Systemic Stimulation of Mesenchymal Stem Cell and Growth Factors Following Trauma

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The candidate confirms that the work submitted is his/her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Bone healing following trauma is known to be associated with an early increase in serum concentrations of several pro-inflammatory and angiogenic growth factors. However, the temporal pattern of growth factors (GFs) involved in bone formation and their relationship with trauma severity has not been explored. Furthermore, to what extent osteogenic progenitors, including mesenchymal stem cells (MSCs) are 'mobilized' following trauma is unknown.

This study investigated the systemic levels of four GFs (PDGF-AA, TGF-β2, follistatin and angiogenin) over the first two weeks following trauma in three groups of patients with increasing severity (Isolated trauma (n=15), Polytrauma (n=15), and Head injury (n=14)) and compared to Healthy Controls (n=9). The dynamics of GF release measured by ELISA was correlated with clinical and biochemical inflammatory parameters and the healing outcome assessed by clinical and radiological parameters as well as requirement of surgical re-interventions. Potential MSC mobilization from their iliac crest bone marrow (ICBMA) niches into peripheral circulation was measured by standard colony-forming assay-fibroblast, at least twice following trauma. Further correlations were sought with circulating levels of platelets, PDFG-BB and PDGF-AA.

Growth factors described as anabolic for bone (PDGF-AA and angiogenin) had an initial suppression following trauma (50% and 80% by day 1, respectively), whereas inhibitory GF follistatin was upregulated compared to control (1.5-fold by day 1). This effect was more pronounced with increasing trauma severity. The variability of TGF-β2 was too high to allow differences between trauma groups to be detected. The dynamics of all GFs were not correlated with the inflammatory state of the patients, assessed both clinically (Systemic Inflammatory Response Syndrome score) and biochemically (total white cell count, C-reactive protein and platelet levels). However, there was a significant correlation between levels of time-matched PDGF-AA and platelets ($p<0.01$, $r=+0.61$), independent of trauma severity. A marked suppression of TGF-β2 throughout the time course which reached statistical significance in the first week following trauma (5-fold, $p<0.05$) was observed in patients identified as 'poor healers'; the same group additionally displayed an altered dynamics of follistatin release compared to patients who healed normally.

The numbers of ICBMA MSCs were dynamic over time in the same patient, but did not correlate with trauma severity or patients' inflammatory state. Instead, significant correlations were observed between the changes in ICBMA MSC numbers and changes the levels of PDGF-AA ($p<0.01$, $r=+0.55$), and PDGF-BB ($p=0.03$, $r=+0.38$) and circulating platelets ($p=0.02$, $r=+0.44$). No MSCs were found in patients' peripheral blood at any time point studied.

These data indicated that measuring GFs implicated in BMP signalling pathway may lead to the discovery of novel biomarkers of fracture non union. Measuring patient's inflammatory response following fracture did not correlate with the release of growth factors studied suggesting that these phenomena were independent. Limited MSC mobilization in the bone marrow (but not into PB) did take place but appeared to be related to platelet counts and a possible release of PDGF-AA and PDGF-BB GFs, which are known to be mitogenic for MSCs. It was not linked to trauma severity or predictive of the healing outcome. Further research is needed to investigate the predictive value of TGF-β2 and follistatin in a larger cohort of patients. Whilst this is the first study showing a 'dynamic' nature of the MSC pool in human BM, further work should determine whether the influence of platelets is due to enhanced MSC migration or their proliferation in situ.

Abbreviations

1-CTP	pyridinoline cross-linked telopeptide parts of type-*I* collagen
ANG	angiogenin
APACHE	Acute Physiology and Chronic Health Evaluation
BAMBI	BMP and activin membrane-bound inhibitor homolog
BDNF	brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
BMPs	bone morphogenic proteins
CCL2	chemokine (C-C motif) ligand 2
CSF	cerebrospinal fluid
CNS	central nervous system
CRP	C-reactive protein
CV	coefficient of variation
DNA	Deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
FGFs	fibroblast growth factors
GCS	Glasgow Coma Scale
GDF	growth differentiation factor
GF	growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN-γ	interferon gamma
ICD	International Classification of Diseases
IGFs	insulin-like growth factors
IL	interleukin
ISS	Injury Severity Score
MCP-1	monocyte chemotactic protein-1
M-CSF	macrophage colony-stimulating factor
MMP	matrix metalloproteinases
MSC	mesenchymal stem cell
OD	optical density
PACAP	pituitary adenylate cyclase activating polypeptide
PDGF	platelet-derived growth factor
PICP	carboxy-terminal propeptide of procollagen type I
RANKL	receptor activator of nuclear factor kappa-B ligand
RT	room temperature
SIRS	systemic inflammatory response syndrome
TARN	Trauma Audit Research Network
TBI	traumatic brain injury
TGF-β	transforming growth factor beta
TNF-α	tumour necrosis factor alpha
VEGF	vascular endothelial growth factor
WCC	white cell count

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1. General Introduction

1.1. Fracture healing

Fracture healing represents a physiological process regulated by a variety of mediators and cells. It was initially described as consisting of three stages: an initial inflammatory response followed by the development of osteogenic repair tissue and finally bone remodelling (McKibbin 1978). This progression of events is dependent on factors such as mechanical stability/rigidity and the local biological cell environment. However, currently the fracture healing process is understood to comprise 2 modes of healing; direct (no callus formation) and indirect or secondary (callus formation, with or without formation of cartilage first) (Einhorn 1998), as represented in Figure 1.1-1.The mechanism of fracture repair *in vivo* is reasonably well understood. This involves coordinated action of the inflammatory response and mesenchymal stem cells (MSCs) that lead to an ordered sequence of events including inflammatory removal of debris from the fracture site, angiogenesis and subsequently bone re-modelling leading to repair with various cytokines being involved at different stages (Dimitriou, Tsiridis et al. 2005).

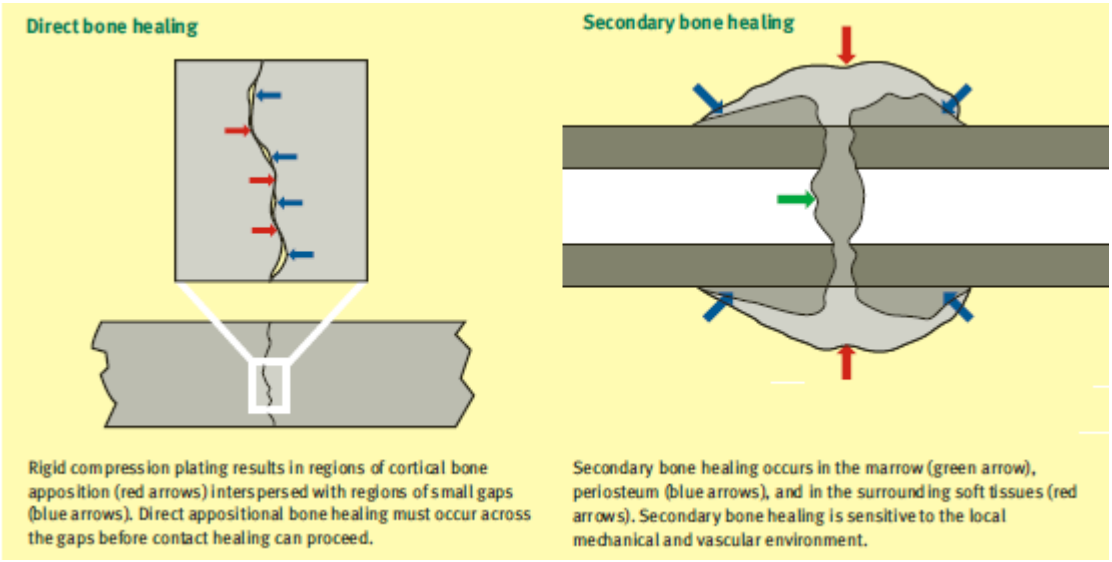

Figure 1.1-1 Schematic representation of Direct and Indirect (Secondary) Bone Healing. (From (McKinley 2003)

1.1.1. Direct bone healing

The process of direct or primary bone healing can only occur in the presence of absolute fracture stability (by rigid internal fixation), with fracture defects of less than 0.01mm (Shapiro 1988) and interfragmentary strain less 2% (Mann and Payne 1989).

This process is initiated by osteoclast bone resorbing cells, which creates tunnels (cutting cones) (McKibbin 1978) across fracture sites. The resultant pathways allow for neo-vascularisation, accompanied by endothelial cells, and perivascular MSCs, which eventually differentiate to osteoblasts (Kaderly 1991). These cutting cones advance across the fracture site at a rate of 50-100μm/day (Marsell and Einhorn 2011). This process concurrently generates new bone and re-establishes the Haversian system. These bridging bony tissues directly remodel into lamellar bone; allowing for fracture healing without callus formation (Marsell and Einhorn 2011).

1.1.2. Indirect (secondary) bone healing

Most fractures however, heal by the indirect process, consisting of endochondral and intramembranous bone healing components (Gerstenfeld, Alkhiary et al. 2006). Endochondral healing occurs outside the periosteum but within proximity of the fracture site (forming soft callus), whereas intramembranous healing occurs within the periosteum (forming hard callus) (Gerstenfeld, Alkhiary et al. 2006). This secondary bone healing undergoes the classically described overlapping stages of an initial inflammatory phase, followed by repair and finally bone remodelling(McKibbin 1978).

Following fracture, a haematoma clot forms on the fracture site. This clot not only functions to limit the amount of blood loss (bleeding), but is a rich source of growth factors and cytokines which function to initiate the cascades of cellular processes involved in fracture healing. Degranulating platelets within the clot release among others, platelet derived growth factors and transforming growth factor-1, both of which are pro-osteogenic growth factors (Lieberman, Daluiski et al. 2002).

The accompanying acute inflammatory process, also results in the release of cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-11 and IL-18 (Gerstenfeld, Cullinane et al. 2003). These cytokines are produced at the local fracture site by the invading macrophages, polymorphonuclear leucocytes and lymphocytes, and incite further downstream response through the recruitment of more inflammatory cells, stimulating vascularisation and extracellular matrix formation (Kon, Cho et al. 2001). Recruited macrophages aid in the resorption and removal of necrotic bone and help the creation of callus. Additionally, TNF-α acts as a chemotactic agent to recruit cells and is involved in the induction of osteogenic differentiation of MSCs (Cho, Kyoung et al.

2006). Among the interleukins, it is believed that IL-1 and IL-6 exert the most influence in the fracture healing process (Kon, Cho et al. 2001). IL-1, which is secreted by macrophages, induces IL-6 production by osteoblasts, initiates the cartilaginous soft callus production and fracture site angiogenesis (Kon, Cho et al. 2001). On the other hand, IL-6 stimulates angiogenesis via the production of vascular endothelial growth factor as well as the differentiation of cells of osteoblastic and osteclastic lineages (Yang, Ricciardi et al. 2007). Overall the inflammatory phase occurs immediately upon fracture, lasting for up to a week, and leading on to the repair phase.

The fracture healing repair phase involves the formation of a cartilaginous tissue within the fracture haematoma, external to the periosteum (Marsell and Einhorn 2011). At the same time, a more direct bony tissue formation occurs through the process of intramembranous ossification, internal to the periosteum (Marsell and Einhorn 2011). These processes occur via recruitment of MSCs from adjacent soft tissues, periosteum, cortical bone and bone marrow. Key roles are played by transforming growth factor-beta 2 and 3, growth differentiation factor 5 in the differentiation into chondrocytes and subsequent process of endochondral ossification of the soft callus (Cho, Gerstenfeld et al. 2002). Similarly, bone morphogenic proteins 5 and 6 have been suggested to be involved in cellular proliferation of the intramembranous ossification process (Cho, Gerstenfeld et al. 2002).

The chondrocytes within the cartilaginous soft tissue progressively become hypertrophied, and begin forming matrix vesicles which migrate to the surrounding matrix and participate in its calcification (Einhorn 2005). Following this process, this calcified cartilage tissue itself becomes replaced with woven bone. The chondrocytes replacement occurs via an ordered process of programmed cellular death (apoptosis) (Lee, Choi et al. 1998), prior to removal by multinucleated cells called chondroclasts. The degradation of the chondrocytes and surrounding calcified matrix enables neo-vascularisation of the callus tissue with concomitant invasion by perivascular MSCs which ultimately differentiate into bone forming osteoblasts (Einhorn 2005). This process occurs between 4 and 6 weeks following fracture.

The final process of remodelling involves the co-ordination of hard callus removal by osteoclasts (mediated by IL-1 and TNF-α (Ai-Aql, Alagl et al. 2008)) and lamellar bone formation by osteoblasts (mediated by molecules from the bone morphogenic protein families (Marsell and Einhorn 2009)). This process may take years to be completed.

Most of the knowledge described above was based on data derived from experiments on animal models, and extrapolated to human. Replication of the animal model studies in humans by serial sampling of tissues from fracture sites would be unethical and unlikely to obtain regulatory approval. Additionally, the effect of different types of fracture location, severity and concomitant injuries on

the fracture healing process has yet to be fully explored. Hence, a method of understanding the dynamics of fracture healing in human would be serial measurements over time of levels of growth factors known to participate in the fracture healing process.

1.2. Growth Factors and Fracture Healing

Various signalling molecules are involved in the process of bone healing. Broadly, they are divided into pro-inflammatory cytokines [(as Interleukin-1β (IL-1β), IL-6, IL-8, IL-12, tumour necrosis factor (TNF) and interferon gamma (IFN-γ)], growth and differentiation factors [(transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), bone morphogenic proteins (BMPs), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs)], matrix metalloproteinases (MMP-9, MMP-14) and angiogenic factors [(vascular endothelial growth factor (VEGF), angiopoietin)]. In addition there are also inhibitory molecules (noggin, follistatin, chordin, BAMBI, Smad) which are believed to play an essential role in regulating the signalling molecules. Each factor exerts its influence at different time points between day 0 and 21 following fracture (Dimitriou, Tsiridis et al. 2005). Table 1.2-1 illustrates the stages of fracture healing discussed earlier (Section 1.1) and the associated expression of signalling molecules.

Table 1.2-1 Stages of Fracture healing and expression of signalling molecules (Adapted from (Ai-Aql, Alagl et al. 2008), (Dimitriou, Tsiridis et al. 2005) and (Phillips 2005))

Stage of Fracture Repair	Biological Processes	Expression of Signaling Molecules and their Proposed Functions
Inflammation (First hours to day 7)	Hematoma	IL-1, IL-6, and TNF-α play a role in initiating the repair cascade.
	Inflammation	TGF-β, PDGF, and BMP-2 expression increases to initiate callus formation.
	Recruitment of MSCs	GDF-8 is restricted to day 1, suggesting its role in controlling cellular proliferation.
Cartilage Formation and Periosteal	Chondrogenesis and endochondral	TGF-β2, -β3, and GDF-5 peak due to their involvement in chondrogenesis and

Response (Days 6-10)	ossification begins	endochondral bone formation.
	Cell proliferation in intramembranous ossification	BMP-5 and BMP-6 rise
	Vascular in-growth/Neo-angiogenesis	Angiopoietins and VEGFs are induced to stimulate vascular in growth from vessels in the periosteum.
Cartilage Resorption and Primary Bone Formation (Days 11-20)	Phase of most active osteogenesis	TNF-α rises in association with mineralized cartilage resorption. This promotes the recruitment of MSCs and induces apoptosis of hypertrophic chondrocytes.
	Bone cell recruitment and woven bone formation	RANKL and M-CSF rise in association with mineralized cartilage resorption.
	Chondrocyte apoptosis and matrix proteolysis	
	Osteoclast recruitment and cartilage resorption	BMP-3, -4, -7, and -8 rises in association with the resorption of calcified cartilage. They promote recruitment of cells of the osteoblastic lineage.
	Neo-angiogenesis	BMP-5 and -6 remain high during this stage, suggesting a regulatory effect on both intramembranous and endochondral ossification. VEGFs are up-regulated to stimulate neo-angiogenesis.
Secondary Bone Formation and Remodeling (Day 21 onwards)	Bone remodeling coupled with osteoblast activity	IL-1 and IL-6 rise again in association with bone remodeling, whereas RANKL and M-CSF display diminished levels.

	Establishment of marrow	Diminished expression of members of the TGF-β superfamily.

As can be seen in the summary table above (Table 1.2-1), growth factors such as PDGF and TGF-β, together with pro-angiogenic factors predominate in the first 10 days following fracture. Molecules from the BMP families would become the prevailing growth factors at a later stage (2^{nd} to 3^{rd} week onwards). Were the BMP molecules initially suppressed by the presence of inhibitory molecules (to BMPs) such as noggin and follistatin? The effect of additional fractures and other injuries (such as traumatic brain injury) to the early dynamics (first 2 weeks) of these molecules remain obscure.

1.3. Cellular component of Fracture Healing

MSCs are multipotent cells capable of differentiating and giving rise to diverse cells such as osteoblasts, chrondrocytes, and adipocytes (Bianco, Cao et al. 2013). As discussed previously, MSCs contribute a significant role in this process through their osteogenic differentiation capacities, supported by a plethora of other cellular phenotypes throughout the different stages of fracture healing. Further details regarding MSCs will be discussed in Chapter 6. Table 1.3-1 summarises the roles played by the key cellular phenotypes found within the fracture site.

Table 1.3-1 Cellular phenotypes involved in the fracture healing process

Cellular phenotypes	Roles
Mesenchymal stem cell	Differentiates into: Chondrocytes: Formation of cartilaginous soft callus as part of the endochondral fracture healing process Osteoblasts: Bone forming cells
Neutrophils	Dominant leucocytes at fracture site within first 24 hours of fracture (Chung, Cool et al. 2006). Secrete several macrophage chemoattractants, such as MCP-1, also known as CCL2, and IL-6 (Kasama, Strieter et al. 1993; Hurst, Wilkinson et al. 2001)
Monocytes	Differentiates into: Macrophages: resorption and removal of necrotic bone and help in the creation of callus. Secrete pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-11 and IL-18 (Gerstenfeld, Cullinane et al. 2003), and growth factors such as fibroblast growth factor, PDGF (Lieberman, Daluiski et al. 2002) Multi-nucleated chondroblasts: resorption and removal of hypertrophied chondrocytes, degradation of its calcified matrix and induction of neo-

	vascularisation (Lee, Choi et al. 1998) Multi-nucleated osteoclasts: resorption of woven bone during remodelling process
Lymphocytes	B-lymphocytes: not found in any stages of fracture healing (Andrew, Andrew et al. 1994) T-lymphocytes: selectively recruited into fracture haematoma and subsequent granulation tissue/soft callus (Andrew, Andrew et al. 1994). Enables osteoblast maturation during early phase of fracture repair (Nam, Mau et al. 2012).
Platelets	Involved in the formation of the fracture haematoma through the clotting cascade. Degranulating platelets are a rich source of cytokines such as PDGF and TGF-β (Lieberman, Daluiski et al. 2002)

The roles of the different cellular phenotypes described above during the fracture healing process occur around the fracture site. What remains unclear is whether the local injury (fracture) exerts a more generalized systemic effect away from site of injury. Particularly, do the cells in similar environments (bone marrow) away from fracture site, get stimulated and up-regulated following a fracture?

1.4. Measurement of Trauma Severity

Efforts have been made previously to quantify the severity of trauma as a tool to predict prognosis and subsequently to improve survival. There are two main groups of trauma scoring systems. The first is developed from the Abbreviated Injury Scale(American Association for Automotive Medicine. Committee on Injury and States 1980) while the second is a disease based database classification system (ICD-9)(1980). Different scoring systems have been suggested such as Injury Severity Score (ISS)(Baker, O'Neill et al. 1974), New Injury Severity Score (Osler, Baker et al. 1997), Trauma Injury Severity Score (Boyd, Tolson et al. 1987), Acute Physiology and Chronic Health Evaluation (Waters, Nightingale et al. 1990) and the International Classification of Diseases (Kim, Jung et al. 2000) based systems. All have been shown to be useful and beneficial. However, most of these systems are complex, not robust enough or inaccurate in the assessment of trauma patients (McAnena, Moore et al. 1992). Table 1.4-1 summarises the main trauma scoring systems that have been previously proposed.

Table 1.4-1 Comparison of Trauma Scoring Systems

Scoring System	Brief description	Strengths	Limitations
Abbreviated Injury Scale (American Association for Automotive Medicine. Committee on Injury and States 1980)	Score of one (minor) to six, one being a minor injury and six being life-threatening	Regarded as "industry standard" for its ability in injury-specific descriptions	Severity scale non-linear, and provides no mechanism to summarize a single patient's multiple injuries into a single score
Injury Severity Score (Baker, O'Neill et al. 1974)	Sum of the squares of the largest AIS values assigned to the three most severe injuries in different body regions.	Taking age into account, it correlates well with mortality and length of stay. Relatively easily to calculate	Requires AIS codes to be available. Does not take into account multiple injuries in same body region
New Injury Severity Score (Osler, Baker et al. 1997)	Sum of the squares of the AIS scores a patient's three most severe injuries.	Takes into account multiple injuries in the same region	Does not discriminate between injury severities in different body regions
Revised Trauma Score (Champion, Sacco et al. 1989)	Physiologic injury severity score based on Glasgow Coma Scale, systolic blood pressure and respiratory rate	Numerically summarizes physiologic injury severity, and have high inter-rater reliability and predict survival/death outcome(Champion, Sacco et al. 1989)	The inclusion of Glasgow Coma Scale, limits application on patients who are intubated/mechanically ventilated, sedated or under influence of alcohol/drugs
Trauma Score and Injury Severity Score (TRISS) (Boyd, Tolson et al. 1987)	Probability of survival score based on Injury Severity Score, Revised Trauma Score and patient's age	Widely used, and is the scoring system adopted by the Trauma Audit and Research Network (TARN)	Weaknesses inherent in its component scores, may over-estimate potentially avoidable death, especially in patients with severe head injury(Hill, Lennox et al. 1992)
Anatomic Profile Score (Copes, Champion et al. 1990)	An anatomy based scoring system, divided into four components: Head/spinal cord, Thorax/anterior neck, other serious injuries, non-serious injuries	Better than ISS in discriminating trauma survivors from non-survivors	Complex to calculate
A Severity Characterization of Trauma (ASCOT) (Champion, Copes et al. 1990)	Probability of survival model based on Anatomic Profile Score, age and Revised Trauma Score	Improved survival prediction over the TRISS model	Not widely used due to complexity of calculation of the Anatomic Profile Score
International Classification of	World Health Organization	Wide availability, as commonly used by	Needs to be converted/adapted to

Disease (ICD) (1980)	sponsored system of diagnostic codes for classifying diseases	hospitals to codify clinical diagnoses	generate injury scores
ICD-9-based Injury Severity Score (ICISS) (Osler, Rutledge et al. 1996)	The product of all the Survival Risk Ratio for each of the individual patient's injuries. The Survival Risk Ratio is calculated from trauma registries; ratio of survivors to total occurrence per ICD-9 code	Data already collected in hospital discharge summaries; does not require separate potentially time and expense consuming AIS-based data collection. Often outperforms other scoring systems on discriminatory ability(Meredith, Evans et al. 2002; Stephenson, Langley et al. 2002)	Relies on robustness of locally collected data. The Survival Risk Ratio calculated for a region may not be representative of another region, and may require derivation of local Survival Risk Ratio databases
Acute Physiology and Chronic Health Evaluation (APACHE) (Waters, Nightingale et al. 1990)	Physiological score designed to measure severity of disease for adult patients admitted to intensive care units (ICU). Calculated based on values on the first ICU admission day	Provides estimated risk of mortality in ICU patients	Not specifically designed for trauma patients, scoring system is computationally complex, and may underestimate mortality risk of trauma patients previously stabilized prior to transfer to ICU

Despite the various trauma scoring system proposed, the ISS is still currently the most widely used and adopted system(Sacco, MacKenzie et al. 1999) and is the system adopted by the national trauma registry (UK TARN). Hence, it is most easily recognisable in communications with colleagues and comparison of data derived from this study based on injury severity is most easily compared using the ISS score.

