytokine levels (IL-4, IL-6, IL-8 and TGFβ) as potential biomarkers of systemic inflammatory response in trauma patients. *International orthopaedics*, *38*(6), 1303-1309.

Yolcu, S., Beceren, G. N., Tomruk, Ö., Doguç, D. K., & Balbaloglu, O. (2014). Can mean platelet volume levels of trauma patients predict severity of trauma?. *Platelets*, *25*(4), 279-284.

Yonge, J. D., Bohan, P. K., Watson, J. J., Connelly, C. R., Eastes, L., & Schreiber, M. A. (2018). The respiratory rate: a neglected triage tool for pre-hospital identification of trauma patients. *World journal of surgery*, *42*(5), 1321-1326.

Yousefzadeh-Chabok, S., Moghaddam, A. D., Kazemnejad-Leili, E., Saneei, Z., Hosseinpour, M., Kouchakinejad-Eramsadati, L., ... & Mohtasham-Amiri, Z. (2015). The relationship between serum levels of interleukins 6, 8, 10 and clinical outcome in patients with severe traumatic brain injury. *Archives of trauma research*, *4*(1).

Zahn, S., Zwirner, J., Spengler, H. P., & Götze, O. (1997). Chemoattractant receptors for interleukin-8 and C5a: expression on peripheral blood leukocytes and differential regulation on HL-60 and AML-193 cells by vitamin D3 and all-trans retinoic acid. *European journal of immunology*, *27*(4), 935-940.

APPENDICES

Appendix I: IREC Approval

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
ELDORET

Reference: IREC/2016/98
Approval Number: 0001683

29th July, 2016

Dr. Rono Dennis Kipkemoi,
Moi University,
School of Medicine,
P.O. Box 4606-30100,
ELDORET-KENYA.

Dear Dr. Rono,

RE: FORMAL APPROVAL

The Institutional Research and Ethics Committee has reviewed your research proposal titled:-

"Inflammatory Cytokines Levels following various Fracture Patterns among Patients Managed at Moi Teaching and Referral Hospital".

Your proposal has been granted a Formal Approval Number: ***FAN: IREC 1683*** on 29th July, 2016. You are therefore permitted to begin your investigations.

Note that this approval is for 1 year; it will thus expire on 28th July, 2017. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Sincerely,

PROF. E. WERE
CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

INSTITUTIONAL RESEARCH & ETHICS COMMITTEE
29 JUL 2016
APPROVED
P. O. Box 4606-30100 ELDORET

cc CEO - MTRH Dean - SOP Dean - SOM
Principal - CHS Dean - SON Dean - SOD

Appendix II: Introductory Letter and Consent Form

Consent Form

RESEARCH TITLE: INFLAMMATORY CYTOKINES LEVELS FOLLOWING VARIOUS FRACTURE PATTERNS AMONG PATIENTS MANAGED AT MOI TEACHING AND REFERRAL HOSPITAL, ELDORET, KENYA

Investigator: Dr. Dennis K. Rono

P.O Box 3118, Kitale, Kenya, Mobile No: 0720475134

I…….of P.O Box……

Tel……..hereby give informed consent to participate in this study at MTRH. The study has been explained to me clearly by Dr. Dennis Rono (or his appointed assistant) of P.O. Box 3118 Kitale.

I have understood that by participating in this study, I shall volunteer information regarding my illness and other comorbidities, undergo medical examination and have 6cc of my blood drawn. I am aware that I can withdraw from this study at any time without prejudice to my right of treatment at MTRH now or in the future. I have also been assured that all information shall be treated and managed in confidence. I have not been induced or coerced by the investigator (or his appointed assistant) to cause my signature to be appended in this form and by extension participate in this study.

Initials of participant……

Signature……

Date……

Name of witness……

Signature……

Date……

Fomu Ya Kibali

MADA YA UTAFITI: Inflammatory Cytokines Levels Following Various Fracture Patterns Among Patients Managed At Moi Teaching And Referral Hospital, Eldoret, Kenya

MTAFITI - Dr. Dennis K. Rono
P.O Box 3118, Kitale, Kenya,
Simu ya Rununu: 0720475134

Mimi _____ wa Sanduku la Posta _____, Nambari ya Simu_____ najitolea kwa hiari yangu mwenyewe kutoa kibali cha kujihusisha katika utafiti uliotajwa hapo juu unaendelezwa katika MTRH. Nimepokea maelezo ya tafsili kuhusu utafiti huu kutoka kwa Daktari Dennis Rono (au watafiti msaidizi wake) katika lugha, kanuni na masharti ninayoelewa vyema. Nimehakikishiwa kuwa, sitadhurika kamwe kutokana na kujihusisha kwangu katika utafiti huu.Ilibainishwa kuwa kujihusisha katika utafiti huu ni kwa hiari na nina uhuru wa kujiondoa wakati wowote ule bila ya kuhujumiwa hasa kuhusu haki yangu ya kupokea matibabu katika MTRH. Zaidi ya hayo, nilihakikishiwa kuwa, kanununi zote za maadili ya utabibu,uhuru, haki, na manufaa zitazingatiwa katika utafiti huu.