Within this study, the purpose of a trauma scoring system is to discriminate between patients who are considered to be multiply injured polytrauma patients and those who have sustained isolated trauma only. Currently, the consensus for the definition of polytrauma is an ISS score of 16 or more (Copes, Champion et al. 1988; Keel and Trentz 2005) as patients with such scores have a mortality risk of 10% (Baker, O'Neill et al. 1974). This study will follow the most universally accepted conventions, and similarly define polytrauma patients as those with ISS≥16. Detailed calculation to derive ISS for each patient is detailed in Figure 1.4-1.

Each injury is assigned an **Abbreviated Injury Scale** (AIS) score and is allocated to one of six body regions (Head, Face, Chest, Abdomen, Extremities (including Pelvis), and External).

AIS score is on a scale of one to six, one being a minor injury and six being life-threatening.

Injury Severity Score (ISS)

The sum of the squares of the largest AIS values assigned to the three most severe injuries in different body regions. Unless the patient has AIS value of 6, regardless of other injuries sustained, they are assigned an ISS of 75

Figure 1.4-1 Calculation of the Injury Severity Scale (Adapted from (Baker, O'Neill et al. 1974)

1.5. Traumatic Head Injury and Fracture Healing

For the past few decades, there has been increasing belief that the additional presence of traumatic brain injury (TBI) in patients with extremity fractures is associated with enhanced rate of fracture healing (Newman, Stone et al. 1987; Perkins and Skirving 1987; Spencer 1987). Early evidence originated from observations that patients with TBI are significantly at risk of developing periarticular heterotopic ossification (Garland, Blum et al. 1980; Mital, Garber et al. 1987; Hendricks, Geurts et al. 2007; Simonsen, Sonne-Holm et al. 2007). There was initial scepticism of the validity of this phenomenon of accelerated fracture healing following TBI when Garland et al observed no significant enhancement of fracture healing in femur (Garland, Rothi et al. 1982) and tibial (Garland and Toder 1980) fractures in association with TBI. However, these studies were single group only (TBI and fracture) observations. Further studies comparing patients with similar fractures demonstrated significantly faster healing rate for long bone fractures such as femur (Perkins and Skirving 1987), tibia (Newman, Stone et al. 1987) and humerus (Spencer 1987) in patients with TBI compared to patients with similar injuries but without TBI.

The clinical observations of enhanced osteogenesis in post-TBI patients have led to further studies to try to elucidate the patho-mechanism of this phenomenon. Cerebrospinal fluids derived from post-TBI patients have demonstrated a significant proliferative effect on osteoblastic type cells (Gautschi, Toffoli et al. 2007). However, the fact that this effect is seen away from the site of injury (for example, tibia and femur) has led to a postulate that the mediator(s) responsible might be found within the peripheral circulation. Several studies in human patients as well as animal models have shown that serum derived post-TBI enhances the *in vitro* proliferation of human fetal osteoblastic cell lines, primary human osteoblasts and MSCs (Bidner, Rubins et al. 1990; Boes, Kain et al. 2006; Cadosch, Gautschi et al. 2009; Gautschi, Cadosch et al. 2009). Further evidence to support these *in vitro* observations were seen when RNA expression of osteoblastic markers were enhanced in cells co-cultured with serum from post-TBI patients (Cadosch, Gautschi et al. 2009; Gautschi, Cadosch et al. 2009). Additionally, serum from patients with the addition of TBI (compared to fracture alone), has been shown to induce an up to 2.5-fold increase in release by T-lymphocytes of pro-fracture healing growth factors such as IL-10, IL-4 and TGF-β (Cadosch, Al-Mushaiqri et al. 2010). Indeed, biomechanical testing (in animal models) showed significantly increased stiffness (Boes, Kain et al. 2006) as well as maximum fracture load and strain values (Ozan, Yildiz et al. 2010) in the post-TBI group compared to fracture alone (Boes, Kain et al. 2006).

Various studies have attempted to elucidate the molecule responsible for this observation. Table 1.5-1 summarises the target molecules studied to date, which attempted to examine the effect that traumatic brain injury exerts on these molecules. Broadly, the molecules studied could be anabolic

growth factors (to fracture healing), bone turnover markers and molecular breakdown of tissue of nervous origin (such as brain). Overall, despite some evidence, the mechanism linking the molecules to the phenomenon of accelerated fracture healing remains inconclusive.

Table 1.5-1 Potential molecular candidates responsible for accelerated fracture healing post traumatic brain injury

	Candidate molecule	Source	Message
Growth Factors	Bone morphogenetic protein-2 (BMP-2) (Wang, Sun et al. 2011)	Serum	Patients with traumatic brain injury (TBI), with consequent heterotopic ossification (HO) had significantly higher levels in first post-injury month compared to those with TBI but no HO
	Basic fibroblast growth factor (bFGF) (Wildburger, Zarkovic et al. 1994; Wildburger, Zarkovic et al. 1995; Andermahr, Elsner et al. 2006)	Plasma (Andermahr, Elsner et al. 2006) Serum (Wildburger, Zarkovic et al. 1994; Wildburger, Zarkovic et al. 1995)	TBI associated with increased circulating levels, but additional presence of long bone fracture did not cause additional elevation of levels (Andermahr, Elsner et al. 2006). Serum levels in patients with TBI and enhanced osteogenesis were not significantly different to ones with normal healing (Wildburger, Zarkovic et al. 1995).
	Interleukin-6 (Beeton, Chatfield et al. 2004)	Serum	TBI and fracture patients had significantly elevated levels of IL-6 compared to fractures only (Beeton, Chatfield et al. 2004).
	Intact parathyroid hormone (Trentz, Handschin et al. 2005)	Serum	Levels significantly higher on days 0 and 1 in combined TBI and fracture patients (Trentz, Handschin et al. 2005)
	Activin A (Phillips, Nguyen et al. 2006)	Serum and CSF	Levels significantly higher in patients with TBI (with or without fracture) compared to healthy controls Levels correlate with surrogate markers of TBI severity (NSE, S-100β)
	Calcitonin (Trentz, Handschin et al. 2005)	Serum	Levels lower in first week post fracture in presence of TBI (Trentz, Handschin et al. 2005)
	Vascular endothelial growth factor (Gong, Hao et al. 2012)	Serum	Serum VEGF of patients with TBI significantly raised in the first 3 weeks post injury compared to healthy control (Gong, Hao et al. 2012)
	Matrix metalloproteinase-9 (Gong, Hao	Serum	Serum MMP-9 of patients with

	et al. 2012)		TBI significantly raised in the first 3 weeks post injury compared to healthy control (Gong, Hao et al. 2012)
Bone turnover markers	Bone specific alkaline phosphatase (B-ALP) (Wildburger, Zarkovic et al. 1994; Trentz, Handschin et al. 2005)	Serum	Combination of TBI and fracture led to significantly elevated serum levels compared to TBI or fracture only (Trentz, Handschin et al. 2005) Levels rose significantly in 2nd week after injury (Wildburger, Zarkovic et al. 1994)
	1-CTP (Trentz, Handschin et al. 2005) (pyridinoline cross-linked telopeptide parts of type-*I* collagen)	Serum	Levels significantly lower with TBI (Trentz, Handschin et al. 2005), reflecting lower osteoclastic activity
	PICP (Wildburger, Zarkovic et al. 1994; Trentz, Handschin et al. 2005) (Carboxy-terminal propeptide of procollagen type I)	Serum	Levels significantly lower with TBI (Trentz, Handschin et al. 2005) Significantly increased level found in 1st week after injury (Wildburger, Zarkovic et al. 1994)
	Osteocalcin (Trentz, Handschin et al. 2005)	Serum	Osteocalcin levels significantly lower in the presence of TBI (Trentz, Handschin et al. 2005).
CNS Breakdown product	PACAP (van Landeghem, Weiss et al. 2007) (pituitary adenylate cyclase activating polypeptide)	Brain tissue	Increased expression of PACAP by neurons and glial cells in pericontusional cortex area.
	Brain derived neurotrophic factor (BDNF) (Chiaretti, Piastra et al. 2003)	Plasma and CSF	Significant increase in CSF levels in children following TBI (Chiaretti, Piastra et al. 2003)
	Substance P (Zacest, Vink et al. 2010)	Brain tissue	Increased immunoreactivity to substance P in cerebral cortex following TBI (Zacest, Vink et al. 2010)
	Protein S-100B (Raabe and Seifert 2000; Romner, Ingebrigtsen et al. 2000; Pelinka, Kroepfl et al. 2004; Stranjalis, Korfias et al. 2004; Gautschi, Toffoli et al. 2007; Honda, Tsuruta et al. 2010; Vos, Jacobs et al. 2010; Bohmer, Oses et al. 2011; Metting, Wilczak et al. 2012)	CSF	Osteoinductive effect of CSF from TBI patient does not correlate with protein S100-B levels (Gautschi, Toffoli et al. 2007).
	Neuron specific enolase (NSE) (Kuroiwa, Tanabe et al. 1993; Yamazaki, Yada et al. 1995; Ross, Cunningham et al. 1996; Vos, Lamers et al. 2004; Honda, Tsuruta et al. 2010; Bohmer, Oses et al. 2011)	CSF (Ross, Cunningham et al. 1996) Serum (Yamazaki, Yada et al. 1995; Ross, Cunningham et al. 1996)	Correlation between serum and CSF levels in patients with major head injury. No correlation between serum levels and APACHE II, ISS, GCS (Ross, Cunningham et al. 1996). Serum levels in non-survivors (of TBI) significantly higher than

			survivors, as it may indicate quantity of brain damage (Yamazaki, Yada et al. 1995)
	Myelin basic protein (Yamazaki, Yada et al. 1995)	Serum (Yamazaki, Yada et al. 1995)	Admission serum levels in non-survivors (of TBI) significantly higher than survivors as it may indicate quantity of brain damage (Yamazaki, Yada et al. 1995)
	Cleaved tau protein (Shaw, Jauch et al. 2002)	Serum (Shaw, Jauch et al. 2002)	Detectable serum levels associated with significant brain injury, more likely to have poor outcome (Shaw, Jauch et al. 2002).
	Glial fibrillary acidic protein (Pelinka, Kroepfl et al. 2004; Vos, Lamers et al. 2004; Honda, Tsuruta et al. 2010; Vos, Jacobs et al. 2010; Bohmer, Oses et al. 2011; Metting, Wilczak et al. 2012)	Serum (Honda, Tsuruta et al. 2010)	Serum levels in first 3 days post injury significantly higher in TBI than in non-TBI (Honda, Tsuruta et al. 2010)

To summarise, bone turnover markers are by-products of bone metabolism (and the fracture healing process) (Mukhopadhyay, Sinha et al. 2011). Changes in levels of these molecules over time are a reflection of changes in the rate of bone metabolism. Hence, these molecules are not considered to directly affect the rate of fracture healing, but instead are a result of changes in rate of fracture healing. Therefore, the association between these molecules and increased osteogenicity observed (Table 1.5-1) does not indicate causation. Similarly, molecules of nervous tissue breakdown would be expected to be elevated following traumatic brain injury. These molecules are more a reflection of severity of brain injury, and levels of these molecules have not been found to directly correlate with rate of bone metabolism and fracture healing. Finally, with molecules from the growth factor categories, there was some evidence of elevated levels associated with traumatic brain injury. However, the levels of these molecules generally were not correlated with rate of fracture healing, compared to healthy controls only or may be secondary to the presence of concomitant injuries (polytrauma).

Therefore, ideally, to delineate the influence of traumatic brain injury on growth factors, patient cohorts required would be Traumatic Brain Injury with fractures, Polytrauma without TBI (to differentiate with additional presence of TBI), Single or Isolated fracture alone (to differentiate effect of multiple injuries to single) and Healthy Control (as baseline). These findings would then need to be correlated with patient outcome in the short term (complications, inflammatory response) and long term (fracture healing).

1.6. Introduction Summary

Thus the basis for this study has been delineated. Currently, despite the knowledge of the roles of various growth factors and cells (particularly MSCs) in the fracture healing process, the effect of different trauma severity and additional presence of traumatic brain injury on the dynamics of growth factors remain unknown. Moreover, the effect on trauma on the systemic mobilisation of MSCs both within the systemic bone marrow and also into the peripheral circulation remains to be explored.

2. Hypothesis

The study proposed in this work aimed to test the following hypotheses:

Hypothesis 1:	Traumatic injury upregulates anabolic growth factors (PDGF-AA, TGF-β2, angiogenin) and suppresses inhibitory factor (follistatin)
Hypothesis 2:	The selected growth factor dynamics is correlated with the inflammatory state of the patients
Hypothesis 3:	Trauma results in systemic mobilisation of MSCs
Hypothesis 4:	Stimulation of MSCs correlates with rise in anabolic growth factors and trauma severity

3. Aims

These hypotheses were tested using the following experimental approach.

Aim 1: To measure the serum levels of anabolic (PDGF-AA, TGF-β2, angiogenin) and inhibitory (follistatin) cytokines following fracture at 6 time points over a 14 day period. Enzyme linked immunoassays (ELISA) will be used to measure the concentrations of these molecules

Aim 2: To compare the dynamics of molecules measured in (Aim 1) with different trauma severity and the additional factor of traumatic head injury.

Both Aims 1 & 2 will be addressed in Chapter 4

Aim 3: To correlate the dynamics of molecules measured in (Aim 1) with the inflammatory state of the patient. Inflammatory state of the patient will be assessed by clinical score (Systemic Inflammatory Response Syndrome) and biochemical parameters.

This will be addressed in Chapter 5

Aim 4: To enumerate MSCs in bone marrow and peripheral circulation over time following trauma. CFU-F assays will be used to enumerate MSCs.

Aim 5: To correlate MSC mobilization with inflammatory response, and fracture healing outcome

Both Aims 4 & 5 will be addressed in Chapter 6

Aim 6: To correlate MSC mobilization with growth factor dynamics on time matched samples

This will be addressed in Chapter 7

4. RESULTS: Growth factor dynamics following trauma

4.1. Introduction

As discussed in the earlier section (General Introduction), fracture healing is a complex physiological process involving osteogenic cells, governed in time by molecular mediators known as growth factors (Dimitriou, Tsiridis et al. 2005).

It is currently understood that the severity of trauma influences the degree of inflammatory response as seen by the pattern of release (into peripheral circulation) of pro-inflammatory cytokines such as tumour necrosis factor (Hensler, Sauerland et al. 2002), interleukin-10(Hensler, Sauerland et al. 2002) and interleukin-6 (Gebhard, Pfetsch et al. 2000). As these pro-inflammatory cytokines have also been associated with the fracture healing process, it is reasonable to speculate on the influence of trauma severity on the kinetics of other growth factor release following fracture/trauma. Currently, the kinetics of release of molecular mediators exerting an effect on the fracture healing process is not well elucidated. Although it has been reported that certain growth factors showed an initial decline followed by gradual rise up to one week, this was limited to single long bone fractures only (Pountos, Georgouli et al. 2013). The effect of worsening trauma severity is still unclear. Moreover, the impact of traumatic head injury on the release of these autocoids remains obscure despite a reported enhanced osteogenic response (Newman, Stone et al. 1987; Perkins and Skirving 1987; Spencer 1987; Giannoudis, Mushtaq et al. 2006).

The 3 growth factors (PDGF-AA, angiogenin and TGF-β2) and 1 inhibitory molecule (follistatin) studied were chosen, as they are known to play an important role in the fracture healing process (Dimitriou, Tsiridis et al. 2005).

4.1.1. PDGF-AA

Platelet-derived growth factor (PDGF) was first identified as a cytokine derived from platelets, which allowed the growth of fibroblasts *in vitro* (Hannink and Donoghue 1989). It contains two different polypeptide chains, A and B, allowing the production of at least three different PDGF-related molecules (A-A, B-B and A-B dimers). PDGF exerts its action by stimulating the PDGF receptor tyrosine kinase, in which two types (alpha and beta) of receptors have been identified (Claesson-Welsh 1994). Specifically, these receptors undergo auto-phosphorylation which ultimately leads to activation of signal transduction, potentially affecting regulation of gene expression and the cell cycle (Claesson-Welsh 1994). PDGF is a major mitogen for MSCs and strongly induces the mitogenic and migratory response of MSCs (Mehrotra, Krane et al. 2004). Of the isoforms, PDGF-BB has been

shown to enhance fracture healing in animal models (Hollinger, Onikepe et al. 2008; Al-Zube, Breitbart et al. 2009). PDGF-AA isoform plays a role in osteoblast replication (Yang, Chen et al. 2000), but the dynamics of its expression *in vivo* following trauma is not well understood.

4.1.2. Angiogenin

Angiogenin is a protein that is found in human plasma (Shapiro, Strydom et al. 1987) and has been previously implicated in angiogenesis of tumour growth (Folkman, Merler et al. 1971). This molecule induces neovascularisation by augmenting the capacity of endothelial cells to digest extracellular matrix components and degrade basement membrane, thereby facilitating the process of endothelial cell invasion and angiogenesis (Hu, Riordan et al. 1994). Specifically, this formation of proteolytic complexes occurs via the binding of angiogenin to actin of endothelium and smooth muscle (Tello-Montoliu, Patel et al. 2006). Although the exact role of angiogenin in fracture healing remains unclear, the obligatory pre-condition of healthy tissue perfusion facilitated by new vessel formation would imply that angiogenin is an important component of the fracture healing process.

4.1.3. TGF-β2

Transforming growth factor-beta (TGF-β) is a cytokine that is part of the Transforming growth factor beta superfamily. It exists in three main isoforms (TGF-β1, TGF-β2 and TGF-β3), and have a variety of regulatory functions in the cell cycle. All three isoforms are secreted by osteoblasts and osteoclasts (Robey, Young et al. 1987) and have been shown to significantly enhance new bone formation in animal models(Mackie and Trechsel 1990). Immunohistochemistry analysis showed that TGF-β1 is found in the region of matrix synthesis and mineralization, whereas TGF-β2 is found in bone formation zones but within pre-osteoblastic cells only and not more differentiated osteoblasts (Toom, Arend et al. 2007). The TGF-β1 isoform has received most interest in its role in fracture healing. Following single long bone fracture, serum levels of TGF-β1 showed an initial decline, followed by a nearly 2-fold rise a week later (Pountos, Georgouli et al. 2013). The serum level of TGF-β1 has been shown to be significantly lower in cases of fracture non-union (Sarahrudi, Thomas et al. 2011) as well as delayed union (Zimmermann, Moghaddam et al. 2007). To date, no studies have looked at the dynamics of the TGF-β2 following fractures and trauma in humans.

4.1.4. Follistatin

Follistatin is an inhibitory protein known primarily for its role in the inhibition of activin activity (part of the TGF-beta superfamily) (Nagamine, Imamura et al. 1998). It acts to block cell signalling by antagonising both type 1 and type 2 receptor binding (Thompson, Lerch et al. 2005). The osteogenic role of activin has been previously shown in both *in vitro* (Gaddy-Kurten, Coker et al. 2002) and *in vivo* studies (Sakai, Miwa et al. 1999). Additionally, follistatin has been shown to have inhibitory effects on various other molecules of the BMP family (Fainsod, Deissler et al. 1997; Otsuka, Moore et al. 2001; Amthor, Christ et al. 2002). Similar to TGF-β2, its dynamics in peripheral circulation following fracture is unknown.

4.2. Methods

4.2.1. Patient Recruitment

Patients were recruited for serum growth factor measurements. Approval from local ethics committee (*Leeds West REC 06/Q1206/127*) was obtained prior to commencement, with all patients providing written informed consent.

To address the role of trauma severity and the additional presence of traumatic head injury on the pattern of growth factor release into the peripheral circulation, patients participating in this study were further divided into 3 groups, Group 1 polytrauma without head injury (ISS≥16, GCS>12), Group 2 polytrauma with head injury (ISS≥16, GCS<12) and Group 3 isolated upper/lower extremity fracture with no head injury (ISS<16, GCS≥12). To simplify description, Group 1 will be referred to as Polytrauma, Group 2 as Head Injury and Group 3 as Isolated Trauma. In addition, healthy volunteers were recruited to form the Control Group, to inform the baseline (non-trauma) levels of the growth factors. The Control Group was selected following recruitment of the trauma group, to allow for appropriate selection to match the age and sex distribution of the trauma group.

Table 4.2-1 Growth Factor Study: Polytrauma Group (n=15)

PATIENT ID	SEX	AGE	ISS	GCS
NT1	M	58	16	14
DL	M	19	32	14
BP2	M	27	25	15
BP4	M	52	40	14
BP6	F	25	27	15
JM2	M	54	29	15
BP15	M	49	24	15
BP16	M	21	24	14
BP12	F	24	29	15
BP18	F	45	38	12
BP19	M	53	24	15

BP20	F	41	34	15
BP27	M	28	27	15
BP28	M	51	24	15
BP31	M	38	27	15
	11M:4F	Median age: 41 Mean age: 39	Median ISS: 27 Mean ISS: 28	Median GCS:15 Mean GCS:14.5

ISS=Injury Severity Score, GCS=Glasgow Coma Scale. Patients in the Polytrauma Group has ISS≥16, GCS>12

Table 4.2-2 Growth Factor Study: Head Injury Group (n=14)

PATIENT ID	SEX	AGE	ISS	GCS
BH1	F	51	18	3
BH3	F	60	27	7
BH4	F	24	27	3
BH6	M	28	29	4
BH8	M	20	27	4
BH9	M	50	27	4
BH10	M	66	16	3
BH11	F	44	43	3
BH13	M	29	25	11
BH16	M	22	27	3
BH17	M	21	50	3
BH18	M	41	29	3
BH20	M	19	20	4
BH21	M	49	34	6
	10M:4F	Median age: 35	Median ISS: 27	Median GCS: 3.5

		Mean age: 37.4	Mean ISS: 28.5	Mean GCS: 4.4

ISS=Injury Severity Score, GCS=Glasgow Coma Scale. Patients in the Head Injury Group has ISS≥16, GCS<12

Table 4.2-3 Growth Factor Study: Isolated Trauma Group (n=15)

PATIENT ID	SEX	AGE	ISS	GCS
BP3	M	25	9	15
BP7	M	53	9	15
BP9	M	21	9	15
BP8	M	63	4	15
BP10	M	22	4	15
JM8	M	21	9	15
JM12	F	18	9	15
JM16	M	35	9	15
BP11	M	45	4	15
BP13	M	51	4	15
BP14	F	32	9	15
BP17	M	38	8	15
BP23	M	31	10	15
BP22	M	20	10	15
BP24	F	19	10	15
	12M:3F	Median age: 31 Mean age: 32.9	Median ISS: 9 Mean ISS: 7.8	Median GCS: 15 Mean GCS: 15

ISS=Injury Severity Score, GCS=Glasgow Coma Scale. Patients in the Isolated Trauma Group has ISS<16, GCS>12

Table 4.2-4 Growth Factor Study: Healthy volunteers (control) (n=9)

PATIENT ID	SEX	AGE	ISS	GCS
BP29	F	19	N/A	N/A
BP30	F	52	N/A	N/A
BP32	M	40	N/A	N/A
CM1	M	63	N/A	N/A
CM2	M	22	N/A	N/A
CM3	M	29	N/A	N/A
CF1	F	63	N/A	N/A
CF2	F	35	N/A	N/A
CF3	F	21	N/A	N/A
	4M:5F	Median age: 35 Mean age: 38.2	N/A	N/A

Overall, all the 3 trauma groups are similarly sized in terms of number of patients recruited. All 4 groups (Polytrauma, Head Injury, Isolated Trauma and Healthy Control) were essentially age (p=0.65, *Kruskal-Wallis*) and sex (p=0.31, *Chi-square*) matched.

4.2.2. Sampling time points

Patient samples were obtained on admission and days 1, 3, 5, 7 and 14 following injury. These sampling time points were chosen to best elucidate the dynamics of the growth factor in circulation as the fracture healing process progresses from the initial inflammatory phase (days 0-3) to a reparative or regenerative phase (days 12-14) (McKibbin 1978).

4.2.3. Specimen acquisition, preparation and storage

Peripheral blood samples (up to 20 ml) were collected in Serum Separator Tubes (BD Vacutainer®, Oxford, England) and allowed to stand for at least 30 minutes, to ensure that they are fully clotted prior to centrifugation at 2000g for 15 minutes. Serum obtained was aliquoted into 1ml cryotubes (1ml each, Sarstedt AG, Leicester, England), labelled appropriately and stored in a -80°C freezer prior to growth factor measurements.

4.2.4. Enzyme-linked immuno sorbent assay (ELISA) analysis

ELISA analysis was carried out with commercially available kits (Quantikine® ELISA kit, R&D Systems, Minneapolis, USA), employing the quantitative sandwich enzyme immunoassay technique. Briefly, a monoclonal antibody specific for the molecule (PDGF-AA, TGF-β2, follistatin or angiogenin) has been pre-coated onto a micro plate. Standards or samples were then pipetted into the wells where molecules of interest were bound by relevant immobilized antibodies. After washing away any unbound substances, a further enzyme-linked polyclonal antibody specific for the study molecule (Conjugate) was then added to the well. Following further wash to remove any unbound antibody-Conjugate, a Colour reagent (Substrate solution) was then added to the wells and colour would then develop in proportion to the amount of study molecule bound. The colour development was then stopped by adding 2N sulphuric acid before the colour intensity is read by Plate Reader (Multiskan Ascent, Thermo Electron Corporation, Langenselbold, Germany). Figure 4.2-1 illustrates the steps described. However, as each molecule has slight variations in protocol in terms of time/temperature/dilution/sample activations, ELISA protocols for each of the four molecules are described separately. Table 4.2-5 summarises the differences in protocol for 4 molecules studied.

Table 4.2-5 Summary of ELISA protocol variations

	PDGF-AA	**TGF-β2**	**ANGIOGENIN**	**FOLLISTATIN**
Sample preparation	10-fold dilution	Activation with HCl followed by neutralisation with NaOH/HEPES	200-fold dilution	No prior preparation
1st incubation period	2 hours RT	2 hours RT	1 hour RT	2 hours (2-8°C)
Wash after 1st incubation	4 times	3 times	3 times	4 times
2nd incubation period (after addition of Conjugate)	2 hours RT	2 hours RT	1 hour RT	2 hours (2-8°C)
Wash after 2nd incubation	4 times	3 times	3 times	4 times
3rd incubation period (after addition of Substrate)	30 minutes RT	20 minutes RT	20 minutes RT	30 minutes RT

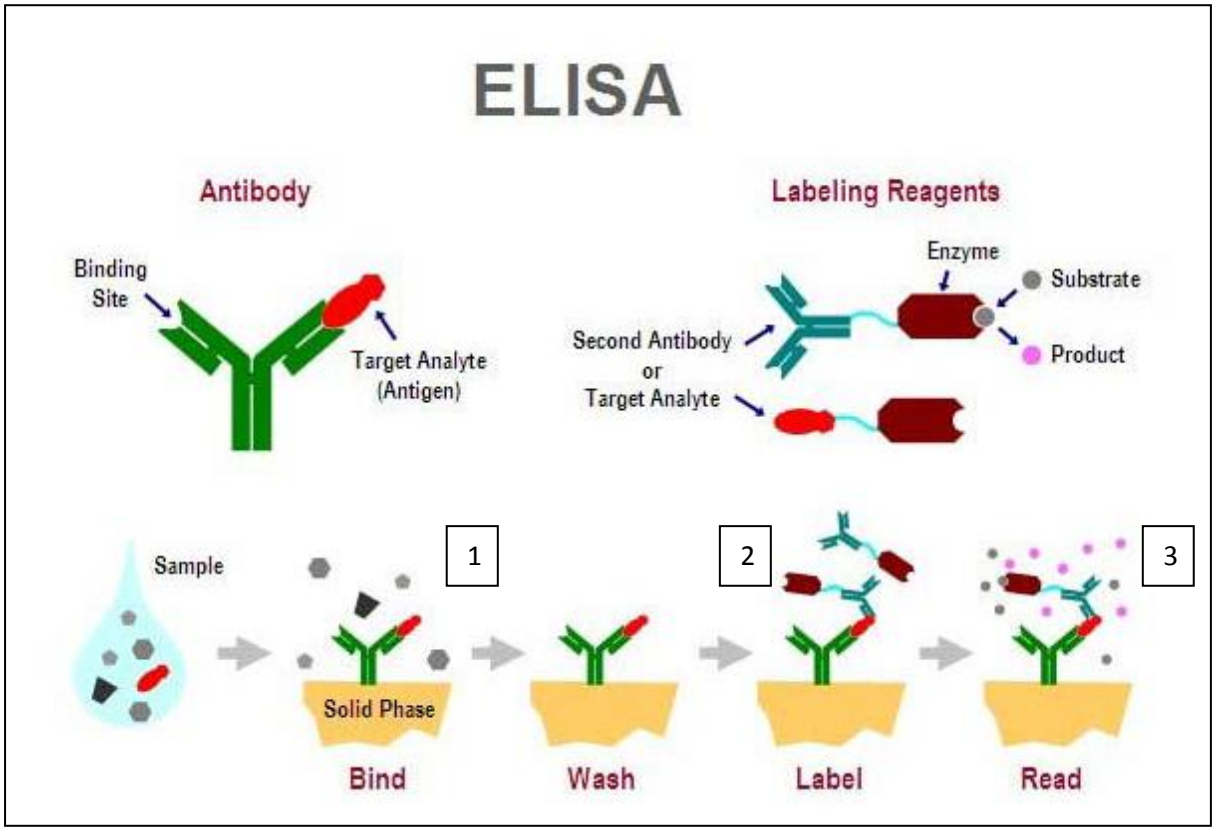

Figure 4.2-1 Double-Sandwich ELISA. Plate is pre-coated with a capture antibody; (1) sample is added, and any antigen present binds to capture antibody; (2) enzyme-linked secondary antibody is added, and binds to captured antigen; (3) substrate is added, and is converted by enzyme to detectable form. Adapted from BioSystem® Development Website (biosystemdevelopment.com)

4.2.4.1. ELISA protocol for measuring PDGF-AA

Samples were thawed at room temperature (RT) and ensured to be thoroughly mixed in a roller. Reagent preparation was carried out per manufacturer's instructions. In brief, samples were diluted 10-fold as recommended by the manufacturer to allow samples to fall within sensitivity of calibration curve. 50µL of Standard or sample was added to 100µL of Assay Diluent (containing buffered protein base) per well, covered and incubated for 2 hours at RT. Standard was prepared earlier from recombinant human PDGF-AA as per manufacturer's manual, and serial dilutions carried out to allow for standard range from 1000pg/ml to 0 pg/ml. Each well was next aspirated and washed with Wash Buffer for a total of 4 times. The Wash buffer is a solution of buffered surfactant, and this step was done to wash away any unbound substances. 150µL of Human PDGF-AA Conjugate (containing monoclonal antibody against PDGF-AA) was added to each well, covered and incubated for further 2 hours at RT. PDGF-AA Conjugate was next aspirated and washing steps were repeated 4 times, to ensure removal of any unbound antibody-Conjugate. At the final stage, 200µL

of Substrate Solution was added to each well, covered (kept from light) and incubated for 30 minutes at room temperature. Substrate Solution contains stabilized hydrogen peroxide and stabilized chromogen (tetramethylbenzidine) which reacts with bound antibody-Conjugate giving an initial blue colour. Finally, 50µL of Stop solution (containing sulphuric acid) was added to arrest further activation of Substrate solution, changing the colour in the wells from blue to yellow. This yellow colour allows reading to be performed using 450 and 570 nm optical filters.

4.2.4.2. ELISA Steps for TGF-β2

Samples were thawed at RT and ensured to be thoroughly mixed in a roller. In serum, TGF-β2 exists in its latent form, and therefore requires the acid activation and neutralization to convert into its immuno-reactive form. Samples are activated by adding 125µL of sample to 25µL 1N HCl, mixed and incubated at RT for 10 minutes. Then, 25µL of 1.2N NaOH/0.5M HEPES was then added and mixed. Finally 800µL Calibrator Diluent (containing buffered protein base) was added to each sample, giving a dilution factor of 7.8. 100µL of Standard (range 0-2000 pg/ml recombinant human TGF-β2) or sample was added to 100µL of Assay Diluent per well, covered and incubated for 2 hours at RT. Each well was aspirated and washed with Wash Buffer for a total of 3 times. As for all the other cytokines, the Wash Buffer is a solution of buffered surfactant, and this step was done to wash away any unbound substances. 200µL of TGF-β2 Conjugate (containing polyclonal antibody against TGF-β2) was then added to each well and incubated for further 2 hours at RT. TGF-β2 Conjugate was next aspirated and washing steps were repeated 3 times, to ensure removal of any unbound antibody-Conjugate. 200µL of Substrate Solution (the same as for all the other cytokines) was added to each well, covered (kept from light) and incubated for 20 minutes at RT. Finally, 50µL of Stop solution was added and reading was performed using 450 and 570nm optical filters.

4.2.4.3. ELISA Steps for Angiogenin

Samples were thawed at RT and mixed. Samples were diluted 200-fold as per manufacturer's manual to allow measurements to fall within sensitivity of the calibration curve. 200µL of Standard (range 0-5000pg/ml) or sample was added to 50µL of Assay Diluent per well, covered and incubated for 1 hour at RT. Each well was aspirated and washed with Wash Buffer for a total of 3 times to wash away any unbound substances. 200µL of ANG Conjugate (containing polyclonal antibody against angiogenin) was added to each well, covered and incubated for further 1 hour at RT. Angiogenin Conjugate was next aspirated and washing steps were repeated 3 times, to ensure removal of any unbound antibody-Conjugate. 200µL of Substrate Solution was added to each well, covered and

incubated for 20 minutes at RT. Finally, 50µL of Stop solution was added and reading was performed using 450 and 570 nm filters.

4.2.4.4. ELISA Steps for Follistatin

Samples were thawed at RT and mixed. 100µL of Standard (range 0-16,000 pg/ml) or sample was added to 100µL of Assay Diluent per well, covered and incubated for 2 hours at 2-8°C. Each well was aspirated and washed with Wash Buffer for a total of 4 times to wash away any unbound substances. 200µL of cold Follistatin Conjugate (containing monoclonal antibody against Follistatin) was added to each well, covered and incubated for further 2 hours at 2-8°C. Follistatin Conjugate was next aspirated and washing steps were repeated 4 times. Then, 200µL of Substrate Solution was added to each well, covered and incubated for 30 minutes at RT. Finally, 50µL of Stop solution was added and plated were read using 450 and 570 nm filters.

4.2.4.5. Plate reading and data Analysis for ELISA

For all plates, Optical density (OD) was read within 30 minutes after the addition of Stop Solution, with a microplate reader (running Ascent Software for Multiskan Version 2.6) set to 450nm with wavelength correction at 570nm (latter to correct for contribution by the plastic well, the lamp and optical fluctuations). Read-out was transferred to Microsoft Excel file format for analysis. The duplicate readings at 450nm were averaged and subtracted to the averaged correction wavelength reading OD at 570nm, obtaining delta absorbance. Linearized standard curve was created by plotting the log mean delta absorbance (OD) for each standard on the y-axis against the log concentration (pg/mL) on the x-axis, and drawing a best-fit line. R-value and conversion formula was then obtained. Conversion formula was then applied to the data, taking into account the dilution factors for each individual ELISA molecule. Graphs are generated using GraphPad Prism v4.00 for Windows (San Diego California, USA).

4.2.5. Intravenous fluid

To investigate the influence of initial intravenous fluid resuscitation volume on the concentration of growth factors in peripheral circulation, the total fluid volume transfused over the first 24 hours of admission was recorded from the patients' medical records.

4.2.6. Statistics

Statistical analysis was carried out using PASW Statistics (SPSS) version 17.0.2 and graphing performed using Graph Pad Prism version 4.00 for Windows (San Diego, California, USA). As Gaussian distribution could not be assumed given the small sample size, non-parametric tests were carried out. Mann-Whitney test was used to compare differences between two independent samples. Wilcoxon signed-rank test was used to compare two dependent samples (same patient, two different time points). Chi-square test was used for comparison of nominal data (for example comparing sex distribution between groups). Variability of results was tested by testing the coefficient of variation (ratio of standard deviation to the mean). Pearson's correlation coefficient was used to assess the strength of linear dependence between two variables. Statistical significance is assumed at $p<0.05$.

4.3. Results

4.3.1. Validation of ELISA assay

Standard curves for each cytokine were first assessed for linearity and compared to sensitivity ranges provided by the manufacturer. Commonly, the relationships between optical density (OD) and concentration are not linear. Therefore, standard curves were linearized by taking $\log_{10}$OD versus $\log_{10}$concentration and regression analysis done to obtain the formula for the conversion of OD to growth factor levels. Representative experiments are shown on Figure 4.3-1, Figure 4.3-2, Figure 4.3-3 and Figure 4.3-4.

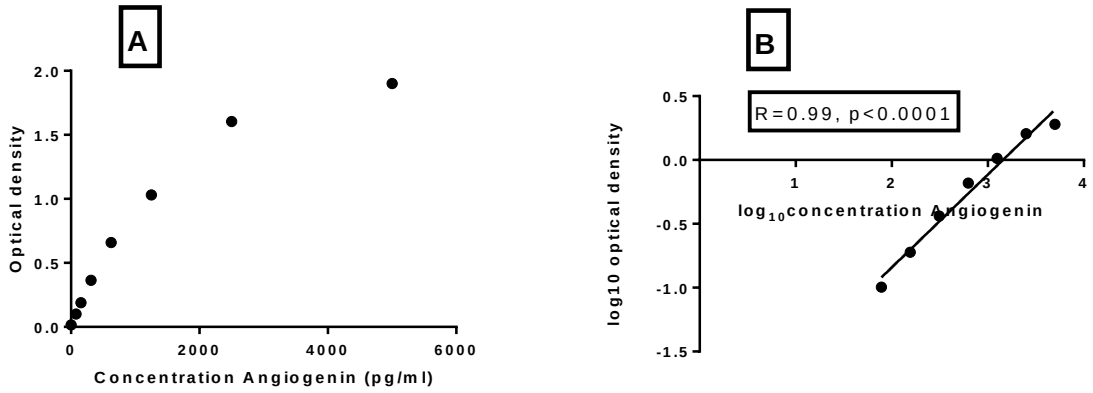

Figure 4.3-1 Sample calibration curve for ELISA study (Angiogenin). The standard curve in between optical density and angiogenin concentration (A) was linearized by applying logarithmic transformation (B) (R=+0.99 and p<0.0001)

Figure 4.3-1 shows the calibration curve (A) and linearized relationship (B) for angiogenin. The OD reading range for the known calibration angiogenin concentrations is 0.017 to 1.9, which is within the sample range, where minimum OD reading is 0.58 and maximum 1.77. According to manufacturer's manual, the minimum detectable dose is 6pg/ml, which would correspond to OD of 0.017.

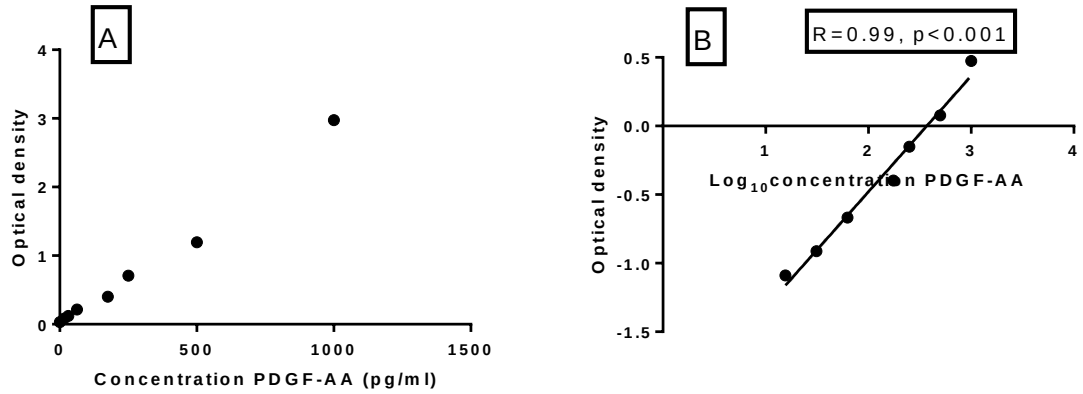

Figure 4.3-2 Sample calibration curve for ELISA study (PDGF-AA). The standard curve in between optical density and PDGF-AA concentration (A) was linearized by applying logarithmic transformation (B) (R=+0.99 and p<0.001)

Similarly, Figure 4.3-2 shows the calibration curve (A) and linearized relationship (B) between OD and PDGF-AA. The OD reading range for the known calibration PDGF-AA concentrations is 0.033 to 2.977, which is within the sample range, where minimum OD reading is 0.033 and maximum 1.115. According to manufacturer's manual, the minimum detectable dose is 2.29pg/ml, which would correspond to OD of 0.013.

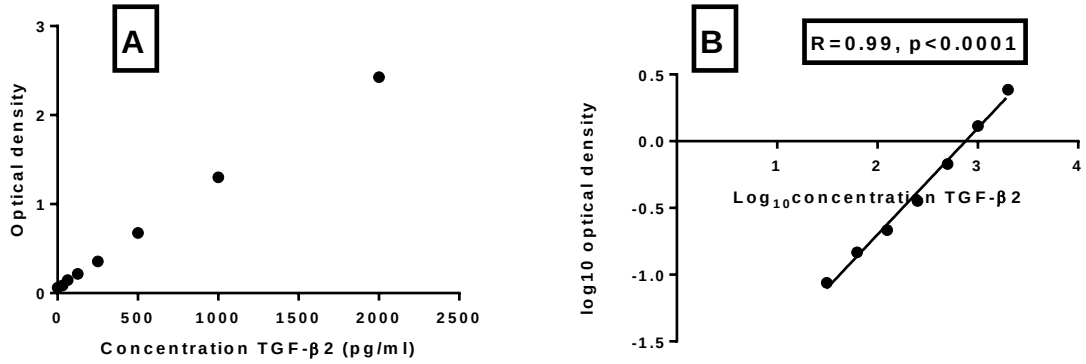

Figure 4.3-3 Sample calibration curve for ELISA study (TGF-β2). The standard curve in between optical density and TGF-β2 concentration (A) was linearized by applying logarithmic transformation (B) (R=+0.99 and p<0.0001)

As per the two earlier figures, Figure 4.3-3 illustrates sample calibration curve for TGF-β2, with (A) showing OD versus known concentration of TGF-β2, and (B) showing the linearized relationship by taking log10 on both x and y-axis. The OD reading range for the known calibration TGF-β2 concentrations is 0.062 to 2.427, which is within the sample range, where minimum OD reading is

0.066 and maximum 0.149. According to manufacturer's manual, the minimum detectable dose is 7.0pg/ml, which would correspond to OD of 0.023.