Jina la Mhojiwa_____

Sahihi _____

Tarehe _____

Jina la shahidi _____

Sahihi _____

Tarehe

_____

Introductory Letter

I am Dr Dennis Rono, a medical doctor currently pursuing my Master's Degree in Orthopedic Surgery and Rehabilitation at Moi University, College of Health Sciences. I am conducting a study on the immunologic response following variable fractures entitled **Inflammatory Cytokines Levels Following Various Fracture Patterns Among Patients Managed At Moi Teaching And Referral Hospital, Eldoret, Kenya.**

You are being asked to take part in the research study. Information on the study and your participation is detailed below. Please read this form carefully. You are free to ask any question during any time of the study. If you decide to participate in the study, you will be given a copy of this introductory letter and the consent form for your records.

Taking part in the study is voluntary. Choosing not to participate in the study will not, in any way, affect the care you receive at MTRH. If you accept to enrol in the study, you will be free to terminate your participation at any time. Any new information concerning the risks and benefits of the study will be communicated to you promptly after which you will be free to opt out or continue with the study.

The purpose of this study is to assess the level inflammation following different fractures. The process of your participation will involve clinical and radiological examination to evaluate the type of injury/fractures sustained. In addition, 6 millilitres of venous blood will be drawn for laboratory analysis for inflammatory markers. Your involvement in the study will be for one sitting. There will be no follow-up required for the purpose of the study.

The information you provide will be kept confidential at all times and there will be no use of identifiers that may trace back to you.

For more information concerning your rights as a research participant, you may contact the Moi University/MTRH Institutional Research Ethics Committee (IREC) on telephone number 053 – 33471 ext 3008.

Yours faithful,

_____

Dr Dennis K. Rono

P.O.Box 3118 (30200)

Kitale.

Barua Ya Utangulizi

Mimi ni daktari Dennis K. Rono. Nimehitimu kama daktari na nimesajiliwa na Bodi ya Madaktari ya Kenya. Kwa sasa, ninasomea shahada ya juu (masters) ya udaktari wa upasuaji wa magonjwa ya mifupa katika Chuo Kikuu cha Moi. Ninafanya utafiti kuhusu viwango vya chemichemi vya kingamiilini baada ya kuvunjika kwa mifupa tofauti.

Ninaomba ujiunge na utafiti huu. Maelezo yafuatayo yanahusu utafiti wangu. Ningependa usomee na iwapo unamaswali yoyote kwa sasa ua baadaye kuwa huru kuuliza.

Kujiunga kwako ni kwa hiari. Kutojiunga hakutaathiri matibabu yako. Una huru wa kujiondoa kutoka kwa utafiti huu wakati wowote. Iwapo kutatokea maelezo zaidi kuhusu utafiti huu tutakueleza na utapata fursa ya kuamua iwapo ungependa kuendelea na kujihusisha na utafiti huu.

Utafiti huu unachukuza viwango vya chemichemi vya kinga mwilini baada ya kuvunjika kwa mifupa tofauti. Kuhusishwa kwako, utakuwa kwa kupimwa na daktari, kupigwa picha na kiwango kidogo (mililita 6) cha damu utatolewa kutoka kwa mshipa wa mkono na ufanyiwe uchunguzi kwenye maabara.

Umechaguliwa kuhusishwa na utafiti kwa sababu jeraha uliyonayo ni jeraha mojawapo zinazotafitiwa. Hakutakuwa na wakati wa kufuatiliwa kwa minajili ya utafiti kwani tutamaliza shughuli ya utafiti kwa siku moja.

Maelezo yote utakayotoa yatahifadhiwa vyema na kwa njia ya siri. Pia, hatutatumia maelezo yoyote ambayo yanawezesha kukufahamisha.

Iwapo utahitaji maelezo zaidi, waweza kuwasiliana na kikundi kinachoangazia utafiti na usawa wake wa IREC katika nambari ya rununu 053 – 33471 (ext 3008)

Mimi wako mwaminifu,

_____

Daktari Dennis K. Rono

SLP 3118 (30200) Kitale

Nambari ya Rununu 0720475134

Appendix III: Data Collection Sheet

1. Bio-demographic Data

Subject Code…..... Age..... Gender: male ☐ female ☐

Patient hospital number.....Residence.....

Mobile number.....

2. Have you been diagnosed with any of the following? (Tick Appropriately)

Asthma:	Yes	No
Recurrent (On and Off) skin rash	Yes	No
Recurrent (On and Off) joint pain	Yes	No
Any other autoimmune disease:	Yes	No
Any infection within the last 1 week:	Yes	No

3. Examination to rule out active infection or autoimmune disease

   Is any of the following signs present: skin rash, lymphadenopathy or joint deformity? Yes No

4. Date and Time of Injury ……

   Date and Time this form is being filled ……

   Time elapsed ……..

5. Characterization of Fractures:

NO	Site	Classification/Pattern	Closed of Open
1			
2			
3			
4			
5			
6			

6. Systemic Inflammatory Response syndrome Status:
    a. Body Temperature <36°C or > 38°C Y ____ N ____
    b. Heart rate >90 beats per minute Y ____ N ____
    c. Respiratory rate >20 breaths per minute Y ____ N ____
    d. Hyperventilation Y ____ N ____
    e. White blood cell count <4,000/mm3 or >12,000/mm3 Y ___ N ____
    f. Immature neutrophils >10% Y ____ N ____

    Patient positive for at least any two above? Y ____ N ____

7. Level of associated soft tissue injury:
    ☐ Minimal ☐ Moderate ☐ Extensive

	Region	Injury Description	Abbreviated Injury Score (0-6)	Square of Abbreviated Injury Score
1	Head and Neck			
2	Face			
3	Chest			
4	Abdomen			
5	Extremity			
6	External			
	New Injury Severity Score (Sum of Square of Top Three)			