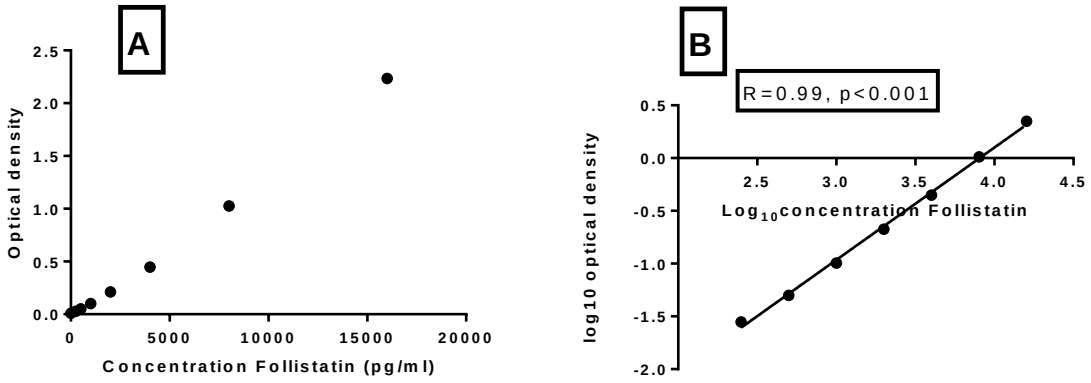

Figure 4.3-4 Sample calibration curve for ELISA study (Follistatin). The standard curve in between optical density and follistatin concentration (A) was linearized by applying logarithmic transformation (B) (R=+0.99 and p<0.001)

Finally, Figure 4.3-4 shows (A) OD against follistatin concentration, which linearity is improved by taking log10 (OD) against log10 (follistatin concentration) (B). The OD reading range for the known calibration follistatin concentration is 0.0095 to 2.2345, which is within the sample range where minimum OD reading is 0.0695 and maximum reading of 0.7035. According to manufacturer's manual, the minimum detectable dose is 29pg/ml, which would correspond to OD of 0.0025.

4.3.2. Inter-individual variability (coefficient of variation)

The coefficient of variation (CV) was first used to investigate patient-to-patient differences. CV is defined as the ratio of the standard deviation (SD) to the mean, which is then multiplied by 100 to allow expression as a percentage. The analysis is done separated into the 4 individual cytokines measured, and the three separate patient groups, to allow different injury types for the possible variations in CV.

Table 4.3-1 summarises the CV for all the growth factor trauma groups and Table 4.3-2 for the Control groups. The following tables (in Appendix) illustrates the individual results for admission and days 1, 3, 5, 7 and 14 following injury/admission for growth factors PDGF-AA (Table 10.1-1), Angiogenin (Table 10.1-2), Follistatin (Table 10.1-3) and TGF-β2 (Table 10.1-4).

Table 4.3-1 Collated Coefficient of variation (%)

	TRAUMA GROUPS	Day 0	Day 1	Day 3	Day 5	Day 7	Day 14
PDGF-AA	Head Injury	57.2	47.4	60.2	39.4	43.7	60.2
	Isolated Trauma	25.3	32.4	30.5	24.3	21.6	51.3
	Polytrauma	59.1	33.0	45.8	44.6	27.1	62.2
ANGIOGENIN	Head Injury	40.3	35.7	39.3	36.2	34.4	57.8
	Isolated Trauma	23.9	26.3	18.6	29.0	22.9	18.5
	Polytrauma	23.3	27.2	24.0	28.6	31.6	31.4
FOLLISTATIN	Head Injury	49.7	41.3	91.2	78.9	59.8	36.9
	Isolated Trauma	33.6	54.6	71.4	64.6	57.6	36.8
	Polytrauma	57.6	39.7	45.3	79.4	37.2	56.3
TGF-β2	Head Injury	43.0	38.5	45.8	43.3	45.2	34.3
	Isolated Trauma	104.0	106.7	90.8	81.3	121.4	62.7
	Polytrauma	136.4	142.3	215.5	158.2	149.8	139.3

Table 4.3-2 Inter-individual variations in control groups

	Mean (pg/ml)	Standard Deviation	Coefficient of variation (%)
PDGF-AA	4108.04	943.8	23.0
Angiogenin	419035.2	83421.3	19.9
Follistatin	1832.8	1211.3	66.1
TGF-β2	1383.0	1433.4	103.6

Manufacturer's range (R&D Systems):

[PDGF-AA (2156 – 5818 pg/ml), Angiogenin (196,000 - 437,000 pg/ml), Follistatin (889 – 11123 pg/ml),

TGF-β2 (not detectable – 873 pg/ml)]

Analysis of growth factor results on admission, angiogenin appeared to have the lowest CV (23.3-40.3%) for the three groups of patients, whereas TGF-β2 had the highest (43-136.4%). Over time (Day 7), the CV for all molecules stayed fairly consistent; with molecules/patient groups with low CVs remaining low and high CVs remaining high.

This variability showed similar trends with samples from patients in the control groups. The mean values for all 4 cytokines in the control groups were within the manufacturer's range.

4.3.3. Growth factor dynamics over time

To allow for comparison of the trends in growth factor dynamics over time from injury, all 4 growth factors, were normalized to Day 1=100, to allow for comparisons of trends in growth factor levels over time from injury, regardless of the variability of individual baseline levels, as shown in Table 4.3-1. These comparisons of trends over time within individual trauma groups for all 4 growth factors are as illustrated in Figure 4.3-5.

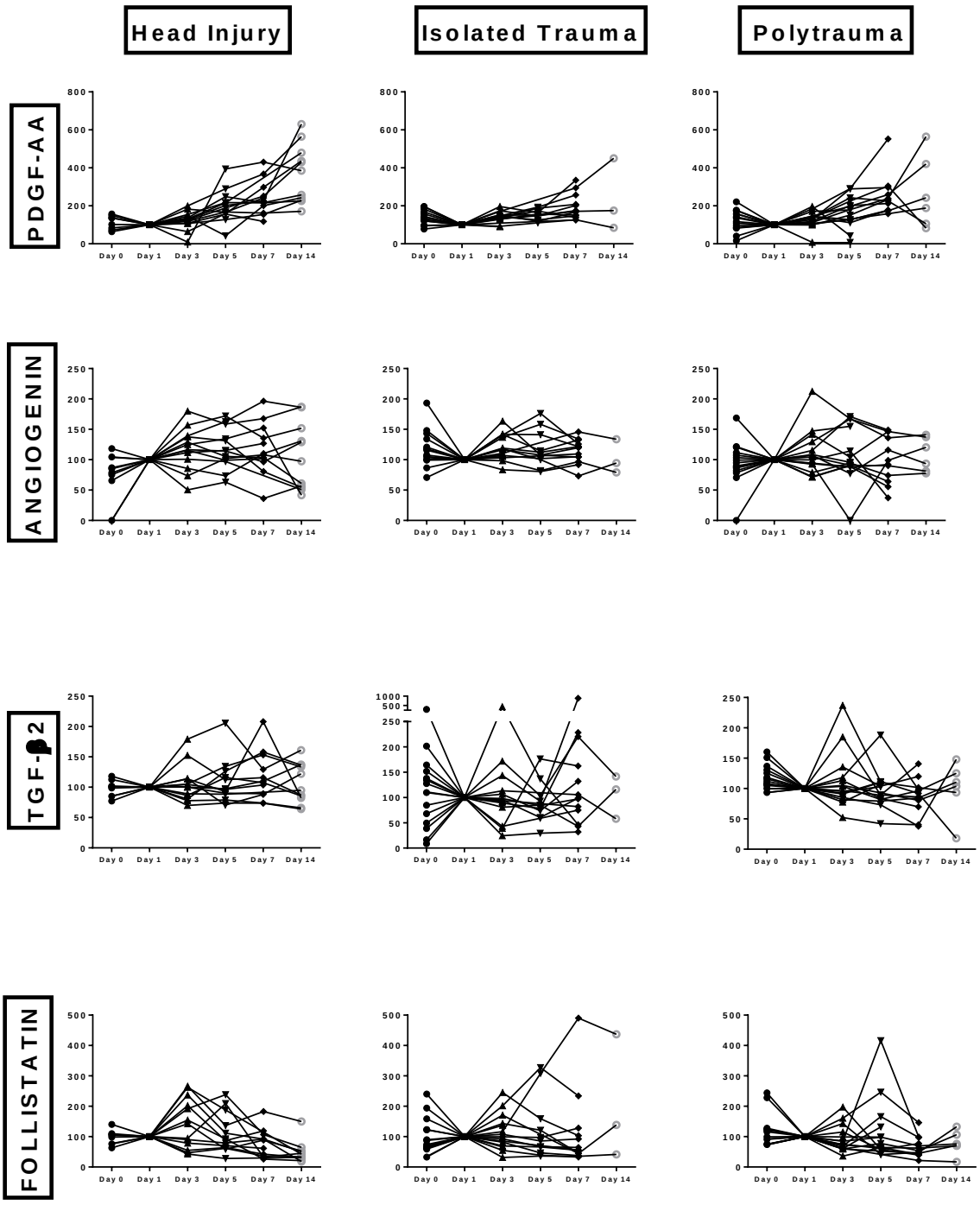

Figure 4.3-5 Individual ELISA results normalized to Day 1. Y-axis represents normalized value to Day 1=100%. Data normalized to Day 1, as not all patients have Day0 samples obtained.

PDGF-AA

Over the period of study, the serum levels of PDGF-AA show an increasing trend over time. Compared to Day 1, these changes were statistically significant ($p<0.05$) across most groups and most time points as shown in Table 4.3-3 below (individual data shown in Appendix Section, Table 10.1-1). The changes were most pronounced in the Head Injury group, with an average 2.4-fold by Day 7 and 3.7-fold by Day 14 compared to Day 1 levels. Similarly, the Isolated Trauma group showed an almost 2-fold increase by Day 7. And finally, the Polytrauma group exhibited a rise of 2.5 and 2.7 fold over Day 1 levels on Days 7 and 14 respectively.

Table 4.3-3 PDGF-AA changes compared to Day 1

	Day0 vs Day1	Day1 vs Day3	Day1 vs Day5	Day1 vs Day7	Day1 vs Day14
Head Injury	p=0.26	p=0.06	p<0.05*	p<0.05*	p<0.05*
Isolated Trauma	p<0.05*	p<0.05*	p<0.05*	p<0.05*	p=0.29
Polytrauma	p=0.43	p<0.05*	p<0.05*	p<0.05*	p=0.08

Wilcoxon, p<0.05 denotes statistical significance. Analysis was carried out against Day 1, as not all patients have Day0 samples obtained.

Angiogenin

The chronological trend for angiogenin serum level was rather muted. As shown in Table 4.3-4 below (individual data shown in Appendix Section Table 10.1-2), over the observed time period, the Head Injury showed only a 1.1 fold increase on Day 7 compared to Day 1, but failed to reach statistical significance. Interestingly, within the Isolated Trauma group, although the average increase was only 1.1 fold by Day 7, this trend was consistent enough to reach statistical significance. The Polytrauma group on average was relatively unchanged over time.

Table 4.3-4 Angiogenin changes compared to Day 1

	Day0 vs Day1	Day1 vs Day3	Day1 vs Day5	Day1 vs Day7	Day1 vs Day14
Head Injury	p=0.16	p=0.09	p=0.09	p=0.12	p=0.93
Isolated Trauma	p<0.05*	p<0.05*	p=0.11	p<0.05*	p=1.00
Polytrauma	p=0.78	p=0.36	p=0.64	p=0.94	p=0.46

Wilcoxon, p<0.05 denotes statistical significance

Follistatin

Follistatin showed a decreasing trend over time across all trauma groups. This reached statistical significance by Day 7 for the Polytrauma (average 70% of Day 1 value)) and Head Injury group by Day 14 (average 79% of Day 1 value), as shown in Table 4.3-5 (individual data shown in Appendix Section Table 10.1-3). The presence of an extreme outlier on Day 7 for the Isolated Trauma group probably caused the changes for this group to remain not significant (p=0.25), unlike the other 2 groups.

Table 4.3-5 Follistatin changes compared to Day 1

	Day0 vs Day1	Day1 vs Day3	Day1 vs Day5	Day1 vs Day7	Day1 vs Day14
Head Injury	p=0.89	p=0.15	p=0.97	p=0.10	p<0.05*
Isolated Trauma	p=0.46	p=0.51	p=0.86	p=0.25	p=0.59
Polytrauma	p=0.87	p=0.36	p=0.39	p<0.05*	p<0.05*

Wilcoxon, p<0.05 denotes statistical significance

TGF-β2

Finally, the serum levels of TGF-β2 did not show any significant (p>0.05) changes, apart for Polytrauma Day 0 to Day 1 comparison. The dynamics within the group displayed considerable variability, especially marked in the Isolated Trauma group. As previously shown in Table 4.3-1 (individual data shown in Appendix Section Table 10.1-4), this molecule exhibited high variability (CV range 38.5-149.8%). Altogether these data showed highly variable TGF-β2 levels in all patient groups and at all the time points studied.

Table 4.3-6 TGF-β2 changes compared to Day 1

	Day0 vs Day1	Day1 vs Day3	Day1 vs Day5	Day1 vs Day7	Day1 vs Day14
Head Injury	p=0.67	p=0.86	p=0.86	p=0.24	p=0.48
Isolated Trauma	p=0.61	p=0.64	p=0.21	p=0.55	p=0.47
Polytrauma	p<0.05*	p=0.91	p=0.36	p=0.12	p=0.60

Wilcoxon, p<0.05 denotes statistical significance

4.3.4. Influence of trauma severity and head injury

In order to analyse the influence of trauma severity and the additional presence of traumatic head injury on the dynamics and pattern of growth factor release, the growth factors are analysed individually, and comparisons are made at identical time points (admission, days 1, 3, 5, 7 and 14 post injury) across the different trauma groups (Head Injury, Isolated trauma, Polytrauma) and Healthy Control group.

PDGF-AA

The analysis of PDGF-AA levels across the trauma groups are shown in Figure 4.3-6 and further detailed in Table 10.1-5. This showed an initial statistically significant ($p<0.05$) suppression across all 3 trauma groups compared to the control levels. Across the trauma groups, both Head Injury (1740 pg/ml) and Polytrauma (2593 pg/ml) groups had statistically ($p<0.05$) more pronounced suppression compared to Isolated Trauma Group (3491 pg/ml). The levels in all trauma groups then started to rise over time, but remains statistically suppressed compared to controls up to Day 3. By Day 7, the levels across all groups have risen, and in the Polytrauma and Isolated Trauma groups, levels were higher than control. By Day 14, levels of PDGF-AA across all trauma groups were higher than control, although this rise did not reach statistical significance compared to control. I would conclude from the figure that the levels were suppressed post trauma, in correlation with trauma severity, and by the end of 2 weeks reached the control levels.

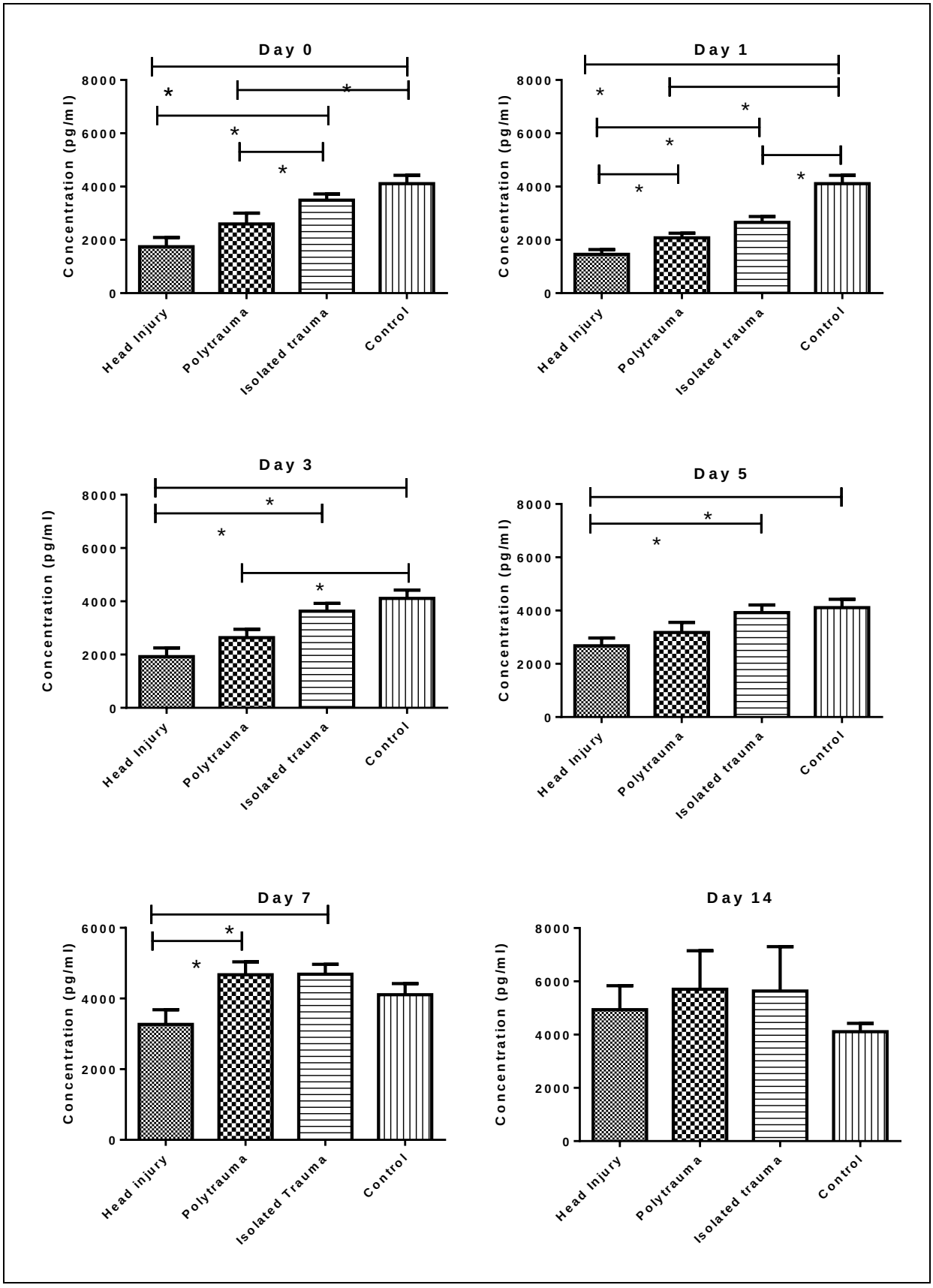

Figure 4.3-6 Comparison of PDGF-AA levels across trauma groups (*denotes statistically significant, p<0.05, Mann-Whitney) over 6 intervals between Day 0 and 14. There was an initial suppression of PDGF-AA levels rising to levels of control by Day 7.

Angiogenin

On admission, the serum levels of angiogenin were suppressed in both the Head Injury (359751 pg/ml) and Polytrauma (343304 pg/ml) group (Figure 4.3-7) compared to Control (419035 pg/ml) and Isolated Trauma (435025 pg/ml) groups, although they were only statistically significant ($p<0.05$) in the Polytrauma Group. While the levels in the Polytrauma group appeared to increase over time, by Day 7 post injury, the levels has reduced significantly ($p<0.05$) compared to all the other trauma groups (Head Injury and Isolated Trauma) as well as Control. By the end of the second post injury week, this group remained depressed, with the levels from the Head Injury group similarly depressed. Interestingly, the Isolated Trauma group appeared to have increased above the Control levels, but failed to reach statistical significance ($p=0.76$). Overall the levels are less variable over time across groups and compared to controls compared to PDGF-AA.

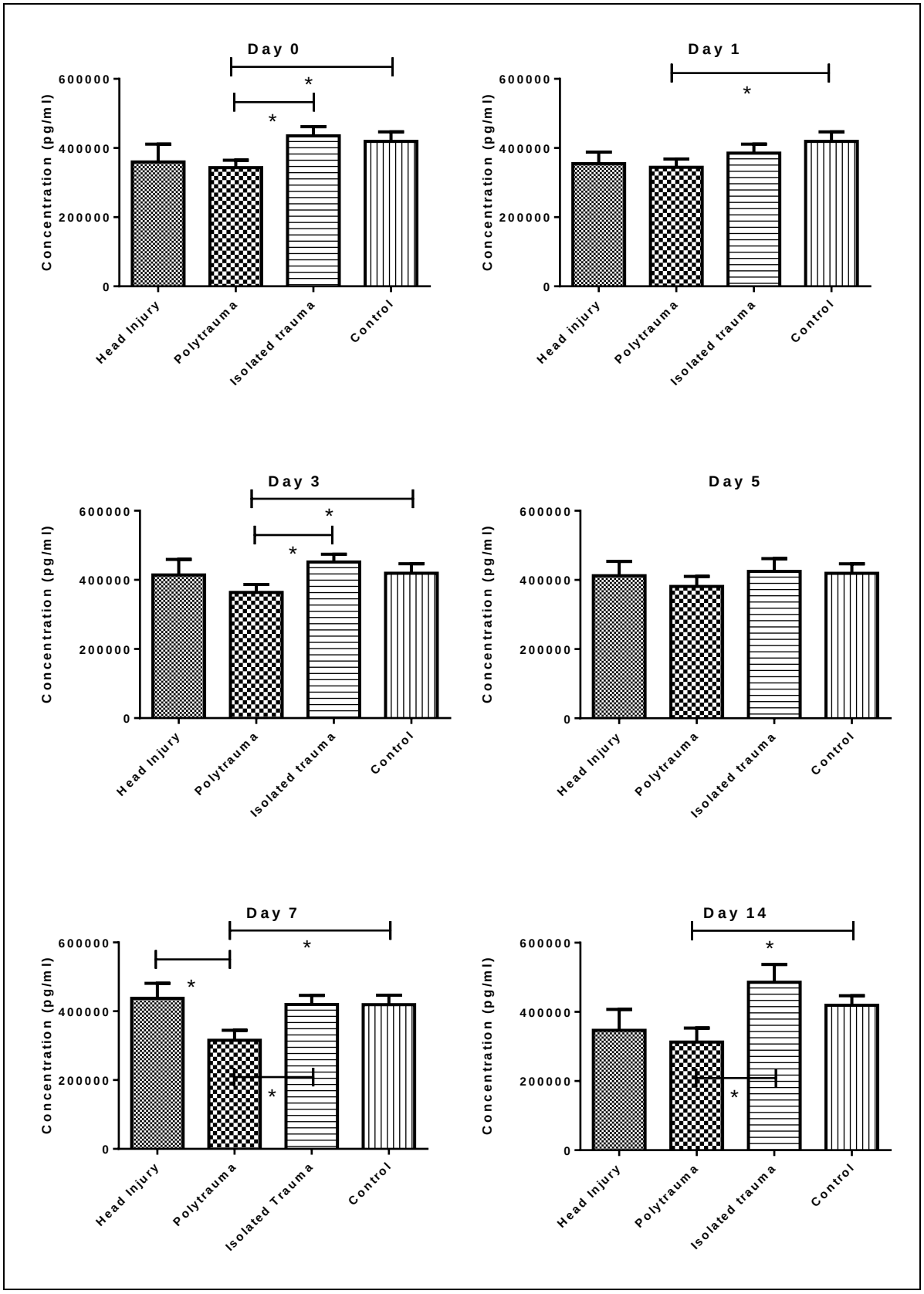

Figure 4.3-7 Comparison of Angiogenin levels across trauma groups (*denotes statistically significant, p<0.05, Mann-Whitney) over 6 intervals between Day 0 and 14. There was an initial suppression of angiogenin levels in Polytrauma and Head Injury group rising to levels of control by Day 5.