Final Score: Abbreviated Injury Score (for Group 1 and 2) or New Injury Severity Score (for Group 3) _____

Appendix IV: Budget

Item Description	**Unit Cost**	**Quantity**	**Total cost**
Printing papers	400	2	800
Note books	300	1	300
Inflammatory Kit Reagents for Flow Cytometry Bead Assay from BD (This includes Human Inflammatory Cytokine PE Detection Reagent, Capture Beads, Buffers)	265,000	1	265,000
AMPATH Reference Laboratory Bench Fees	40,000	1	40,000
Laboratory supplies: Vacutainers, Micropipettes and test tubes	40,000	1	40,000
TOTAL			**346,100**

Appendix V: Work Plan

Year: 2016-2019

YEAR	January-May 2016	April 2017-December 2018	January 2019 – April 2019	August 2019
ACTIVITY				
Proposal writing				
Proposal Presentation				
Data Collection				
Data Analysis				
Thesis Writing				
Draft Presentation				
Thesis Submission				

Appendix VI: Abbreviated Injury Score and Injury Severity Score Guidelines

TRAUMA CHART
INJURY SEVERITY SCALE (*GUIDELINES)*

AIS SCORE	1	2	3	4	5
	MINOR	**MODERATE**	**SEVERE NOT LIFE THREATENING**	**SEVERE LIFE THREATENING**	**CRITICAL SURVIVAL UNCERTAIN**
EXTERNAL	**Abrasion/confusion** Superficial or unspecified/ ≤ 25cm^{2-} on face or 50 cm^2 on body. **Superficial or unspecified laceration** i) Not into subcutaneous tissue but ≤ 5cm on face or ≤10cm on body. 1st burn up to 100% 2nd or 3rd burn < 6% total body.	**Major abrasion/confusion** > 25cm^2 on face > 50cm^2 on body Deep laceration (into subcutaneous tissue) and >10cm on body or > 5cm on face 2° or 3° burn to 6-15% total body.	2° or 3° burn to 16-35% total body.	2° or 3° burn to 26-35% total body.	2° or 3° burn to 36-90% total body.
HEAD (*includes FACE (F)*)	**Awake on admission or initial observation** No prior unconsciousness but may have headache/dizziness 2° to head trauma **Ear canal injury** **Eyes (F)** Conjunctiva abrasion/ confusion/ laceration	**Awake on admission or initial observation** Prior unconsciousness but length of time unspecified. Amnesia (no recollection of crash) Unconsciousness < 15min **Lethargic, suporous, obtuded on admission or initial observation (can be aroused by verbal stimuli)** No prior unconsciousness	**Awake on admission or initial observation** Prior unconsciousness but length unspecified/ amnesia. Unconsciousness 15 mins with neurological deficit. Unconsciousness 15-59 mins. **Lethargic, suporous, obtuded on admission or initial observation (can be accused by verbal stimuli)** No prior /unconsciousness < 15 min with neurological deficit.	**Awake on admission or initial observation** Unconscious 15-59 min with neurological deficit. Lethargic, stuporous, obtunded on admission or initial observation (can be aroused by verbal stimuli) Unconsciousness 15-59 min/ prior unconsciousness for unspecified length of time/ unspecified loss of	**Unconscious on admission or initial observation (unresponsive to verbal stimuli)** Inappropriate movements (decerabrate, decorticate, flaccid, no response to pain- no matter the length of unconsciousness)

	Corneal abrasion/confusion Lid abrasion/confusion/laceration Vitreous/retina/canaliculis (tear duct) laceration Choroid rutpture Urea injury **Gingiva (F)** (gum) confusion /laceration Lip (F) confusion/ laceration (no matter how extensive) **Mandible (F)** Fracture unspecified Ramus fracture **Nose (F)** fracture **Teeth (F)** avulsion/ dislocation (loosened) fracture **Superficial tongue (F) laceration**	Unconsciousness <15 min **When level of construction** Unconsciousness < 15 min **Medical diagnosis listed as concussion with no other description** Fracture of vault (frontal, occipital, parietal sphenoid, temporal or unspecified) closed, undisplaced, diastatic, linear, simple, unspecified **Ear** Inner/middle ear injury Ossicular bone dislocation Lympanic membrane rupture Avulsion of pinna (outer ear) **Eye (F)** Cornea laceration Sclera laceration **Alveolar ridge (bone) (F)** fracture with or without tooth injury. **Avulsiongingiva/ lid/ lip (F)** **Mandibular fracture (F)** Ramus if open/displaced/ comminuted Body with or without ramus involvement Subcondylar **Maxilla fracture (F)** closed/ unspecified/ Le Fort 1/ zygomatic fracture **Tongue (F)** deep + / or extensive laceration	Unconsciousness 15-59 min Prior unconsciousness/loss of consciousness unspecified. **Unconsciousness on admission or initial observation (can be aroused by verbal stimuli)** Length of unconsciousness unspecified. Unconsciousness < 1 hr **When level of consciousness on admission or initial observation is unknown** Unconsciousness 15-59 min. Unconsciousness < 15 mins with neurological deficit. **Fracture of base (**basilar ethmoid, orbital roof, sphenoid, temporal) without CSF leak. **Comminuted compound, depressed or displaced fracture of vault** **Cerebellum or cerebrum** Confusion Injury involving any of the following but no further anatomic description (subarachnoid hemorrhage, edema, brain swelling, subpial hemorrhage, hygroma, ischemia, infarction) **Zygomatic fracture (F)** open/ displaced/ comminuted **Eye (F)** Avulsion Optic nerve avulsion /laceration Tear **Mandibular fracture (F)** Ramus involvement/ mandible fracture	consciousness involving neurological deficit. **Unconscious on admission or initial observation (unresponsive to verbal commands)** 1-24 hr (includes 1 calender day when hrs cannot be estimated) Appropriate movements but only upon painful stimuli (no matter the length of unconsciousness) Length of unconsciousness unspecified/ unconscious < 1 hr involving neurological deficit. **When level of consciousness on admission or initial observation is unknown, but unconscious for:** 1-24 hrs (includes 1 calender day when hrs cannot be estimated) 15-59 min involving neurologic deficit **Fracture of base** (basilar ethmoid, orbital roof, sphenoid, temporal) with CSF leak or pneumocephalus **Fracture of vault** (frontal occipital, parietal, sphenoid,	1-24 hr (includes 1 calender day when hours cannot be estimated) /appropriate movements but only upon painful stimuli (no matter the length of unconsciousness with neurological deficit) **When level of consciousness on admission or initial observation is unknown, but unconscious for:** 1-24 hrs (includes 1 calender day when hours cannot be estimated) with neurological deficit ≥ 24 hrs **Brain stem** Compression/ confusion/ injury involving hemorrhage **Cerebellum or cerebrum** Hematoma, epidural/ subdural > 100 cc diffuse brain injury (white matter shearing injury)