Follistatin

Serum follistatin levels was initially elevated post injury, with the more severely injured groups [Head Injury (2888 pg/ml) and Polytrauma (2723 pg/ml)] exhibiting statistically significant ($p<0.05$) higher levels compared to Control (1833 pg/ml) by Day 1 post injury (See Figure 4.3-8). Across groups, these levels started to drop down to the levels of Control by Day 7. The levels of serum follistatin in the Isolated Trauma group started to rise again over the course of the second week, and were significantly ($p<0.05$) elevated compared to the other 2 trauma groups as well as control. In conclusion, the levels were upregulated post trauma across groups in correlation with trauma severity, and returned back to baseline level by day 7 post trauma. Levels start to rise again inversely related to trauma severity in the second week post trauma.

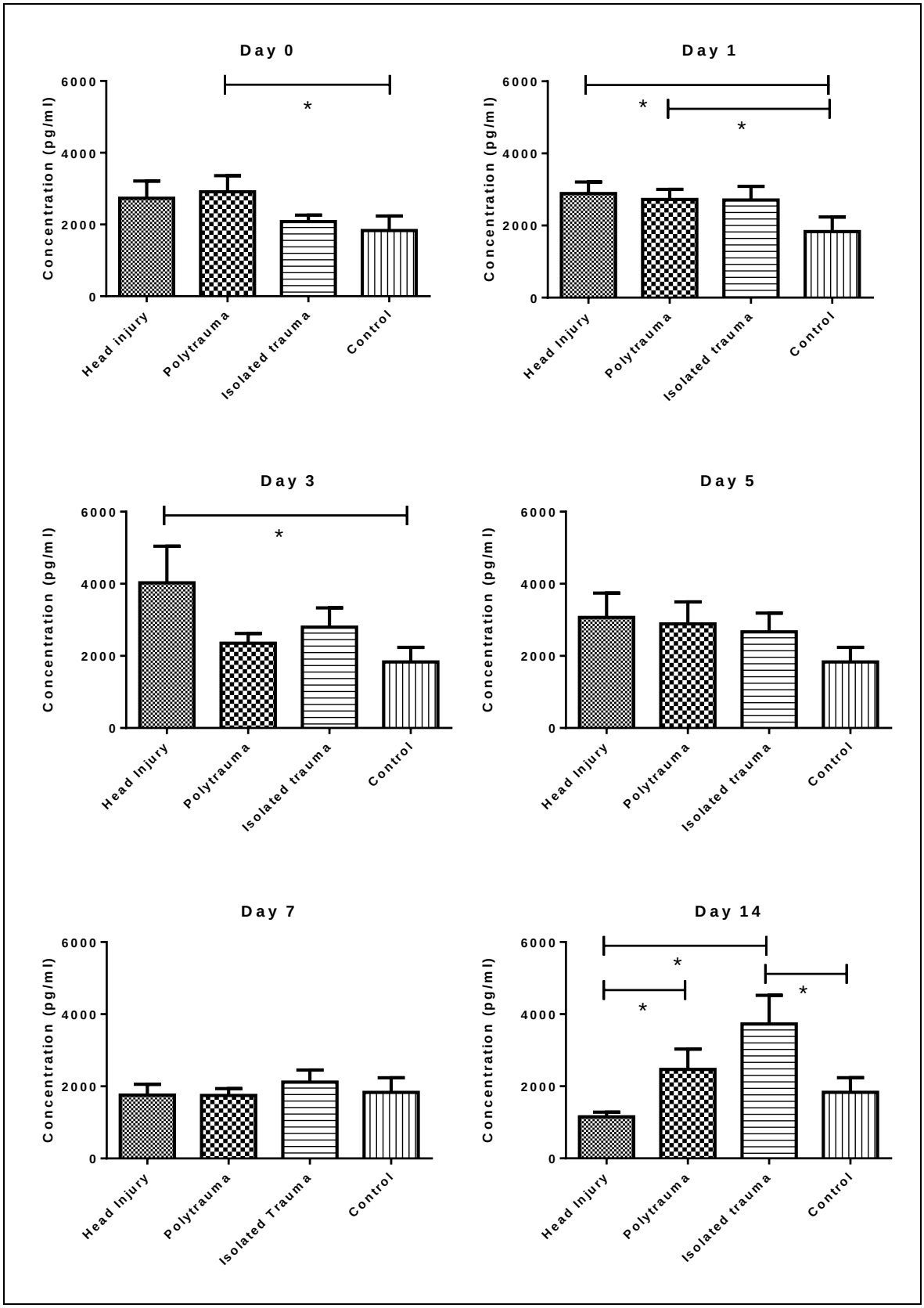

Figure 4.3-8 Comparison of Follistatin levels across trauma groups (*denotes statistically significant, p<0.05, Mann-Whitney) over 6 intervals between Day 0 and 14. There was an initial upregulation of follistatin levels which return to levels of control by Day 7.

TGF-β2

Finally, the serum concentration of TGF-β2 within each trauma group has displayed a wide coefficient of variation, as shown previously (Table 10.1-4). The Head Injury group has displayed consistently suppressed levels compared to Control across all observed time points (Figure 4.3-9). Similarly, although statistically significant differences ($p<0.05$) were found between Polytrauma and Control groups for Days 1, 3, and 5, the levels for the Polytrauma group exhibited high variability for each of these time points. The Isolated Trauma group displayed a progressive increase in levels over the first post-injury week, but was then depressed by the end of second week, although the changes in concentrations over time failed to reach statistical significance compared to Control.

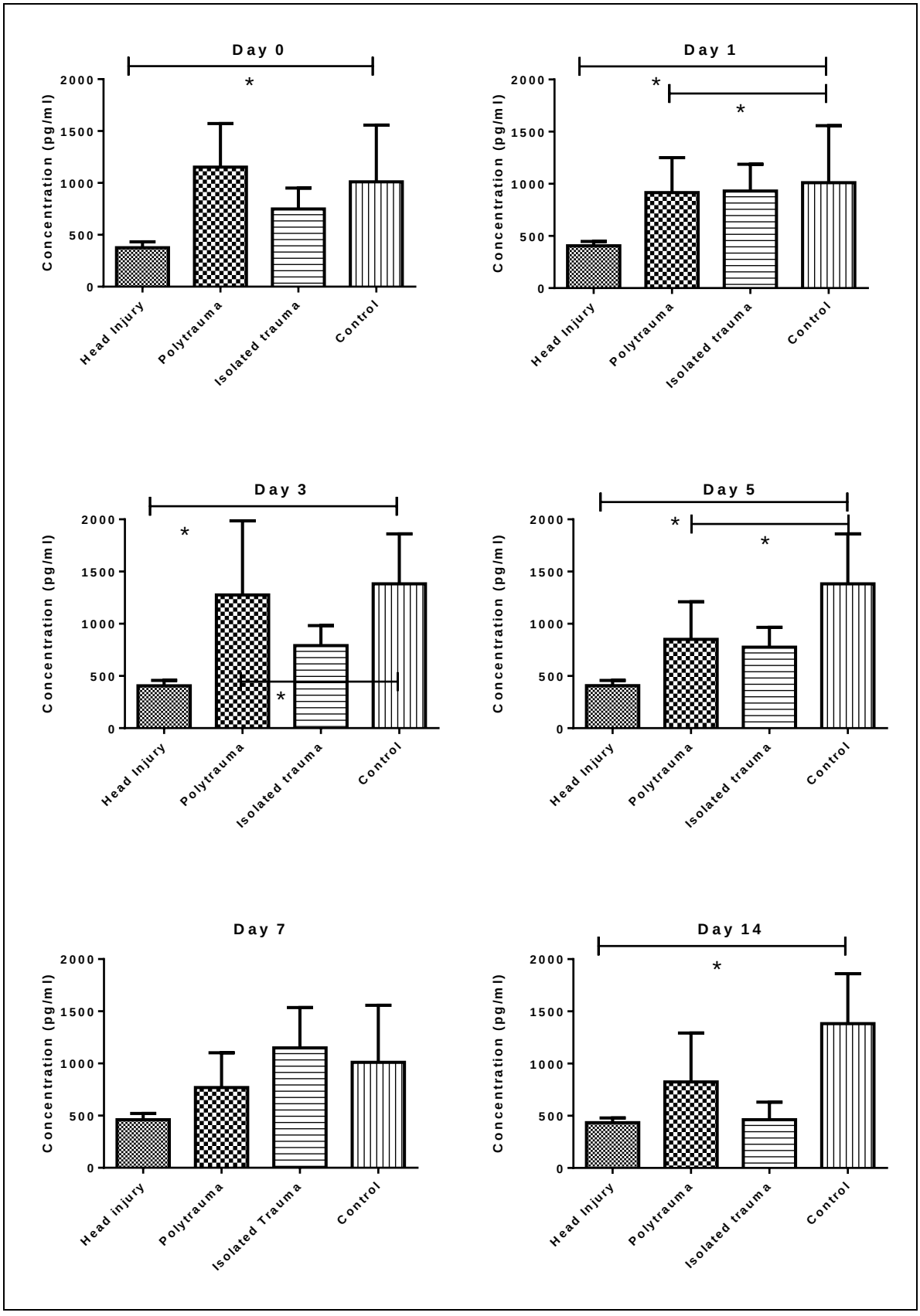

Figure 4.3-9 Comparison of TGF-β2 levels across trauma groups (*denotes statistically significant, p<0.05, Mann-Whitney) over 6 intervals between Day 0 and 14. There was a consistent suppression of TGF-β2 levels compared to controls in the Head Injury group.

4.3.5. Influence of fluid dilution

Patients admitted following trauma often receive intravenous fluid as part of their resuscitation. In fact, the Advanced Trauma Life Support principle advocates rapid infusion of up to two litres of intravenous crystalloid solutions (2012). Fluid resuscitation requirement often reflects the severity of trauma. A comparison of intravenous fluid infusion in the first 24 hours following presentation to hospital (See Figure 4.3-10) revealed that the Head Injury group of patients received the highest volume, with median of 3.4 litres (range 0.3-8.1), Polytrauma group 3.0 litres (range 1.4-9.5), and Isolated trauma 2.0 litres (range 1.0-3.0). The difference in fluid resuscitation was not statistically significant between Head Injury and Polytrauma groups (p=0.79, Mann-Whitney), but reach statistical significance between both Head Injury (p=0.05, Mann-Whitney) and Polytrauma (p<0.01, Mann-Whitney) compared with Isolated trauma. Therefore, the observed depression in the initial admission concentrations of growth factors (PDGF-AA, TGF-β2, angiogenin) could be due to the differences in fluid volume transfused.

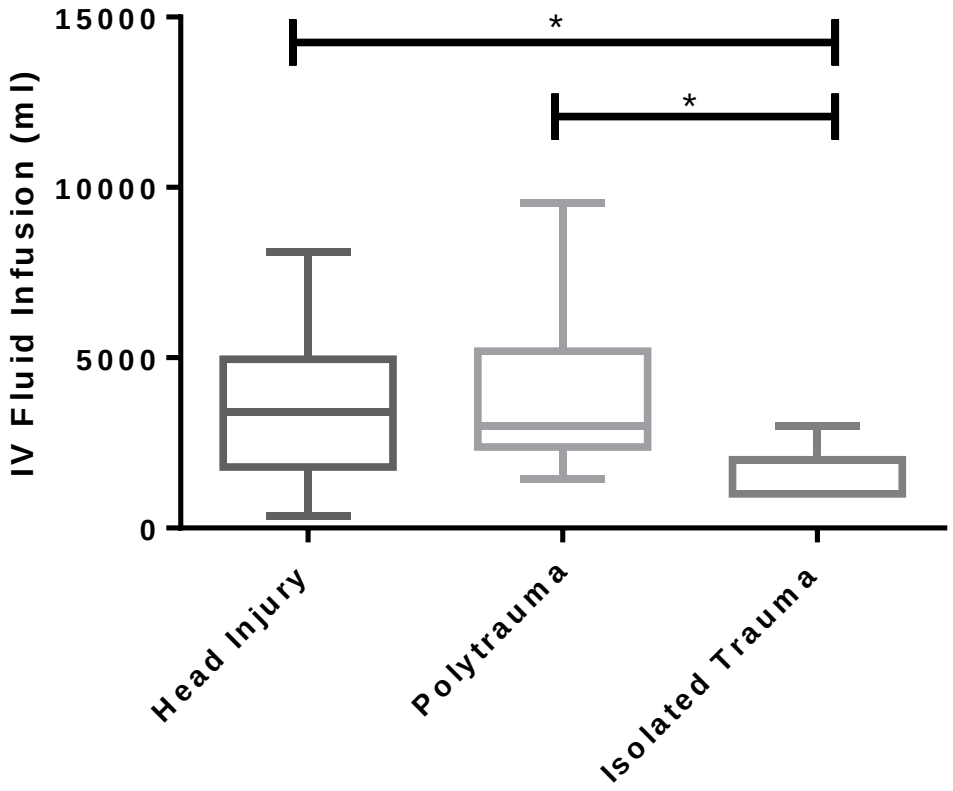

Figure 4.3-10 Box and Whisker plot of total volume of intravenous fluid infused in the first 24 hours of admission. Top line represents maximum value, bottom line represents minimum value. Middle line inside box represents median value. *Denotes statistical significance between median values, p<0.05, Mann-Whitney

The blood sample for Day 0 was obtained as soon as possible after admission. Therefore, an analysis of association between concentrations of growth factors (PDGF-AA, angiogenin, TGF-β2, follistatin) would be erroneous, as most of the intravenous fluid transfusion would have occurred after the Day 0 blood sample was taken. On the contrary, the blood sample taken for Day1 would have occurred soon after the first 24 hours of admission (and hence fluid infusion). Therefore, analyses were carried out to compare the concentrations of growth factors on Day 1 versus total fluid infusion in first 24 hours (just prior to Day 1 blood sample).

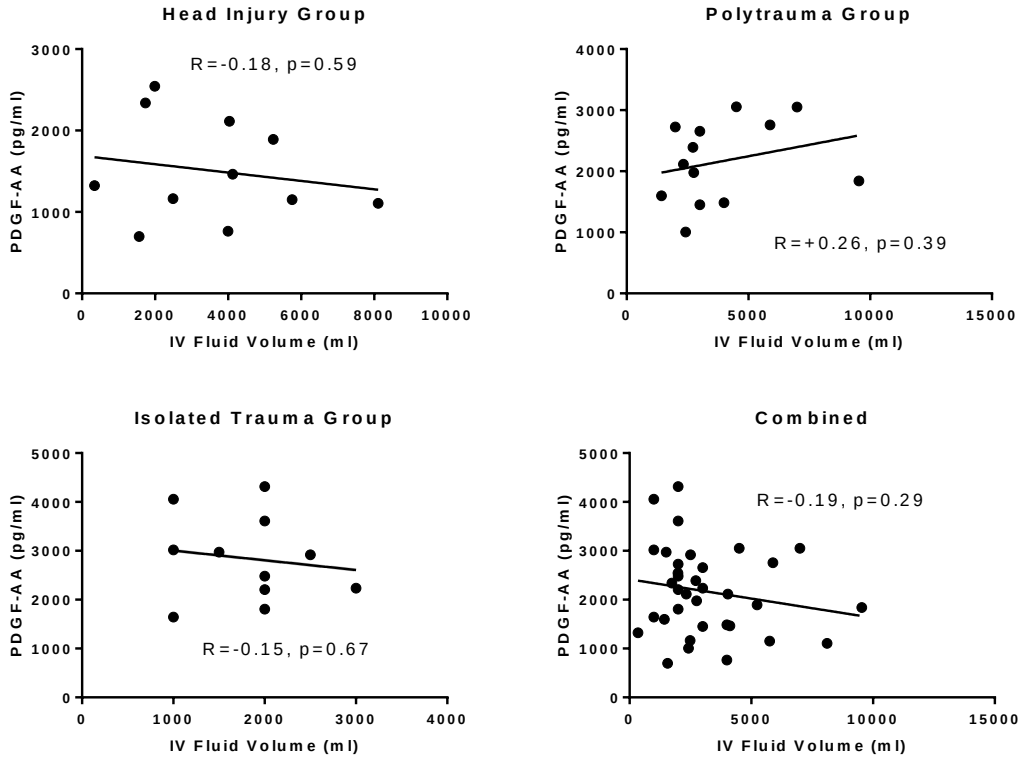

Figure 4.3-11 Association between PDGF-AA concentration in peripheral circulation on Day 1 against total fluid transfused in the first 24 hours following admission. No significant correlation was found across all trauma groups

As can be seen in Figure 4.3-11, there are no significant (p>0.39) associations between levels of PDGF-AA on Day 1 and the volume of intravenous fluid transfused in the previous 24 hours, across all trauma groups.

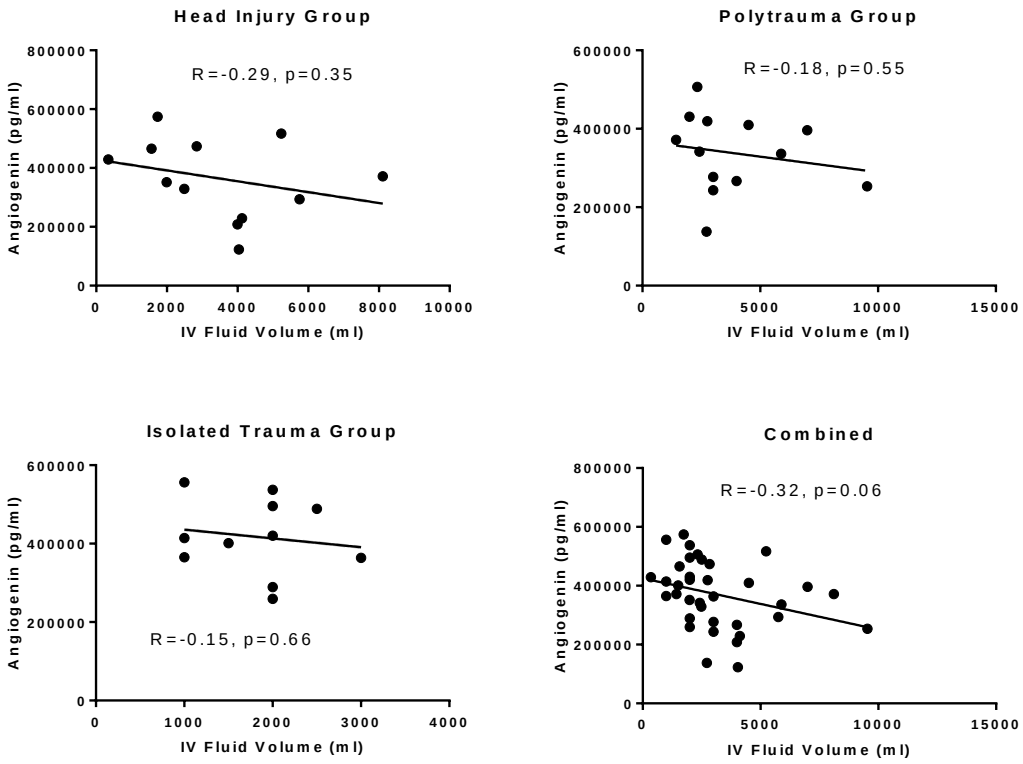

Figure 4.3-12 Association between Angiogenin concentration in peripheral circulation on Day 1 against total fluid transfused in the first 24 hours following admission. No significant correlation was found across all trauma groups.

Similarly, there appears to be no correlation between initial fluid transfusion volume (first 24 hours) and levels of angiogenin in the peripheral circulation at 24 hours (Day 1) following admission (Figure 4.3-12). This trend was comparable across all trauma groups.

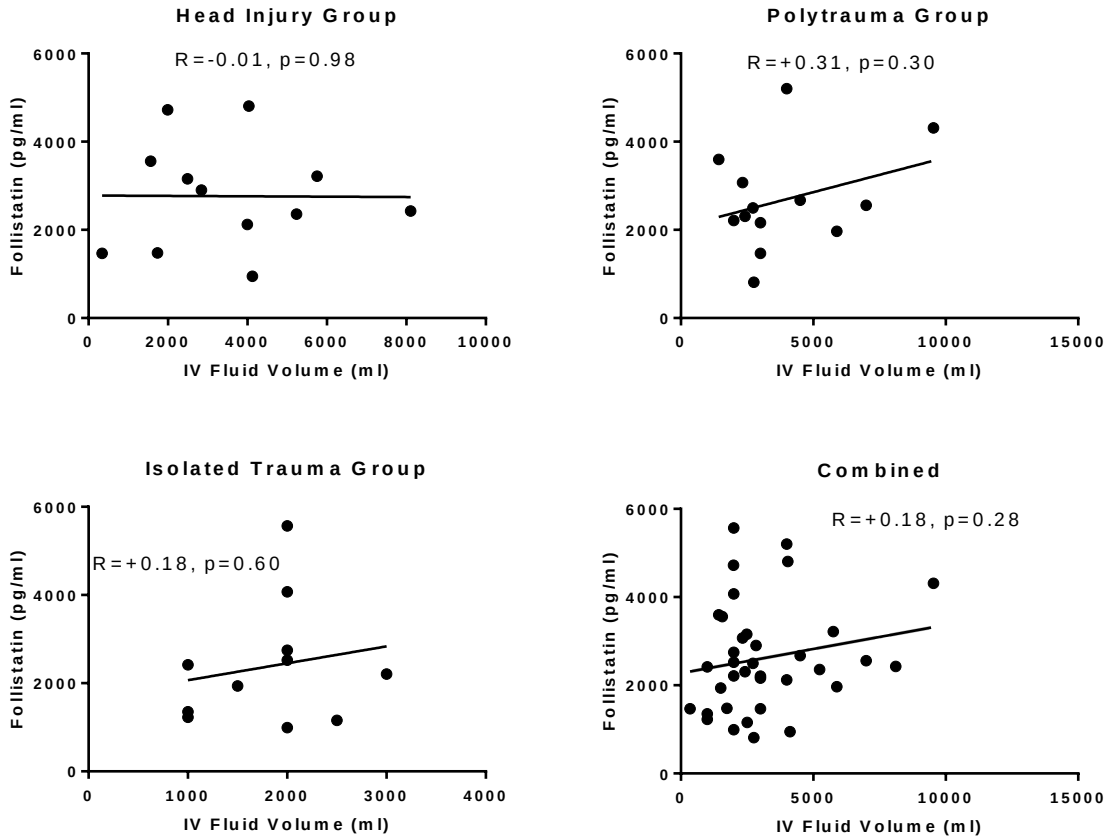

Figure 4.3-13 Association between Follistatin concentration in peripheral circulation on Day 1 against total fluid transfused in the first 24 hours following admission. No significant correlation was found across all trauma groups.

The results of the analysis for follistatin (Figure 4.3-8) were similar to those shown previously for PDGF-AA and angiogenin, with no significant ($p>0.28$) correlations between follistatin concentrations and total intravenous fluid transfusion, across all trauma groups.

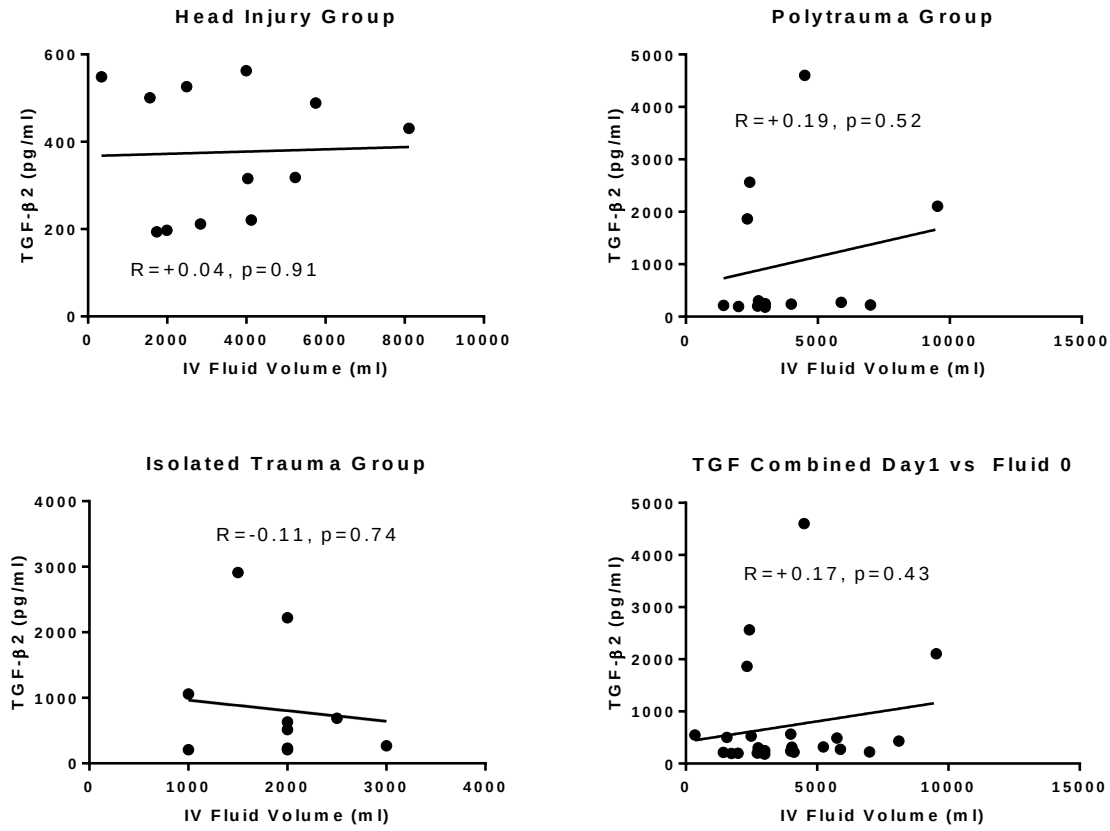

Figure 4.3-14 Association between TGF-β2 concentration in peripheral circulation on Day 1 against total fluid transfused in the first 24 hours following admission. No significant correlation was found across all trauma groups

Finally, analysis of the concentration of TGF-β2 (Figure 4.3-9) also showed no association with amount of fluid transfused, in keeping with findings of the other molecules (PDGF-AA, angiogenin, follistatin) detailed above.

In summary, there was statistically significant ($p<0.05$) higher volume of initial intravenous fluid transfused in both the Head Injury and Polytrauma Groups compared to the Isolated Trauma Group (as shown in Figure 4.3-10). However, within the individual trauma groups, the variability in volume of fluid transfused did not correlate with the variability in concentrations for all 4 measured growth factors (PDGF-AA, angiogenin, follistatin, TGF-β2).

4.4. Discussion

4.4.1. Study population

Across all trauma groups, there is a positive skew towards male patients, with an average of 3-fold more males compared to females. This male predominance has been previously reported in other polytrauma (Edwards, Di Bartolomeo et al. 2007; Christensen, Ridley et al. 2008) as well as traumatic head injury (Fuller, Bouamra et al. 2011) studies. However, all four patient groups were age and sex matched, allowing valid comparisons across groups. Additionally, the Injury Severity Score between the Polytrauma and Head Injury group was similar, thus allowing us to speculate that any differences observed within the growth factor kinetics measured to be attributed to the additional presence of traumatic brain injury alone.

4.4.2. Serum levels of growth factors in Healthy Controls

In this study, all 4 growth factors studied were detected at measurable levels in healthy non trauma controls. The mean serum concentrations of our control group were the following; PDGF-AA 4108 pg/ml (range 2482-5261, standard deviation 944), angiogenin 419,035 pg/ml (range 232,385-528,411, standard deviation 83421), follistatin 1833 pg/ml (range 865-2180, standard deviation 1211) and TGF-β2 1383 pg/ml (range 452-4727, standard deviation 1433). This is in agreement with the data from the commercial kit used to analyse these samples, which reported a mean serum concentration in healthy controls of PDGF-AA 4208 pg/ml (range 2156-5818), angiogenin 360,000 (range 196,000-437,000), follistatin 2483 pg/ml (range 889-11,123) and TGF-β2 386 pg/ml (range not detectable to 873) in healthy controls. The levels of these growth factors in our control group are by and large also in keeping with the literature. Serum follistatin levels in healthy control have been previously reported to range between 3500pg/ml (Sakamoto, Shintani et al. 1996) to 13,300pg/ml (Wakatsuki, Shintani et al. 1996). Previous publications reported the average serum angiogenin in healthy control to range between 177,000 and 336,000pg/ml (Molica, Vitelli et al. 2004; Dziankowska-Bartkowiak, Gerlicz-Kowalczuk et al. 2011). Although Doupis et al (Doupis, Lyons et al. 2009) reported a mean control population serum PDGF-AA levels of 23200pg/ml, his population cohort differs slightly from ours (older age, on medication for hypertension, hypercholesterolemia), and measured with different assay techniques (Luminex), which may contribute to this observed difference. The detection and presence of these growth factors in the serum of healthy controls naturally reflect their roles in maintaining the balance and homeostasis in daily human physiological functions. And finally, TGF-β2 in healthy control have been reported to range from 83.3 (Kapetanakis, Drygiannakis et al. 2010), 10,500(Wu, Wu et al. 2010) to 59,410 (Ma, Miao et al. 2013)

pg/ml. Although the published levels differ, this may be due to dilution or calculation error on their part, as the levels found within this study agrees with those provided by the manufacturer.

4.4.3. Influence of Fluid Dilution

I have shown that there was a statistically significant ($p<0.05$) larger volume of fluid resuscitation in the Head Injury and Polytrauma groups compared to Isolated Trauma group. Therefore, it was possible that the observed initial depression (compared to control) in serum concentration of PDGF-AA and angiogenin was due to fluid dilution. However, further analysis did not show any statistically significant ($p>0.05$) correlations between volume of fluid and growth factors concentrations, across all trauma groups, for all growth factors (Figure 4.3-11, Figure 4.3-12, Figure 4.3-13, Figure 4.3-14). Furthermore, should the dilutional factor be fully accountable for my observations, then I should similarly expect a depression of follistatin (compared to control); which was contrary to measured follistatin values in my cohort of patients (Figure 4.3-8).

Additionally, the *in vivo* half-lives of the molecules measured are relatively short [PDGF-AA (2 minutes) (Cianciolo, Stefoni et al. 1999), follistatin (4 minutes) (Kogure, Zhang et al. 1996), TGF-β2 (2 minutes) (Kaminska, Wesolowska et al. 2005), angiogenin (12 hours) (Hatzi, Bassaglia et al. 2000)]. This implies that the concentrations of these growth factors detected represented what was being actively synthesised and released into the peripheral circulation at that moment in time. The volume of fluid transfused would then have less of influence on the concentrations.

Therefore, the observed initial depression of growth factors noted in the more severely injured group was unlikely to be due to the influence of fluid dilution, but rather a depression in the rate of synthesis and release into the peripheral circulation.

4.4.4. Inter-individual variability

As presented earlier, within the same trauma group and time points, all 4 growth factors exhibited variations in baseline levels, as well as changes over time. Within each time point/trauma group, this coefficient of variation (CV) varied between 21.6-215.5% (Table 4.3-1). Comparisons of CVs between trauma groups showed that the Isolated Trauma group was least variable followed by the Head Injury group with the Polytrauma group to be most variable. Most patients recruited from the Isolated Trauma group presented with a single long bone fracture (most likely of the tibia) and underwent relatively similar post-injury course and treatment. In contrast, the Polytrauma Group is far more heterogeneous in terms of the assortment of injuries that each patient suffered. These

differences in type, locations, severity and post-injury management may account for the high CVs observed in the major trauma groups especially Polytrauma. This is borne out by the differences in the Injury Severity Score range of 4-9 in the Isolated Trauma group, versus 16-40 in Polytrauma and 16-50 in the Head Injury Group.

The other possible reason for the observed inter-individual variability could be due to the known circadian variations in levels of various cytokines in humans (Chan, Spieth et al. 2012). This effect has been minimized by ensuring that, apart from admission samples, the remaining samples were taken fasted in the morning.

Finally, the role of patients' own inherent (genetic) response following injury may play a role. The influence of genetic polymorphisms on the systemic inflammatory response following trauma has been well documented (Waterer and Wunderink 2003; Imahara and O'Keefe 2004). In fact, patients with certain polymorphism (to the NOG and SMAD6 genes) are significantly associated with a greater risk of fracture non-union (Dimitriou, Carr et al. 2011). Therefore, taking into account the influence of genetic polymorphism on the initial post-injury response through to fracture union rate, it would be reasonable to infer that similarly, some of the inter-individual responses observed in this study, is due to the individual patient's inherent response following trauma. This could also explain the variation in growth factors levels in the healthy control population found in this study as well.

4.4.5. Comparison against hypothesis – Anabolic growth factors

As previously mentioned in this chapter, the molecules PDGF-AA, angiogenin and TGF-β2 are known to be anabolic growth factors for the bone fracture healing process.

My study has shown that following trauma, serum levels of PDGF-AA rise over time, with an incremental rise observed in association with the patients in the severe trauma groups (Head Injury and Polytrauma). However, this conclusion would have been erroneous as, compared to our age and sex matched Healthy Control group, initially, the actual serum concentration of PDGF-AA across all groups was significantly ($p<0.05$) lower; with more pronounced suppression in Head Injury group, followed by Polytrauma and Isolated Trauma Groups respectively. In fact, over the period of one week, levels of serum PDGF-AA would rise to recover back to baseline (Healthy Control) levels. As previously discussed, PDGF-AA exerts its effect on MSCs and plays an important role in osteoblast replication. Based on McKibbin's (McKibbin 1978) classic model of the phases of fracture healing, the first week following fracture coincides with the inflammatory phase, to be followed by the osteogenic repair phase from day 12 onwards. Therefore, PDGF-AA would be expected to exert its maximal effect during this repair phase, hence a rise in level from initial trauma. What remains

unclear is the mechanism responsible for causing the initial suppression of serum PDGF-AA levels. This will be addressed in Section 5.3.1, in which PDGF-AA and platelet levels are measured in parallel, in the same cohort of patients.

In addition, activation of the PDGF-A receptor has been found to contribute towards impairment of the blood brain barrier (Ma, Huang et al. 2011). Therefore, following traumatic brain injury, it would be reasonable to speculate on the possible downregulation of PDGF-AA ligand production in order to induce a neuroprotective effect.

A previous study has reported post-fracture serum angiogenin to exhibit an early peak (1 week), with a steady decline over a 24-week period (Weiss, Zimmermann et al. 2009). Moreover, a DNA micro-array analysis in a murine model showed that the gene cluster responsible for angiopoiesis is upregulated from days 10 to 21 post fracture (Khan, Solaris et al. 2008). This may explain the relatively muted response observed in our cohort of patients, as we only measured levels up to one week post fracture. Additionally, angiogenin is predominantly synthesized in the liver, colon and small intestine (Strydom 1998), and as previously stated, the average healthy control concentration of angiogenin in this study was on average 420,00pg/ml . Therefore, even if there was an increase in angiogenin level in response to trauma (fracture), this increase may be too small to be detectable statistically.

TGF-β2 has been shown to be expressed locally on sites of fracture callus formation (Robey, Young et al. 1987) and plays a significant role in new bone formation (Mackie and Trechsel 1990). Its role as a topical bone graft augmentation agent has been previously demonstrated(Dailiana, Kantzanou et al. 2004). Interestingly, in the presence of SIRS, TGF-β2 level in peripheral circulation was reported to be significantly lower than healthy controls (Stoiser, Knapp et al. 1998). A similar observation was made in this study within the Polytrauma group with a significant ($p<0.05$) decrease in the first 24 hours following admission. Compared to bone marrow, TGF-β2 has shown high levels of expression in cardiac (5-fold) and skeletal (4-fold) muscle cells (GeneAtlas U133A, BioGPS). Therefore, the high variability (up to 10-fold difference) seen within the control group, could be due to the differences in expression/secretion in cells of cardiac/skeletal muscle origin. The invariable muscular cellular, and sometimes cardiac cellular injuries associated with trauma may thus be responsible for the observations seen in this study. Little else is known about the dynamics of circulating TGF-β2 following fractures and trauma in the literature.

Overall, the findings do not support the hypothesis that bone anabolic growth factors are further up-regulated by worsening severity of trauma and the additional presence of traumatic head injury.

4.4.6. Comparison against hypothesis – Inhibitory cytokine

In this study, follistatin was found to be upregulated (compared to healthy control) initially across all trauma groups, and gradually drop to control level at one week post trauma.

The molecule follistatin is known to be an inhibitory molecule in the process of fracture healing (Dimitriou, Tsiridis et al. 2005). Additionally, follistatin has been shown to have inhibitory effects on various other molecules of the BMP family (Fainsod, Deissler et al. 1997; Otsuka, Moore et al. 2001; Amthor, Christ et al. 2002). Similar to angiogenin, the dynamics of its expression following fracture is unknown. However, activin has been found to have neuroprotective effects *in vitro* (Krieglstein, Suter-Crazzolara et al. 1995; Iwahori, Saito et al. 1997) and in animal model experiments (Wu, Lai et al. 1999). The presence of brain injury has been found to up-regulate the expression of activin (Munz, Hubner et al. 1999) and down-regulate expression of follistatin (Wu, Lai et al. 1999) compared to controls in murine models.

Follistatin inhibits the activities of activin by binding the high affinity folistatin-activin complex. The osteogenic role of activin has been previously shown in both *in vitro* (Gaddy-Kurten, Coker et al. 2002) and *in vivo* studies (Sakai, Miwa et al. 1999). The initial rise in levels of follistatin in my trauma groups (compared to control) would imply high levels of free follistatin (not binding to activin) and hence a lower level of activin. The fact that activin is a known acute phase reactant protein (de Kretser, Hedger et al. 1999) that is upregulated by inflammatory response (Phillips, Jones et al. 2001) (such a trauma) appeared contradictory to this conclusion. Could the initial upregulation of follistatin be due to its interaction with the BMPs instead of activin? The concentration of activin would need to be measured in parallel with follistatin to address this issue.

4.4.7. Limitations

Ideally, for the growth factor release study, serial blood samples should be taken for up to two weeks following trauma. However, the duration that the patient is admitted in hospital varies and from experience sometimes gets discharged 5 days post-injury. However, despite this, about half the patients blood samples were obtained to Day 14. The trend over time from initial trauma (either progressive rise or decrease) was also consistent towards Day 14; hence despite the incomplete patient samples obtained, the results obtained for Day 14 would be expected to be similar had the samples been fully collected from all patients.

This study could potentially be limited with regards to the number of patients recruited. However, for the growth factors PDGF-AA, angiogenin and follistatin, the low inter-individual variability seen within the control population, together with the clear trends exhibited by these molecules probably implied that the sample size in this study was sufficient for analysis. However, the growth factor TGF-β2 had up to 10-fold difference in variability within the control population, and hence I was unable to exhibit any clear trends in the trauma groups. Unless the variability within the control group could be addressed or explained, it would be very difficult to address its dynamics following fracture/trauma.

5. Results – Growth factor dynamics and inflammatory response

5.1. Introduction

Following trauma, there is an initiation of a cascade of physiological responses, which has been recognized and defined as both an inflammatory and counter-inflammatory response(Lenz, Franklin et al. 2007). Signalling molecules such as IL-1β, IL-6, IL-8, IL-12, TNF and IFN-gamma (therein referred to as inflammatory cytokines) have been well described and shown to have potential roles in the inflammatory process following trauma (Hoover, Bochicchio et al. 2006). These cytokines, however, have also been shown to be expressed and involved in the early stages of bone healing. However, it is as yet unclear the degree of influence trauma severity plays on the production of these molecules and hence the evolution of bone healing. Given the overlap and cross-over of molecules involved in the trauma inflammatory response and bone healing, it is reasonable to speculate that severity of trauma has local and systemic effects on bone healing via a production of these signalling molecules.

5.1.1. Inflammatory response following trauma

It is currently understood and accepted that there is a positive correlation between the severity of inflammatory response observed clinically and upregulation of inflammatory cytokines in these patients' peripheral circulation (Gebhard, Pfetsch et al. 2000; Hensler, Sauerland et al. 2002). One of the recognized phenomena of a severe degree of inflammatory response is known as the systemic inflammatory response syndrome (SIRS). It is a serious condition, which is associated with complications such as organ dysfunction, organ failure and death.

The criteria for SIRS were set in 1991 by the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference (1992). It contained 3 clinical parameters (core temperature, heart rate and respiratory rate) and one laboratory parameter (white cell count). Table 5.1-1 below details the values within each parameter with any combination of 2 or more would define the presence of SIRS. These parameters were simplified from an earlier study, which reported an association between an increasing APACHE (acute physiology, age, chronic health evaluation) III Score and subsequent risk of hospital mortality(Knaus, Sun et al. 1992). APACHE III is a complex scoring system consisting of 17 physiologic measurements of changes and presence of co-morbidities (Knaus, Wagner et al. 1991). An increasing APACHE III score was found to be correlated with an increasing risk of hospital mortality (Knaus, Wagner et al. 1991). Further analysis of these parameters found that patients who met the criteria for SIRS on the first day of admission to the

intensive care unit accurately predicted (96.9%) patients who were deemed clinically to have developed sepsis (severe systemic response to infection) (Knaus, Wagner et al. 1991).

However, SIRS may have multiple aetiologies of origin apart from infection. It may also be caused by trauma, pancreatitis, and burns. Figure 5.1-1 illustrates the interrelationship between SIRS, sepsis and infection.

Table 5.1-1 Criteria for Systemic Inflammatory Response Syndrome (1992). SIRS is diagnosed when 2 or more of the parameters are present

Parameters	Value
Temperature	<36 °C or >38 °C
Heart rate	>90/min
Respiratory rate	>20/min or P_aCO_2<32 mmHg (4.3 kPa)
WCC	<4x10^9/L (<4000/mm³), >12x10^9/L (>12,000/mm³), or 10% bands (immature neutrophils)

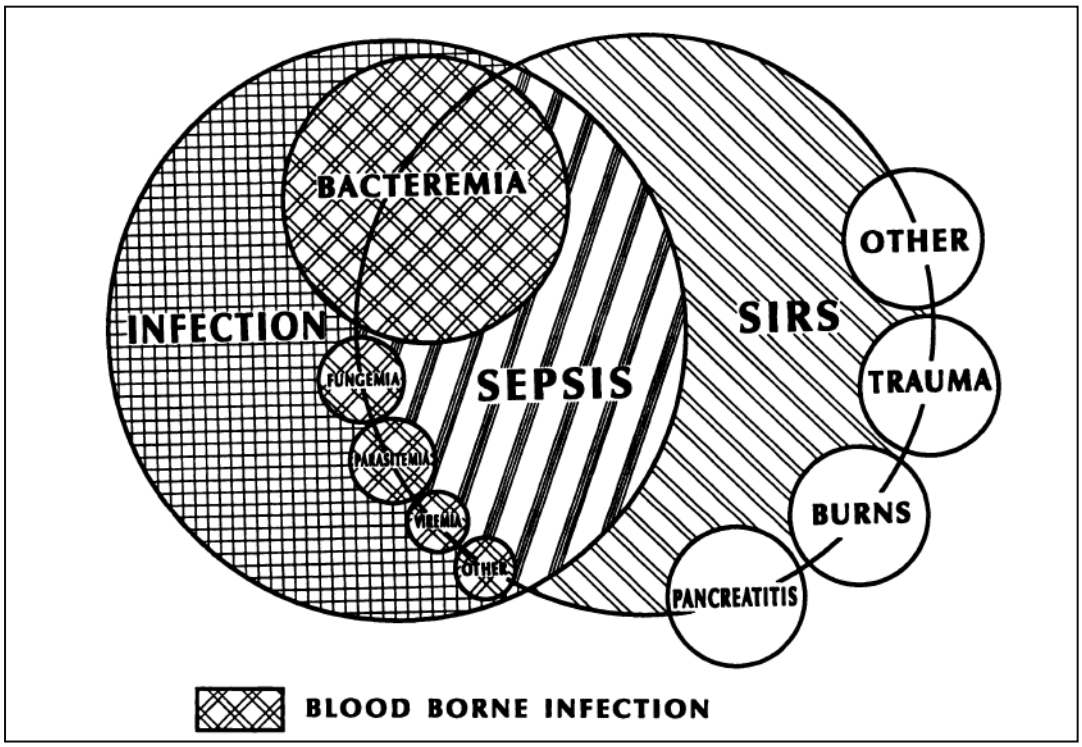

Figure 5.1-1 illustrates the interrelationship between SIRS, sepsis and infection. From Bone et al. (1992)

5.1.2. Hospital blood test for clinical monitoring

Following an admission to the hospital after trauma, patients may undergo various investigations to help with clinical diagnosis, management and monitoring. This may include analysis of patient's blood samples, which provides a window to the patient's physiological state at that moment. Some of the routine blood tests requested in post-traumatic patients are summarized in Table 5.1-2 and Table 5.1-3.

Table 5.1-2 Routine Haematology laboratory parameters. From Wallach's (Williamson, Snyder et al. 2011).