		Nose (F) fracture open/displaced/ comminuted	Subcondylar/ body with or without ramus involvement for any displaced/ comminuted. **Orbit fracture open /displaced, comminuted (F)** **Le Fort II (F)**	temporal, unspecified) open/dura torn/ CSF leak/ pneumocephalus or brain exposed. **Cerebellum or cerebrum** Laceration Hematoma, epidural/ subdural ≤100 cc or unspecified. Hematoma, intracerebral, intracerebellar (including petechial and subcortical hematoma) **Le fort III (F)**	
Neck	**Pharynx** confusion/ laceration/ puncture/ rupture **Throat (inner soft tissue)** abrasion/ confusion/ laceration (not involving major artery) **Tracheal** confusion	**Pharynx** confusion with hematoma/ laceration with hemorrhage Confusion/ esophagus/ larynx/ thyroid gland	**Trachea** crush **Thyroid gland** laceration	**Laceration** of trachea/ carotid artery/ subclavian artery. **Larynx** crush/ fracture/ laceration	**Esophagus/** larynx/ trachea avulsion/ rupture
Thorax	Rib confusion/ fracture	**Rib** fracture open/ displaced/ >2 adjacent ribs up to flail chest. **Sternum…** fracture	**Lung/pericardium** confusion with or without unilateral hemathorax **Lung** laceration superficial or unspecified **Unilateral/hemathorax/ pneumothorax** with rib cage or thoracic cavity injury. **Sternum fracture…** open/ displaced/ comminuted	**Chest wall (soft tissue)** perforation/ puncture **Lung confusion** with hemomediastinum/ pneumomediastinum /bilateral hemothorax or pneumothorax **Myocardium…** confusion **Pericardium…** confusion with hemomediastinum/ pneumomediastinum or tamponade/ perforation/	**Laceration…** aorta /bronchus/ coronary artery/ lung (deep +/or extensive) /pulmonary artery or vein/ superior or inferior vena cava/ pericardium if involving hemomediastinum/ pneumomediastinum or tamponade **Puncture/ rupture** of aorta/ intracardiac valve or septum/ myocardium (involving

				puncture/ rupture/ laceration/ bilateral hemothorax or pneumothorax **Bilateral hemothorax/ pneumomediastinum** Flail chest (“sucking chest” wound) **Lung laceration** superficial or unspecified with hemothorax/ pneumothorax **Inhalation burn**	multiple chambers) /superior or inferior vena cava/ pericardium if involving hemomediastinum/ pneumomediastinum or tamponade **Perforation** of aorta/ bronchus/ myocardium/ pericardium if involving hemomediastinum pneumomediastinum/ tamponade **Rupture bronchus** **Inhalation burn** requiring mechanical respiratory support **Myocardium confusion** if severe or involving hemomediastinum or pneumomediastinum

AIS SCOPE	MINOR	MODERATE	SEVERE NOT LIFE-THREATENING	SEVERE LIFE-THREATENING	CRITICAL, SURVIVAL UNCERTAIN
ABDOMEN/ PELVIC CONTENTS (include all described in parenthesis)	**Superficial or unspecified laceration/ perforation** of abdominal wall (no organ involvement) **Abrasion/ confusion/ superficial or unspecified laceration or perforation** of scrotum/ vagina/ vulva/ perineum Penis… confusion Scrotum… rupture	**Abdominal wall avulsion** Deep +/or extensice laceration or perforation of abdominal wall (no organ involvement) /scrotum **Stomach confusion** **Ureter** confusion/ superficial or unspecified laceration	**Abdominal wall musculature rupture** **Confusion** of biliary tract (gall bladder, hepatic, cystic and common bile ducts) /colon /duodenum/ jejunum/ ileum/ kidney (with or without hematuria) /liver/ bladder/ mesentery (omentum)/ pancreas/ peritoneum/ rectum/ spleen/ urethra/ uterus. **Superficial or unspecified laceration/ perforation of bladder/ penis/ ureter/** diaphragm **Deep +/or extensive laceration/ perforation** of perineum/ ureter/ vagina/ vulva **Avulsion** of scrotum/ ureter **Retroperitoneum…** injury involving hemorrhage or hematoma.	**Superficial or unspecified laceration/ perforation** biliary tract/ colon/ duodenum/ peritoneum/ rectum/ (superficial over entire rectal wall extraperitoneal) **Deep +/or extensive laceration/ perforation** of bladder/ mesentery/ penis/ stomach/ urethra/ uterus **Avulsion** of bladder/ mesentery/ penis/ spleen/ stomach/ testes/ urethra/ uterus (unpregnant or 1st trimester) /ovary **Rupture** of spleen/ stomach/ urethra/ uterus (unpregnant or 1st trimester)/ bladder (intraperitoneal) **Rupture/tear** ovarian/fallopian tube **Spleen laceration**	Avulsion/ deep +/or extensive laceration/ perforation/ rupture of biliary tract/ colon duodenum/ jejunum/ ileum/ kidney/ liver/ pancreas (with or without duodenum involvement) **Deep +/or extensive laceration/ rupture of** peritoneal/ rectum. **Intra-abdominal or intrapelvic major vessel laceration** **Uterus** (in 2nd or 3rd trimester) avulsion/ rupture.

SPINE	Acute strain with no fracture or dislocation cervical/ thoracic/ lumbar spine	**Dislocation (subluxation) + /or fracture** spinous or transverse process for unspecified of cervical, thoracic, lumbar spine **Minor compression fracture** T_{1-12}/ L_{1-5} (≤20% loss of height of anterior vertebral body/ unspecified)	**Cervical cord** confusion with transient neurological signs (muscle weakness, paralysis, loss of sensation) **Disc herniation (rupture) with nerve root damage** of cervical/ thoracic/ lumbar spine **Dislocation (subluxation) +/or fracture** of lamina/ body/ facet/ pedicle/ odontoid of cervical/ thoracic/ lumbar spine **Nerve root/trunk brachial plexus/ lumbar plexus/ sacral plexus** avulsion/ laceration/ rupture, injury with unknown lesion. **Compression fracture of more than one vertebrae +/or >20% loss of height of anterior body** T_{1-12} L_{1-5}	**Cervical cord lesion** incomplete with preservation of significant sensation +/or motor function	Cervical cord crush/ laceration or total transection with or without fracture +/or dislocation C_4 or below Complete cervical cord lesion (quadriplegia or paraplegia) Crush/ laceration/ total transection (paraplegia) of cord/ caudia equina
EXTREMITIES & BONY PELVIS	Confusion/ sprain of acromioclavicular joint/ sternoclavicular joint/ wrist (carpus)/	**Dislocation/ laceration into joint** of acromioclavicular joint/elbow (dislocation of radial head)/ hand (laceration involving flexor or extensor tendons)/ sternoclavicular joint/	**Crush** of acromioclavicular joint/ arm/ forearm/ elbow/ hand/ shoulder/ sternoclavicular joint/ wrist/ ankle/ foot/ heel/ knee/ below knee **Amputation** upper extrimities	Pelvis… crush **Above knee crush/ amputation** (traumatic partial or complete)	

	ankle **Confusion** fibula/ knee **Sprain** finger/ foot /hip /toe **Fracture/dislocation** finger /toe	wrist/ heel (dislocation subtalar: laceration involving achiles tendon)/ patella (laceration or rupture patellar tendon) **Fracture** of clavicle/ acromion/ hand (carpal or metacarpal)/ humerus/ radius (including Colles)/ scapula/ ulna/ fibula (head, neck, shaft or lateral malleolus)/ foot (metatarsal talar, tarsal or unspecified)/ heel (calceneous) patella/ pelvis (closed or unspecified with or without dislocation of any of the combination of the illium, ischium, coccyx, sacrum, pubic ramus)/tibia (shaft, malleolus, plateau, condyles) **Laceration into joint of shoulder/ ankle/ knee** **Muscle** avulsion or laceration of major muscle tendon of upper and lower (except patella and Achilles) extremities **Nerve laceration** of upper (median, radial, ulnar) or lower (femoral, tibial, sciatic or peroneal) extrimities **Dislocation** of foot (subtalar, transtarsal, or transmetatarsal) **Laceration or rupture** of distal biceps tendon **Biceps** muscle rupture **Amputation/crush** of finger/	above or below elbow/ hand/ foot/ heel/ lower extremity below knee (traumatic, partial or complete) **Dislocation** of shoulder/ wrist (radiocarpal, intercarpal, pericarpal)/ ankle/ knee/ elbow/ (if involving olecranon)/ hip (with or without fracture of acetabulum, femoral head, neck or intertrochanteric) **Fracture** of humerus/ radius (including colles)/ ulna (with any one or combination of open, displaced, comminuted or involving radial nerve)/ femur (condyle, head, neck, shaft with or without sciatic nerve involvement). **Fracture** of tibia/ fibula/ or pelvis (closed or unspecified with or without dislocation of any one or combination of the following: illium, ischium, coccyx, sacrum, pubic ramus) with any one or combination of open, displaced or comminuted. **Sacroiliac** fracture +/or dislocation **Symphysis pubis** separation (fracture) **Knee…** rupture of collateral of cruciate ligaments **Ankle…** rupture of collateral		

		toe **Acromioclavicular separation** **Confusion** of fibula with peritoneal nerve injury ("footdrop") **Rupture** of collateral or cruciate ligaments of the knee	ligaments +/or achiles tendon **Laceration** of axillary/ brachial femoral/ popliteal artery **Nerve laceration** of upper (median, radial, ulnar) extremity involving ≥ 2 nerves in same extremities **Muscle avulsion or laceration** of multiple major muscle tendons in upper and lower (except patella or Achilles) extremities **Complete patellar tendon** eration or rupture		

Appendix VII: Procedure for Flow Cytometry Bead Assay

BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit

Instruction Manual

Workflow overview

Workflow

The overall workflow consists of the following steps.