Test	Unit	Normal range	Role/function/implication
Haemoglobin (Hb)	g/dL	13.5-18.0	Respiratory protein for red blood cells. Levels reduced in all anaemias
Total white cell count (WCC)	10^9/L	4.00-11.00	Numerical total of the white cell components: neutrophils (including bands), lymphocytes, monocytes, eosinophils and basophils.
Platelet (Plt)	10^9/L	150-400	Involved in the blood clotting process. May have a reactive rise to inflammatory response such as severe trauma and infection
Prothrombin time (PT)	s	9-14	Assess the coagulation activity of the extrinsic and common coagulation pathways.
Activated partial thromboplastin time (APTT)	s	25-39	Assesses the coagulation activity of the intrinsic and common pathways of coagulation. Best screening test for disorders of coagulation that do not involve factor VII (extrinsic pathway) and platelet function
International Normalized Ratio (INR)	n/a	n/a	Derived as a ratio of patient's prothrombin time to normal control raised to the power of the International Sensitivity Index of the analytical system used(1985).
Derived Fibrinogen (Fib)	g/L	1.6-5.9	A glycoprotein synthesized in the liver, which is modified by thrombin to become fibrin. Derived fibrinogen is calculated based on prothrombin time.

Table 5.1-3 Routine Chemical pathology laboratory parameters. From Wallach's (Williamson, Snyder et al. 2011).

Test	Unit	Normal range	Role/function/implication
Sodium (Na)	mmol/L	135-145	Major extracellular constituent, and plays central role in maintaining water distribution and osmotic pressure
Potassium (K)	mmol/L	3.5-5.0	Primary intracellular ion, the ionic gradient (compared to extracellular) is required for nerve impulse transmission and cardiac/skeletal muscle contractility
Urea	mmol/L	2.1-8.0	Low molecular weight substance, which is a measure of protein breakdown in the body. As urea is excreted by kidneys, excretion of urea may reflect kidney function
Creatinine	µmol/L	80-115	Synthesized at a steady rate from amino acids in the kidney,

(Creat)			liver and pancreas. Urinary and serum creatinine used in conjunction calculates creatinine clearance, a measure of renal function
Alanine transaminase (ALT)	iu/L	<40	Found primarily in liver. Most sensitive tests for acute hepatocellular injury
Total Bilirubin	µmol/L	5-21	By-product of erythrocytes breakdown. Metabolized/excreted by liver. Therefore commonly used to assess liver function
Alkaline phosphatase (ALP)	iu/L	70-300	Family of enzymes that catalyze hydrolysis of phosphate esters at an alkaline pH. Majority of ALP activity from bone and liver (1:1 ratio). Increased in bone and liver disease
Albumin (Alb)	g/L	34-48	Constitutes 55-65% of total plasma protein. Decreased levels may be due to decreased synthesis in liver, acute inflammatory process, and increased loss secondary to pathologies such as burns and trauma.
Adjusted Calcium (Ca^{2+})	mmol/L	2.20-2.60	Also known as ionized calcium. Plays crucial roles in many physiological processes. The majority of calcium in human is unavailable, in bone(Guyton and Hall 2006).
C-reactive protein (CRP)	mg/L	<10.0	Acute phase reactant protein produced by hepatocytes and induced by the release of inteleukin-1 and 6. It reflects activation of systemic inflammation.

Although all the blood investigation parameters may be affected by trauma, as a result of disruption to the normal physiological function of the haematological, renal and liver systems, a few parameters are known to be directly influenced by an inflammatory process. The following parameters have been previously reported to be associated with an acute inflammatory process, such as following trauma.

C-reactive protein

Ever since the discovery of C-reactive protein (CRP) in the 1930s (Tillett and Francis 1930), it has been studied extensively due to its association with the inflammatory process. CRP is produced in the liver (Hurlimann, Thorbecke et al. 1966) and formation is principally induced by the inflammatory cytokine interleukin-6 (IL-6), and enhanced by the additional presence of interleukin-1(IL-1) (Kushner, Jiang et al. 1995). Functionally, CRP is believed to function as a mediator of the inflammatory process, inducing the release of pro-inflammatory cytokines (IL-1, IL-6, IL-18, TNF-α) (Ballou and Lozanski 1992), to activate the complement system (Mold, Gewurz et al. 1999) and to enhance phagocytosis by macrophages (Hokama, Coleman et al. 1962).

Clinically, CRP is a useful tool to the attending clinicians, as a sensitive but non-specific marker of inflammation. Serum CRP concentration rises in response to tissue injury, infection and

inflammation, and are therefore useful as a tool to monitor the patient's disease activity and response to treatment (Thompson, Pepys et al. 1999).

Overall, the influence of CRP on the dynamics of growth factors remains unknown. The inflammatory cytokines (such as IL-6 and IL-1) have been implicated in the early phase of the fracture repair processes (Einhorn, Majeska et al. 1995), and thus may influence subsequent release of other growth factors. CRP, with its close association with the inflammatory cytokines, may by inference also influence the dynamics of growth factors in general.

Total White Cell Count

The total white cell (leucocyte) count is used by the managing physician to assess the patient's immunological status. A rise in total white cell count, or leucocytosis would commonly alert the physician to the possible presence of an infectious pathology (Kumar and Clark 2009). However, the phenomenon of leucocytosis can also occur following other inflammatory processes such as trauma (Keel and Trentz 2005). A brief description of the cellular constituents that make up the total white cell count is shown in Table 5.1-4. Further discussion would be limited to the leucocyte response following trauma.

Table 5.1-4 Constituents of the Total White Cell Count. Adapted from (Alberts 2002) and (Kumar and Clark 2009)

Cell type	Typical concentration in human blood (cells/litre)	% of total white cell count	Brief description of function
Neutrophil	5×10^9	57.9	Phagocytosis and destruction of invading bacteria
Monocyte	4×10^8	4.6	Phagocytosis of microorganisms, foreign body and damaged cells
Lymphocyte B-cells	2×10^9	23.1	Production of antibodies
Lymphocyte T-cells	1×10^9	11.6	Plays central role in cell-mediated immunity
Basophil	4×10^7	0.5	Release histamine
Eosinophil	2×10^8	2.3	Anti-parasitic activities and modulation of allergic response

The leucocyte response following trauma is secondary to a complex cascade of inter-dependent signalling molecules. In brief, trauma and fracture cause local cellular death. This causes the dead cells to release their intracellular contents, including molecules known as damage-associated molecular patterns (DAMPs). Example of DAMPS include the high mobility group box 1 protein (HMGB1) (Scaffidi, Misteli et al. 2002), heat shock proteins (HSPs) (Prohaszka, Singh et al. 2002),

monosodium urate (Shi, Evans et al. 2003), mitochondrial damage associated protein (Zhang, Raoof et al. 2010), and even (previously intracellular) double stranded deoxyribonucleic acid (DNAs) (Ishii, Suzuki et al. 2001). These molecules then exert their effects in two different ways. Firstly, DAMPs are recognized by receptors (such as Toll-like receptors (Piccinini and Midwood 2010)) on leucocytes and other cells, which are then stimulated to release pro-inflammatory cytokines such as IL-1. These cytokines initiate an increase in the permeability of the local vasculature, dilatation of arterioles and venules thus giving rise to an overall increase in blood volume and delivery of plasma and leucocytes to the injured region (Kono and Rock 2008). Secondly, upregulation of pro-inflammatory cytokines, stimulated by DAMPs, stimulates the activation, migration and accumulation of leucocytes (Keel and Trentz 2005) and hence leucocytosis. However, sometimes during severe inflammatory response (such as SIRS mentioned above), leukopenia (drop in total white cell count) may occur instead. This has been postulated to be related to increased apoptosis, triggered by stress and cell death proteins (Keel and Trentz 2005).

Platelets

Platelets are non-nucleated disc shaped cells found within the peripheral circulation. They are produced by bone marrow megakaryocytes, regulated physiologically by thrombopoietin(Burstein 1997). Functionally, platelets play a central role in haemostasis, via the formation of the haemostatic plug(Kumar and Clark 2009). Additionally, platelets contain storage compartments known as alpha granules, which contain a plethora of cytokines (See Table 5.1-5). These stored cytokines are released following platelet activation in the process of haemostatic plug formation.

Following trauma, platelet numbers in peripheral circulation are known to be raised, a process known as reactive thrombocytosis (Kumar and Clark 2009). This process is determined by elevated levels of thrombopoietin, interleukin-6 and other cytokines produced by the accompanying inflammatory process. Table 5.1-6 summarises the main cytokines that are known stimulators of platelet production.

Although platelet production is known to be stimulated by trauma, the effect of different trauma severity is less clear. Platelet number in peripheral circulation is not only affected by rate of production, but also rate of platelet destruction, pooling, consumption and blood loss. As such, the effect of circulating platelets and its cytokine function and influence following trauma of different severity remains to be studied.

Table 5.1-5 Platelet Alpha Granule Constituents (From (McNicol and Israels 2008))

Adhesive Proteins	Chemokines	Other
Fibrinogen α chain Fibrinogen β chain Fibrinogen γ chain Fibronectin Thrombospondin 1 Vitronectin Von Willebrand Factor (vWF)	Connective Tissue-Activating Peptide (CTAPIII; CXCL7) Epithelial Neutrophil Activating Peptide (ENA-78; CXCL5) GRO-α (CXCL1) I-309 (CCL1) Interleukin-8 (IL-8; CXCL8) Macrophage Inflammatory Protein 1α (MIP-1α; CCL3) Monocyte Chemoattractant Protein-1 (MCP-1; CCL2) Monocyte Chemoattractant Protein-3 (MCP-3; CCL7) Neutrophil-Activating Peptide-2 (NAP-2; CXCL7) Platelet Basic Protein (CXCL7) Platelet Factor (PF4; CXCL4) Platelet Factor 4 variant 1 (PF4alt; CXCL4L1) Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES; CCL5) Stromal Cell-Derived Factor (SDF-1; CXCL12) Thymus and Activation-Regulated Chemokine (TARC; CCL17) β-Thromboglobulin (β-TG; CXCL7)	Albumin Amyloid β-A4 protein Angiopoietin-1 Angiostatin Clusterin Endostatin Factor V Factor XI Factor XIII High-molecular weight kininogen (HMWK) Matrix metalloproteinase-2 (MMP-2) Metalloproteinase inhibitor-1 Multimerin Osteonectin Plasminogen Plasminogen activator inhibitor (PAI-1) Protein C inhibitor Protein S Secretory granule proteoglycan core protein (SGPCP) Thrombocidin-1 (TC-1) Thrombocidin-2 (TC-2) Thymosin β-4 Tissue inhibitor of metalloproteinase (TIMP-4) von Willebrand antigen-II α2 macroglobulin α2-antiplasmin α-actinin 1 α-actinin 2 α-actinin 4
Cytokines	**Growth Factors**	
Interleukin-1β (IL-1β) High Mobility Group Box Chromosomal Protein-1 (HMGB1)	Basic fibroblast growth factor (bFGF) Epidermal growth factor (EGF) Hepatocyte growth factor (HGF) Insulin-like growth factor-1 (IGF-1) Insulin-like growth factor-2 (IGF-2) Platelet-derived endothelial cell growth factor (PD-ECGF) Platelet-derived growth factor (PDGF) Transforming growth factor (TGF-β) Vascular endothelial growth factor A (VEGF-A) Vascular endothelial growth factor C (VEGF-C)	

Table 5.1-6 Cytokines known to stimulate thrombocytosis (From (Klinger and Jelkmann 2002))

Cytokines
Stem cell factor
Thrombopoietin
Granulocyte-monocyte colony stimulating factor
Granulocyte- colony stimulating factor
Interleukin-1
Interleukin-3
Interleukin-6
Interleukin-11
Leukaemia inhibitory factor
Interferon gamma
Erythropoietin
Oncostatin

In summary, the previous results chapter has shown that the growth factor dynamics for the molecules PDGF-AA, angiogenin, follistatin and TGF-β2 changes over time following trauma. Differences were also observed between the different trauma groups, with the more severely injured (Head Injury and Polytrauma) showing marked initial suppression (PDGF-AA) or elevation (follistatin) compared to the less severely injured group (Isolated Trauma). As discussed above, worsening severity of trauma is associated with increased inflammatory response.

This chapter therefore aims to elucidate if these differences in growth factor dynamics observed was influenced by the degree of inflammatory response both biochemically (CRP, WCC, platelets) and clinically (SIRS score). In addition, this chapter also aims to clarify if the early dynamics of growth factors measured (PDGF-AA, angiogenin, follistatin, TGF-β2) had an influence on eventual fracture healing outcome.

5.2. Methods

5.2.1. Patient recruitment, serum sampling and growth factor analysis

Details of the method are as described in Chapter 4. Briefly, the patient population recruited is divided into Traumatic Head Injury, Polytrauma and Isolated Trauma groups. Blood sampling time points are at admission, and Days 1, 3, 5, 7 and 14 post-injury. Growth factors analysed are PDGF-AA, angiogenin, TGF-β2 and follistatin. Hospital laboratory parameters analysed were C-reactive protein, total white cell count, platelet.

5.2.2. Hospital blood laboratory results

Patients admitted to our unit following injury frequently have blood samples taken and sent for analysis in the hospital laboratory as part of their clinical care, as required. Therefore, not all time points captured in the Growth Factor analysis have time-matched samples from the hospital laboratory analysis. Available data was obtained from the Leeds Teaching Hospital Results Service System. Routine laboratory analyses undertaken are briefly divided into the Haematology and Chemical Pathology Department. Details of parameters captured as well as brief explanation of the role of these tests are shown in Table 5.1-2 for Haematology and Table 5.1-3 for Chemical Pathology. However, for the purpose of analysis, only the parameters identified to be associated with an acute inflammatory process will be reported.

5.2.3. Clinical data

Clinical data was collected to match with the blood sample (growth factor) time points. In addition to total white cell count (obtained from the Leeds Teaching Hospitals Haematology laboratory), the parameters of core temperature, heart rate and respiratory rate were obtained daily. The results were then tabulated and SIRS was diagnosed when two or more criteria were met (Table 5.1-1). Requirement of intensive or higher dependency care was also noted together with the total duration of hospitalization.

Additionally, following discharge from hospital, patients who were followed up in the Leeds Teaching Hospitals outpatient fracture clinic department also had their longer term outcome recorded; specifically, rate of fracture healing and requirement for re-operation due to non-healing of fractures.

Fracture healing can be ascertained radiologically or clinically. However clinical diagnosis of fracture healing can be subjective. Therefore, the radiological method is used to ascertain fracture healing in this study. A fracture is considered healed when bridging callus across the fracture site is seen on two orthogonal views on plain film X-rays. For the purpose of analysis, patients requiring 24 weeks or more to achieve radiological healing would be considered delayed union (Calori, Phillips et al. 2008).

5.2.4. Statistical analysis

Statistical analysis was carried out using PASW Statistics (SPSS) version 17.0.2 and graphing performed using Graph Pad Prism version 4.00 for Windows (San Diego, California, USA). As Gaussian distribution could not be assumed given the small sample size, non-parametric tests were carried out. Mann-Whitney test was used to compare differences between two independent samples, in cases of three or more groups. Kruskal-Wallis one-way analysis of variance was used (3 different trauma groups). Wilcoxon signed-rank test was used to compare two dependent samples (same patient, two different time points). Chi-square test was used for comparison of nominal data (for example comparing sex distribution between groups).

5.3. Results

In order to further understand the relationship between serum concentrations of the measured growth factors (PDGF-AA, follistatin, angiogenin and TGF-β2) and the clinical course and outcome of the patients, the growth factors were analysed against time matched hospital laboratory parameters as well as patients' early (SIRS) and late (non-union) complications.

5.3.1. Comparison against Hospital laboratory parameters

Patients admitted to the hospital following acute trauma and injury undergo regular sampling of their peripheral blood for analysis of their physiological state. Details have been explained in Section 5.1.2 above. It has been hypothesised that the growth factor dynamics in peripheral circulation is positively influenced by the degree of inflammatory response. Within the routine blood tests carried out in the hospital for these patients, the following parameters have been associated with changes in the inflammatory state of the body (as discussed in Section 5.2.2): C-reactive protein, total white cell count, and platelet. It must be noted, however that these data derived from the hospital laboratory measurements do not cover all time points as measured in the growth factor study, as these tests were carried out based on the individual patients' needs. Therefore, analyses were carried out on a sub-group of patients recruited.

C-reactive protein (CRP)

C-reactive protein levels matching growth factor measurements were available on 13 patients from Head Injury, 11 from Polytrauma and 6 from Isolated Trauma Group, as detailed in Table 5.3-1. As can be seen in Figure 5.3-1, CRP in these patients showed an overall increasing trend from admission, peaking between days 3 and 5 before subsiding. Overall, the Polytrauma Group appeared to have the highest mean CRP values across all time points studied, with peak value of up to 4-fold admission values.

To enable analysis of any correlations between growth factors and CRP values, only matched growth factor values were used. Figure 5.3-2 summarizes the growth factor values for the CRP sub-group of patients.

Each individual growth factor was then further analyzed to ascertain the relationship (if any) between matched CRP and growth factor levels. Data were analyzed separated into individual trauma groups, as well as overall. As the aim of this analysis was to determine relationship between two matched values, therefore, values for all time points within each group were analysed

collectively. Additionally, data was analysed for the influence of the different trauma groups, and also collectively.

Table 5.3-1 Patients with matched Growth Factor and CRP data

Trauma Groups	Patient ID
Head Injury (n=13)	BH1, BH3, BH4, BH6, BH8, BH9, BH10, BH11, BH13, BH16, BH17, BH18, BH21
Polytrauma (n=11)	NT1, DL, BP2, BP4, BP6, BP12, BP15, BP16, BP18, BP20, BP27
Isolated Trauma (n=6)	BP8, BP9, BP10, BP11, BP13, BP14

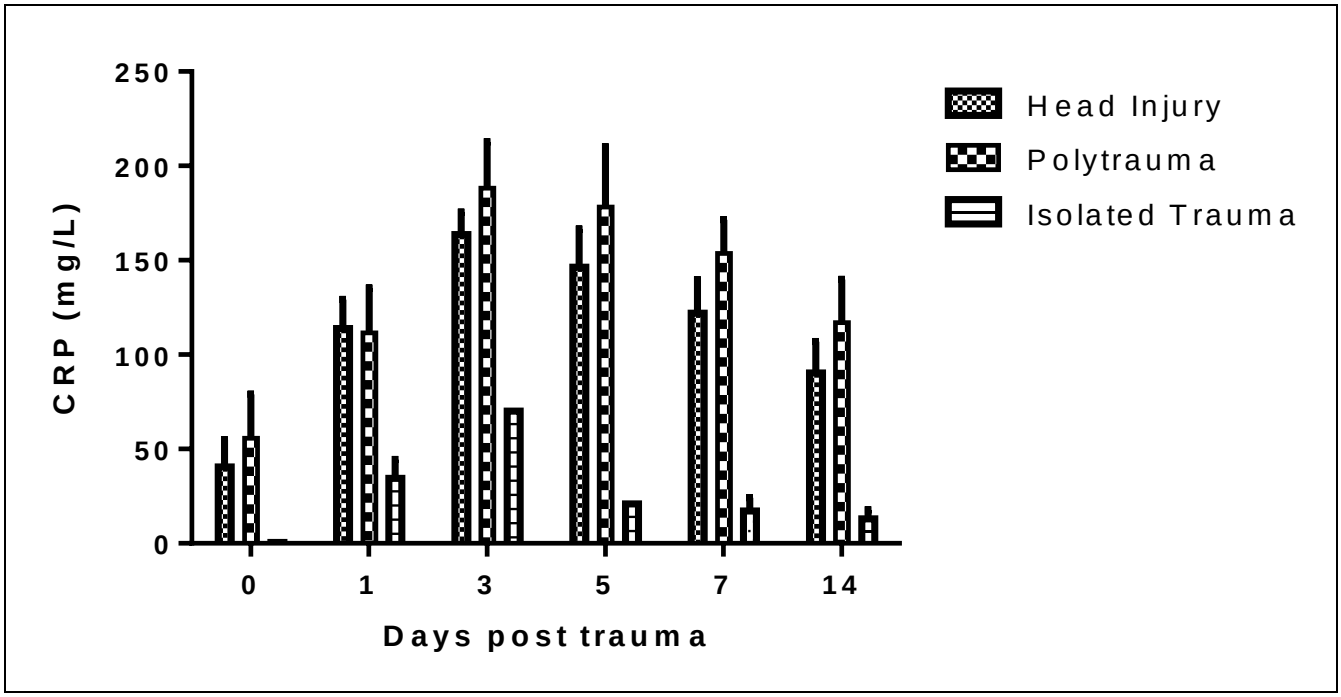

Figure 5.3-1 Mean CRP values over time across trauma groups. All values were above 10mg/L. Error bars represent one standard deviation. Overall trends showing CRP rising over time reaching peaks at day 3-5 before gradually subsiding

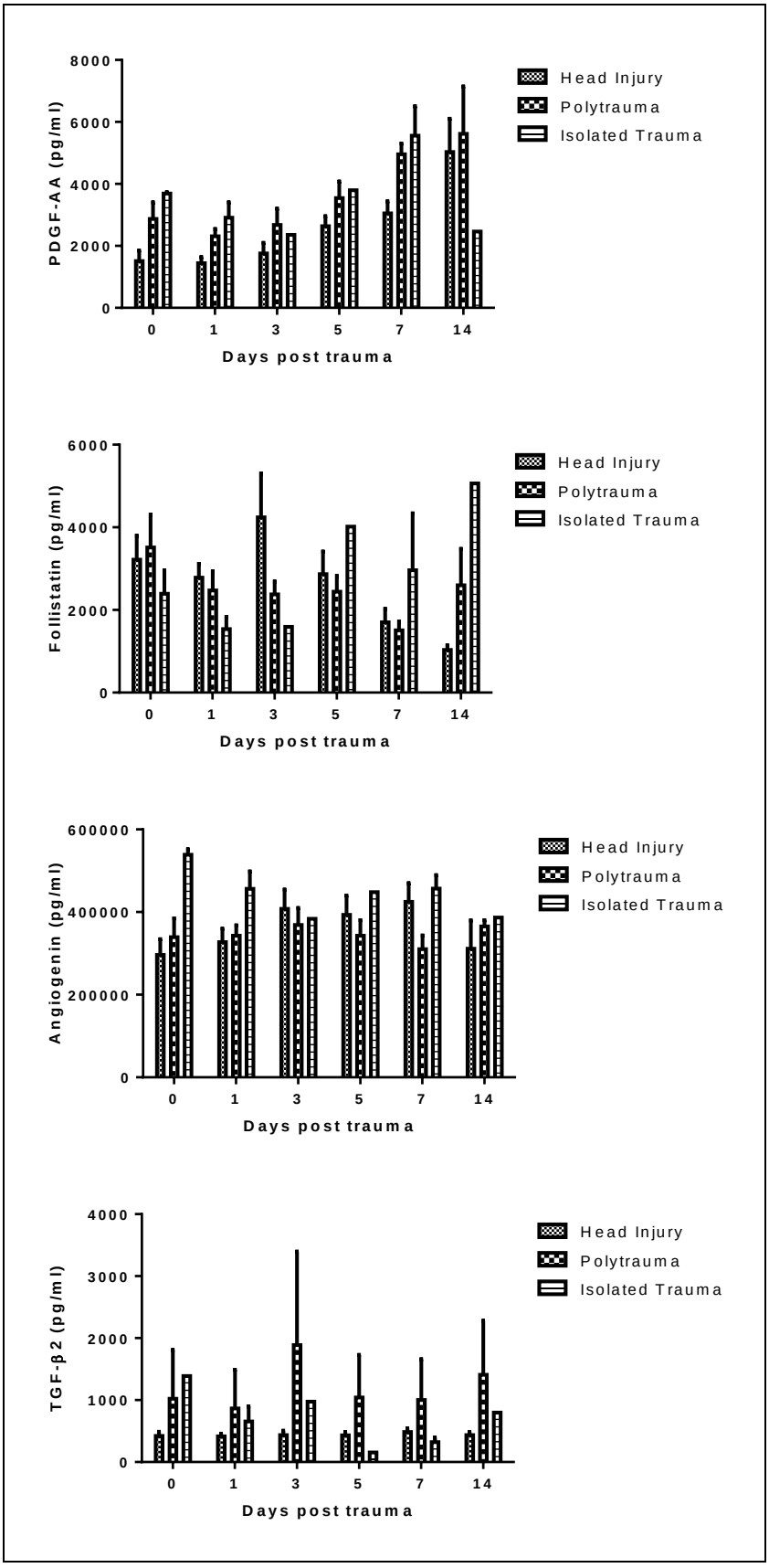

Figure 5.3-2 CRP sub-group: Trends of growth factors over time across trauma groups. Error bars represent one standard deviation. No obvious trends were seen across all 4 growth factors over the 6 time intervals measured.