Step	Description
1	Preparing Human Inflammatory Cytokines Standards (page 18)
2	Mixing Human Inflammatory Cytokine Capture Beads (page 20)
3	Diluting samples (page 22)
4	Performing instrument setup with Cytometer Setup Beads (instructions can be found at bdbiosciences.com/cbasetup) **Note:** Can be performed during the incubation in step 5.
5	Performing the Human Inflammatory Cytokine Assay (page 24)
6	Acquiring samples (instructions can be found at bdbiosciences.com/cbasetup)
7	Data analysis (page 30)

Incubation times

To help you plan your work, the incubation times are listed in the following table.

Procedure	Incubation time
Preparing standards	15 minutes
Preparing mixed capture beads (when analyzing serum or plasma samples only)	30 minutes

Preparing Cytometer Setup Beads	30 minutes
Performing the assay	3 hours

Required materials

Materials required but not provided

In addition to the reagents provided in the BD CBA Human Inflammatory Cytokines Kit, the following items are also required: λ A dual-laser flow cytometer equipped with a 488- nm or 532-nm and a 633-nm or 635-nm laser capable of distinguishing 576-nm, 660-nm, and >680-nm fluorescence. The following table lists examples of compatible instrument platforms.

Flow cytometer	Reporter channel	Bead channels
BD FACSArray™	Yellow	Red
BD FACSCanto™ platform BD™ LSR platform BD FACSAria™ platform	PE	APC
BD FACSCalibur™ (single laser) BD FACSCalibur (dual laser)	FL2	FL3 FL4
Note: Visit bdbiosciences.com/cbasetup for setup protocols.		

- Falcon® 12 × 75-mm sample acquisition tubes for a flow cytometer (Catalog No. 352008) λ
- 15-mL conical, polypropylene tubes (Falcon, Catalog No. 352097), or equivalent λ
- FCAP Array software (Catalog No. 641488 [PC] or 645447 [Mac®])

Materials required for plate loader equipped flow cytometers

- Millipore MultiScreen$_{HTS}$-BV 1.2 µm Clear non- sterile filter plates [Catalog No. MSBVN1210 (10 pack) or MSBVN1250 (50 pack)]

- Millipore MultiScreen$_{HTS}$ Vacuum Manifold, (Catalog No. MSVMHTS00)
- MTS 2/4 Digital Stirrer, IKA Works, VWR (Catalog No. 82006-096)
- Vacuum source
- Vacuum gauge and regulator (if not using the recommended manifold)

PreparingHumanInflammatoryCytokinesStandards

Purpose of this procedure

The Human Inflammatory Cytokines Standards are lyophilized and should be reconstituted and serially diluted immediately before mixing with the Capture Beads and the PE Detection Reagent.

You must prepare fresh standards to run with each experiment. Do not store or reuse reconstituted or diluted standards.

Procedure

To reconstitute and serially dilute the standards:

1. Open one vial of lyophilized Human Inflammatory Cytokine Standards. Transfer the standard spheres to a 15-mL polypropylene tube. Label the tube "Top Standard."
2. Reconstitute the standards with 2 mL of Assay Diluent.

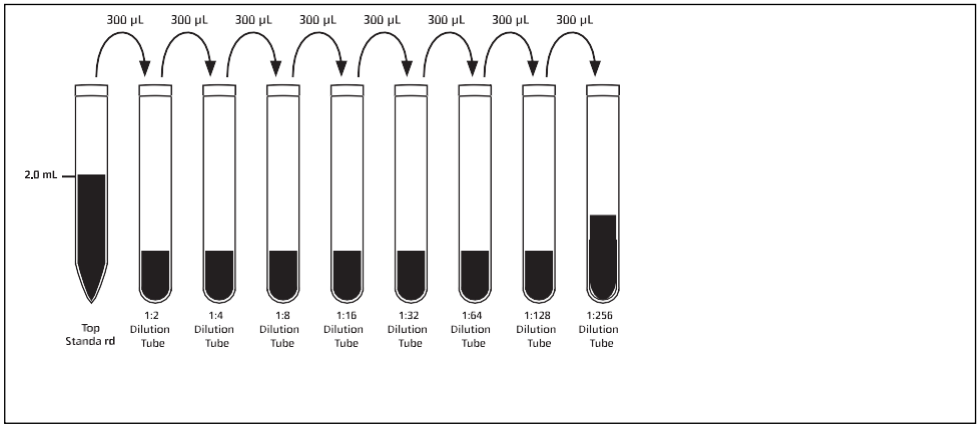
a. Allow the reconstituted standard to equilibrate for at least 15 minutes at room temperature.

b. Gently mix the reconstituted protein by pipette only. Do not vortex or mix vigorously.

3. Label eight 12 × 75-mm tubes and arrange them in the following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256.
4. Pipette 300 µL of Assay Diluent in each of the 12 × 75-mm tubes.
5. Perform a serial dilution:

a. Transfer 300 µL from the Top Standard to the 1:2 dilution tube and mix thoroughly by pipette only. Do not vortex.

b. Continue making serial dilutions by transferring 300 µL from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube.



6. 6. Prepare one 12 × 75-mm tube containing only Assay Diluent to serve as the 0 pg/mL negative control.

Concentration of standards

See the Performing the Human Inflammatory Cytokine Assay (page 24) for a listing of the concentrations (pg/ mL of all six recombinant proteins in each standard.

Next step

Proceed to Mixing Human Inflammatory Cytokine Capture Beads (page 20).

Purpose of this procedure

The Capture Beads are bottled individually (A1–A6). You must pool all six bead reagents immediately before using them in the assay.

Mixing the beads To mix the Capture Beads:

1. Determine the number of assay tubes (including standards and controls) that are required for the experiment (for example, 8 unknowns + 9 standard dilutions + 1 negative control = 18 assay tubes).

2. Vigorously vortex each Capture Bead suspension for 3 to 5 seconds before mixing.

Note: The antibody-conjugated beads will settle out of suspension over time. Vortex the vial immediately before taking a bead-suspension aliquot.

3. Add a 10-µL aliquot of each Capture Bead, for each assay tube to be analyzed, into a single tube labeled “Mixed Capture Beads” (eg, 10 µL of IL-8 Capture Beads × 18 assay tubes = 180 µL of IL-8 Capture Beads required).

4. Vortex the bead mixture thoroughly.

Resuspending the beads

If you are using serum or plasma samples, you must perform this procedure to reduce the chances of falsepositive results due to serum or plasma proteins. This procedure is optional for all other sample types.

To resuspend the Capture Beads in Serum Enhancement Buffer:

1. Centrifuge the mixed Capture Beads at 200g for 5 minutes.

2. Carefully aspirate and discard the supernatant.

3. Resuspend the mixed Capture Beads pellet in Serum Enhancement Buffer (equal to the volume removed in step 2) and vortex thoroughly.

4. Incubate the mixed Capture Beads for 30 minutes at room temperature, protected from light.

Next step

The mixed Capture Beads are now ready to be transferred to the assay tubes. Discard excess mixed Capture Beads. Do not store after mixing.

To begin the assay, proceed to Performing the Human Inflammatory Cytokine Assay (page 24). If you need to dilute samples having a high-protein concentration, proceed to Diluting samples (page 22)

Diluting samples

Purpose of this procedure

The standard curve for each protein covers a defined set of concentrations from 20 to 5,000 pg/mL. It might be necessary to dilute test samples to ensure that their mean fluorescence values fall within the range of the generated standard curve. For best results, dilute samples that are known or assumed to contain high levels of a given protein. This procedure is not required for all samples.

Procedure

To dilute samples with known high-cytokine concentration:

1. Dilute the sample by the desired dilution factor (for example, 1:2, 1:10, or 1:100) using the appropriate volume of Assay Diluent. Optimal recovery from serum samples typically requires a 1:4 dilution.

2. Mix sample dilutions thoroughly.

Next step

Perform instrument setup using the Cytometer Setup Beads. For details, go to bdbiosciences.com/cbasetup and select the appropriate flow cytometer under CBA Kits: Instrument Setup.

Or, if you wish to begin staining your samples for the assay, proceed to Performing the Human Inflammatory Cytokine Assay (page 24), and you can perform instrument setup during the 3-hour staining incubation.

Performing the Human Inflammatory Cytokine Assay

Before you begin

1. Prepare the standards as described in Preparing Human Inflammatory Cytokines Standards (page 18).

2. Mix the Capture Beads as described in Mixing Human Inflammatory Cytokine Capture Beads (page 20).

3. If necessary, dilute the unknown samples. See Diluting samples (page 22).

Procedure for supernatant samples

To perform the assay:

1. Vortex the mixed Capture Beads and add 50 µL to all assay tubes.

2. Add 50 µL of the Human Inflammatory Cytokine Standard dilutions to the control tubes as listed in the following table.

Tube label	Concentration (pg/mL)	Standard dilution
1	0 (negative control)	no standard dilution (Assay Diluent only)
2	20	1:256
3	40	1:128
4	80	1:64
5	156	1:32
6	312.5	1:16

7	625	1:8
8	1,250	1:4
9	2,500	1:2
10	5,000	Top Standard

1. Add 50 µL of each unknown sample to the appropriately labeled sample tubes.
2. Add 50 µL of the Human Inflammatory Cytokine PE Detection Reagent to all assay tubes.
3. Incubate the assay tubes for 3 hours at room temperature, protected from light.

Note: If you have not yet performed cytometer setup, you may wish to do so during this incubation.

4. Add 1 mL of Wash Buffer to each assay tube and centrifuge at 200*g* for 5 minutes.
5. Carefully aspirate and discard the supernatant from each assay tube.
6. Add 300 µL of Wash Buffer to each assay tube to resuspend the bead pellet.

Procedure for serum/plasma samples

To perform the assay:

1. Vortex the mixed Capture Beads and add 50 µL to all assay tubes.
2. Add 50 µL of the Human Inflammatory Cytokine Standard dilutions to the control tubes as listed in the following table.

Tube label	Concentration (pg/mL)	Standard dilution
1	0 (negative control)	no standard dilution (Assay Diluent only)
2	20	1:256
3	40	1:128
4	80	1:64
5	156	1:32
6	312.5	1:16

7	625	1:8

Tube label	Concentration (pg/mL)	Standard dilution
8	1,250	1:4
9	2,500	1:2
10	5,000	Top Standard

3. Add 50 µL of each unknown sample to the appropriately labeled sample tubes.

4. Incubate the assay tubes for 1.5 hours at room temperature, protected from light.

Note: If you have not yet performed cytometer setup, you may wish to do so during this incubation, or during the incubation in step 8.

5. Add 1 mL of Wash Buffer to each assay tube and centrifuge at 200*g* for 5 minutes.

6. Carefully and consistently aspirate and discard the supernatant, leaving approximately 100 µL of liquid in each assay tube.

7. Add 50 µL of the Human Inflammatory Cytokine PE Detection Reagent to all assay tubes. Gently agitate the tubes to resuspend the pellet.

8. Incubate the assay tubes for 1.5 hours at room temperature, protected from light.

9. Add 1 mL of Wash Buffer to each assay tube and centrifuge at 200*g* for 5 minutes.

10. Carefully aspirate and discard the supernatant from each assay tube.

11. Add 300 µL of Wash Buffer to each assay tube to resuspend the bead pellet.

Procedure for filter plates for supernatant samples

To perform the assay:

1. Wet the plate by adding 100 µL of wash buffer to each well.
2. Place the plate on the vacuum manifold.
3. Aspirate for 2 to 10 seconds until the wells are drained.
4. Remove the plate from the manifold, then blot the bottom of the plate on paper towels.
5. Add 50 µL of each of the following to the wells in the filter plate:
   - Capture Beads (vortex before adding)
   - Standard or sample (add standards from the lowest concentration to the highest, followed by samples)
   - Human Inflammatory Cytokine PE Detection Reagent
6. Cover the plate and shake it for 5 minutes at 1,100 rpm on a plate shaker.
7. Incubate the plate for 3 hours at room temperature on a non-absorbent, dry surface.

   Note: Place the plate on a non-absorbent, dry surface during incubation. Absorbent or wet surfaces can cause the contents of the wells to leak.
8. Remove the cover from the plate and apply the plate to the vacuum manifold.
9. Vacuum aspirate for 2 to 10 seconds until the wells are drained.

   Remove the plate from the manifold, then blot the bottom of the plate on paper towels after aspiration.
10. Add 120 µL of wash buffer to each well to resuspend the beads.
11. Cover the plate and shake it for 2 minutes at 1,100 rpm before you begin sample acquisition.

Procedure for filter plates for serum/plasma samples

To perform the assay:

1. Wet the plate by adding 100 µL of wash buffer to each well.
2. Place the plate on the vacuum manifold.
3. Aspirate for 2 to 10 seconds until the wells are drained.

4. Remove the plate from the manifold, then blot the bottom of the plate on paper towels.

5. Add 50 µL of each of the following to the wells in the filter plate:

- Capture Beads (vortex before adding)
- Standard or sample (add standards from the lowest concentration to the highest, followed by samples)

6. Cover the plate and shake it for 5 minutes at 1,100 rpm on a plate shaker.

7. Incubate the plate for 1.5 hours at room temperature on a non-absorbent, dry surface.

Note: Place the plate on a non-absorbent, dry surface during incubation. Absorbent or wet surfaces can cause the contents of the wells to leak.

8. Remove the cover from the plate and apply the plate to the vacuum manifold.
9. Vacuum aspirate for 2 to 10 seconds until the wells are drained.
10. Remove the plate from the manifold, then blot the bottom of the plate on paper towels after aspiration.
11. Add 200 µL of wash buffer to each well. Cover the plate and shake for 2 minutes at 1,100 rpm.
12. Repeat step 8 through step 10.
13. Add 100 µL of assay diluent to each well.
14. Add 50 µL of Human Inflammatory Cytokine PE Detection Reagent to each well.
15. Cover the plate and shake it for 5 minutes at 1,100 rpm on a plate shaker.
16. Incubate the plate for 1.5 hours at room temperature on a non-absorbent, dry surface.

Note: Place the plate on a non-absorbent, dry surface during incubation. Absorbent or wet surfaces can cause the contents of the wells to leak.

17. Repeat step 8 through step 10.
18. Add 120 µL of wash buffer to each well to resuspend the beads.
19. Shake the plate for 2 minutes at 1,100 rpm before you begin sample acquisition.

Next step

Acquire the samples on the flow cytometer. For details, go to bdbiosciences.com/cbasetup and select the appropriate flow cytometer under CBA Kits: Instrument Setup.

CBA samples must be acquired on the same day they are prepared. Prolonged storage of samples, once the assay is complete, can lead to increased background and reduced sensitivity.

To facilitate the analysis of samples using FCAP Array software, we recommend the following guidelines:

- Acquire standards from lowest (0 pg/mL) to highest (Top Standard) concentration, followed by the test samples.
- If running sample dilutions, acquire sequentially starting with the most concentrated sample.
- Store all FCS files (standards and samples) in a single folder.

When you are finished acquiring samples, proceed to Data analysis (page 30).

Data analysis

How to Analyse

Analyze BD CBA Human Inflammatory Cytokines Kit data using FCAP Array software. For instructions on analysis, go to bdbiosciences.com/cbasetup see the *Guide to Analyzing Data from BD CBA Kits Using FCAP Array Software*.