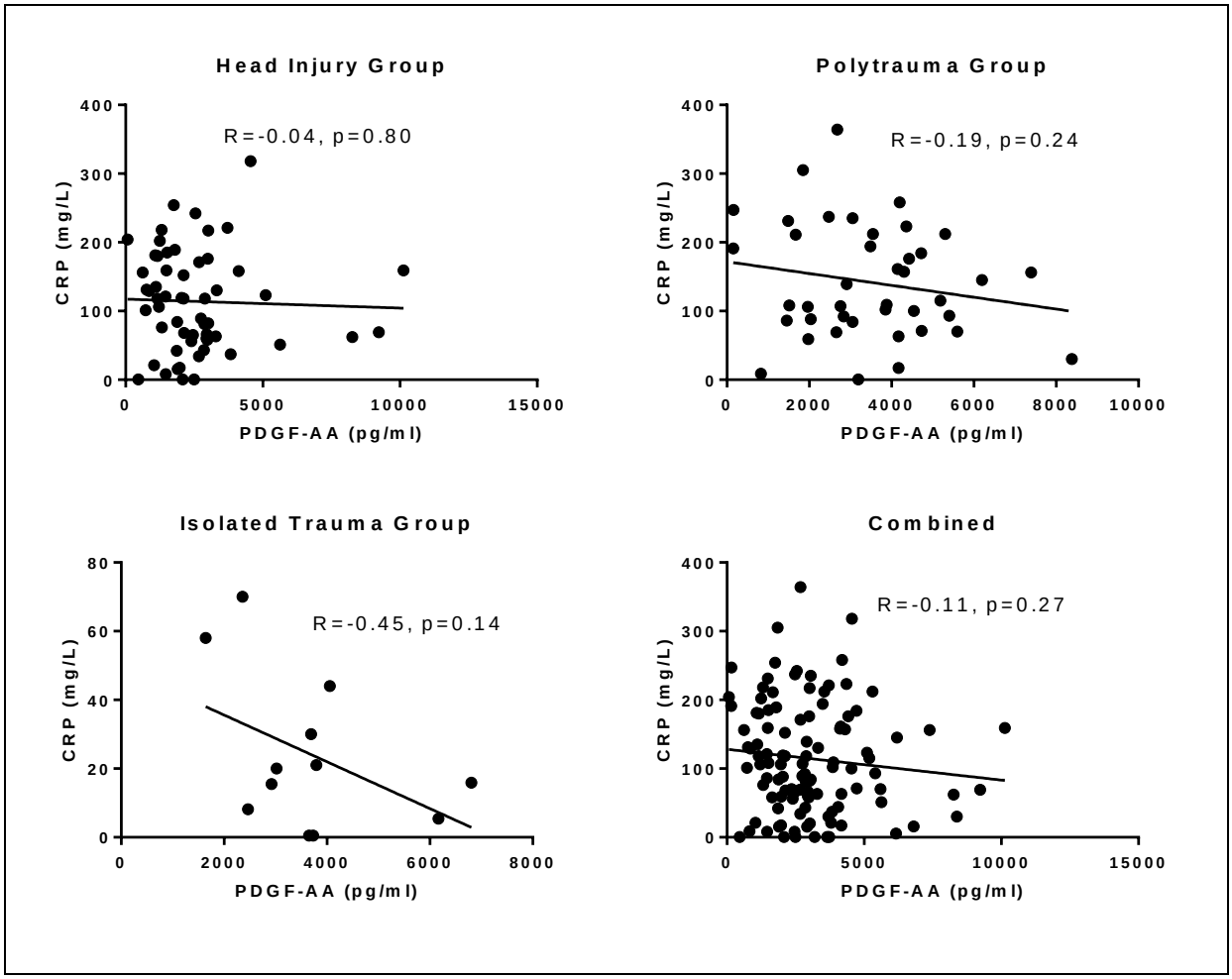

Figure 5.3-3 Relationship between matched CRP and PDGF-AA levels. No statistically significant correlations were found between CRP and PDGF-AA levels across all trauma groups.

As can be seen in Figure 5.3-3, across all trauma groups there are only very weak correlations between the values for CRP and PDGF-AA. Application of simple linear regression shows a negative correlation throughout, with R values ranging from -0.45 (Isolated trauma group) to -0.04 (Head Injury group).

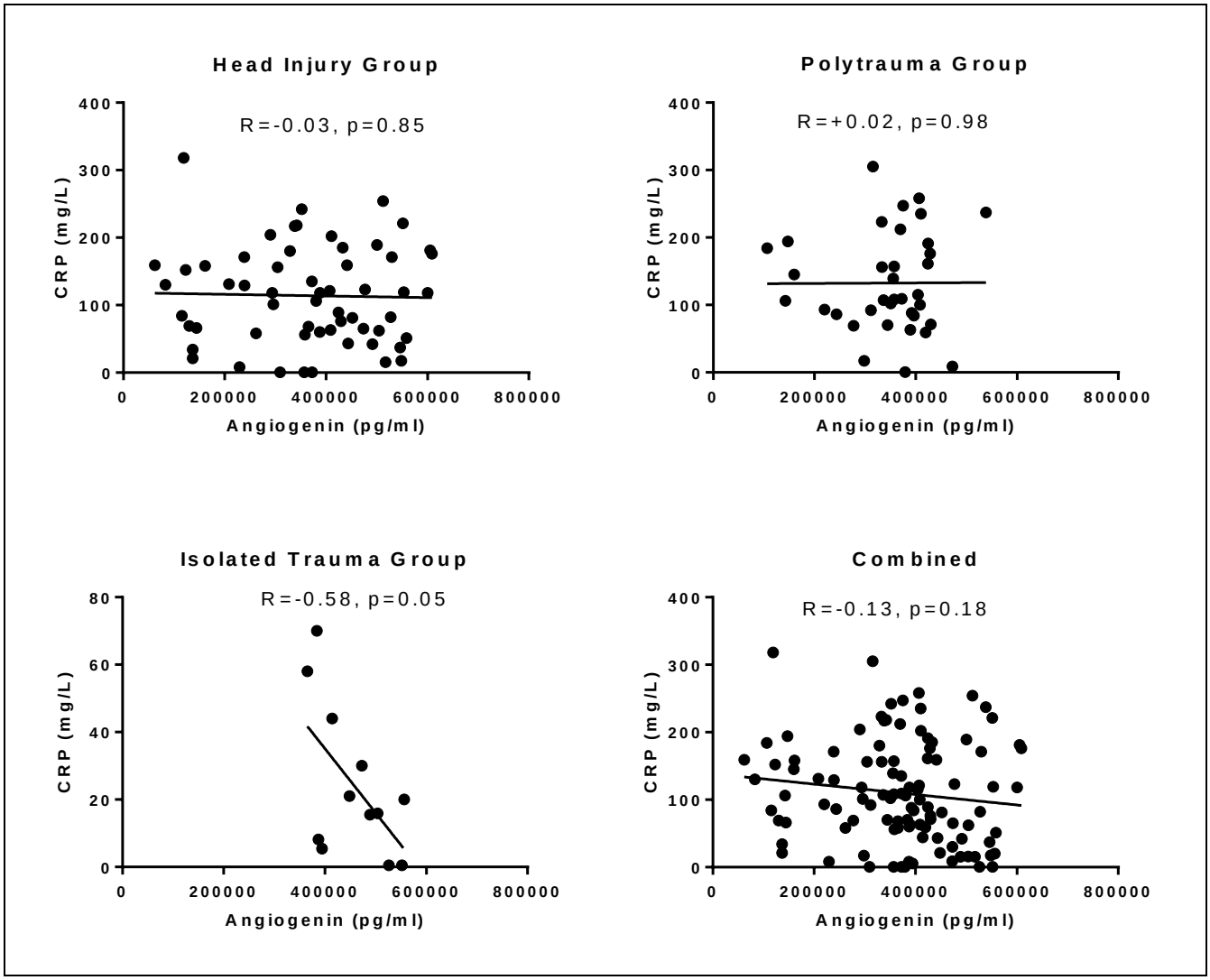

Figure 5.3-4 Relationship between matched CRP and Angiogenin levels. CRP was weakly correlated with angiogenin in the isolated trauma group, but overall, no correlations were found.

Analysis of the values for angiogenin (Figure 5.3-4), however, revealed a significant negative correlation within the isolated trauma group (R=-0.58, p=0.05). This finding may have to be interpreted with caution, however, as relatively low numbers of matched time points in this trauma group may give rise to statistical sampling errors. This observed correlation was not repeated with the other 2 trauma groups, or with the combined analysis.

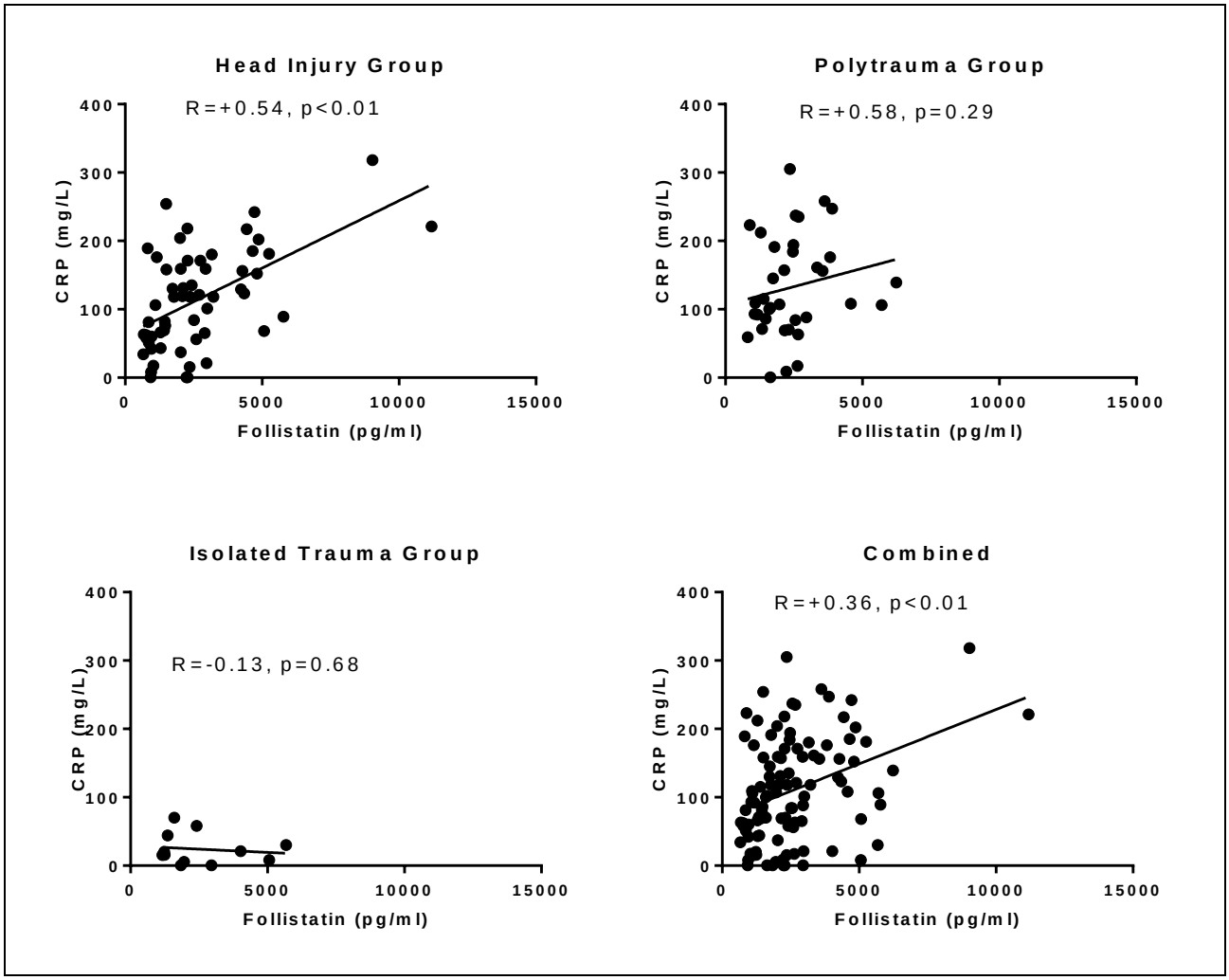

Figure 5.3-5 Relationship between matched CRP and Follistatin levels. CRP was weakly correlated with follistatin in the more severely injured group (Head Injury, Polytrauma)

Patients in both the Head Injury and Polytrauma Group appeared to show a positive statistically strong correlation between follistatin and CRP levels (Figure 5.3-5). Both groups exhibit similar positive R values (0.54 for Head Injury, 0.58 for Polytrauma) on regression analysis, which in combination with data from Isolated trauma group, gave a weak positive correlation in the combined analysis (R=+0.36, p<0.01). Although the association was relatively weak, these analysis suggest that the levels of follistatin were more influenced by the degree of inflammatory response reflected by CRP levels, in patients from the more severely injured trauma groups compared to Isolated Trauma alone.

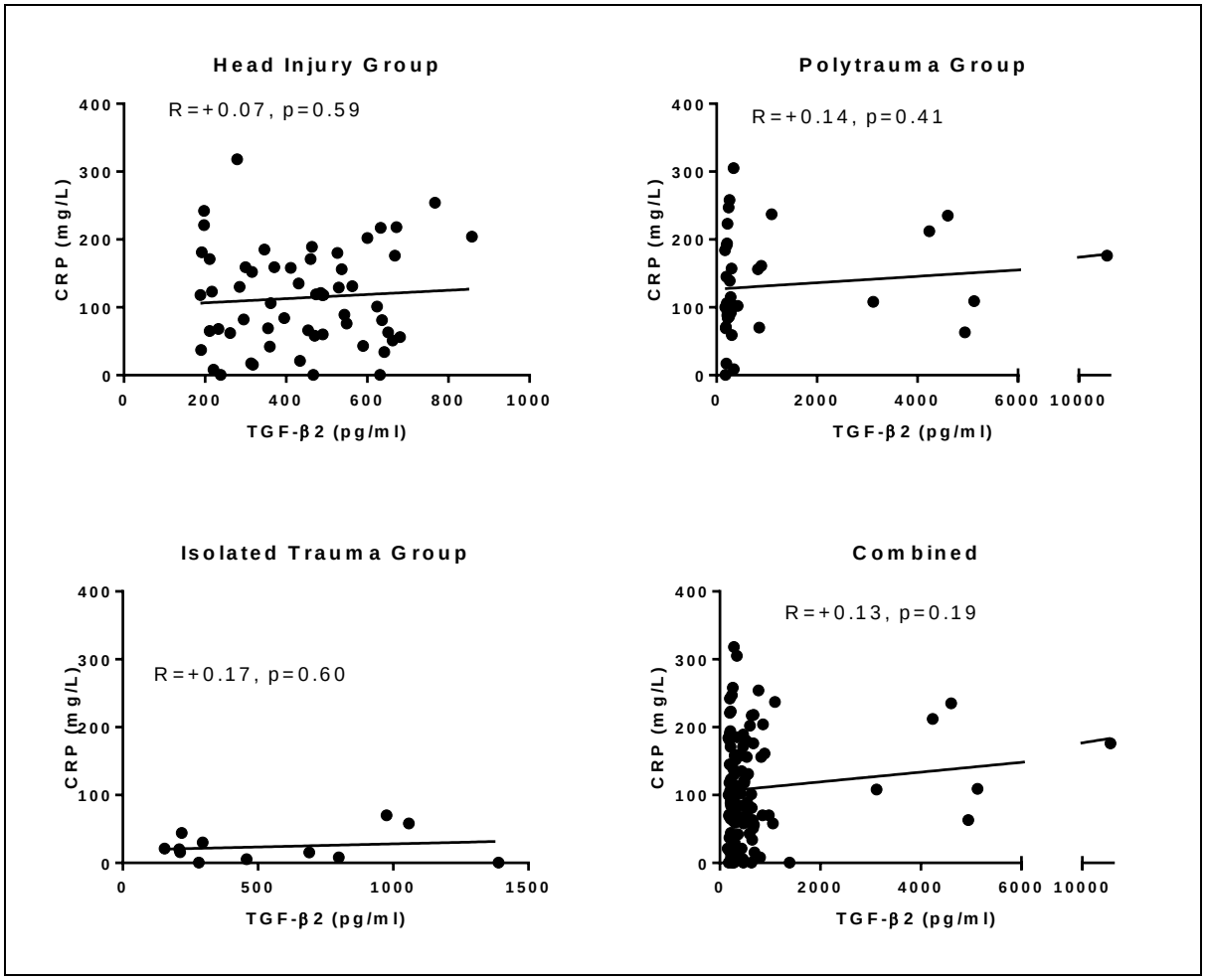

Figure 5.3-6 Relationship between matched CRP and TGF-β2 levels. No correlations were observed between CRP and TGF-β2 across all trauma groups.

And finally, association analysis between TGF-β2 and CRP levels (Figure 5.3-6) revealed that the 2 parameters are unlikely to be correlated with R values ranging from 0.07 to 0.14. This is especially evident in the combined analysis, with CRP values appearing to be independent of TGF-β2.

The relationship between the growth factors, trauma groups and CRP levels are summarised in Table 10.2-1. In summary, no correlations were found between CRP and measured growth factors across all trauma groups, except a weakly positive correlation with follistatin in the more severely injured group.

Total White Cell Count (WCC)

Similarly, data for total white cell count which are time-matched to growth factor measurements are available for 14 patients in Head Injury, 14 in Poly trauma and 12 in Isolated Trauma patients respectively. (Table 5.3-2)

Table 5.3-2 Patients with matched Growth Factor and WCC data

Trauma Groups	Patient ID
Head Injury (n=14)	BH1, BH3, BH4, BH6, BH8, BH9, BH10, BH11, BH13, BH16, BH17, BH18, BH20, BH21
Polytrauma (n=14)	NT1, DL, BP2, BP4, BP6, BP12, BP15, BP16, BP18, BP19, BP20, BP27, BP28, BP31
Isolated Trauma (n=12)	BP3, BP7, BP8, BP9, BP10, BP11, BP13, BP14, BP17, BP22, BP23, BP24

Figure 5.3-7 summarises the overall trends of WCC values over time across the 3 trauma groups. There is an initial acute rise on admission across all trauma groups which then subsided by day 3 before rising again towards day 14 post trauma. The Isolated Trauma appeared to have highest initial values for WCC in contrast to the Polytrauma group, which rose to the highest value across groups towards day 14 post trauma.

As not all time points for the growth factor measurements has matched WCC data, only matched growth factor measurements are used for analysis. Figure 5.3-8 shows the summary trends over time across all trauma groups for the growth factors measured.

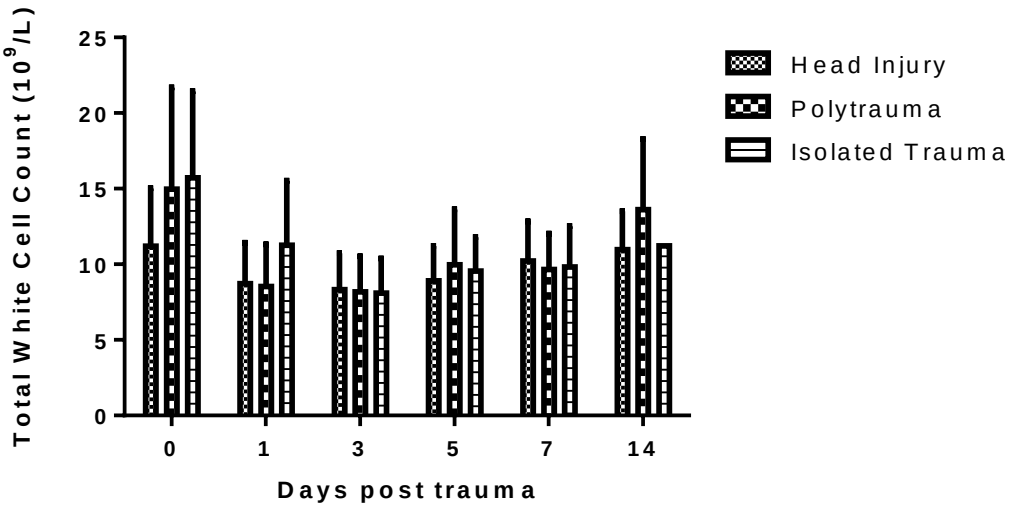

Figure 5.3-7 Mean WCC values over time across trauma groups. Normal WCC values 4-11X10^9/L. Error bars represent one standard deviation

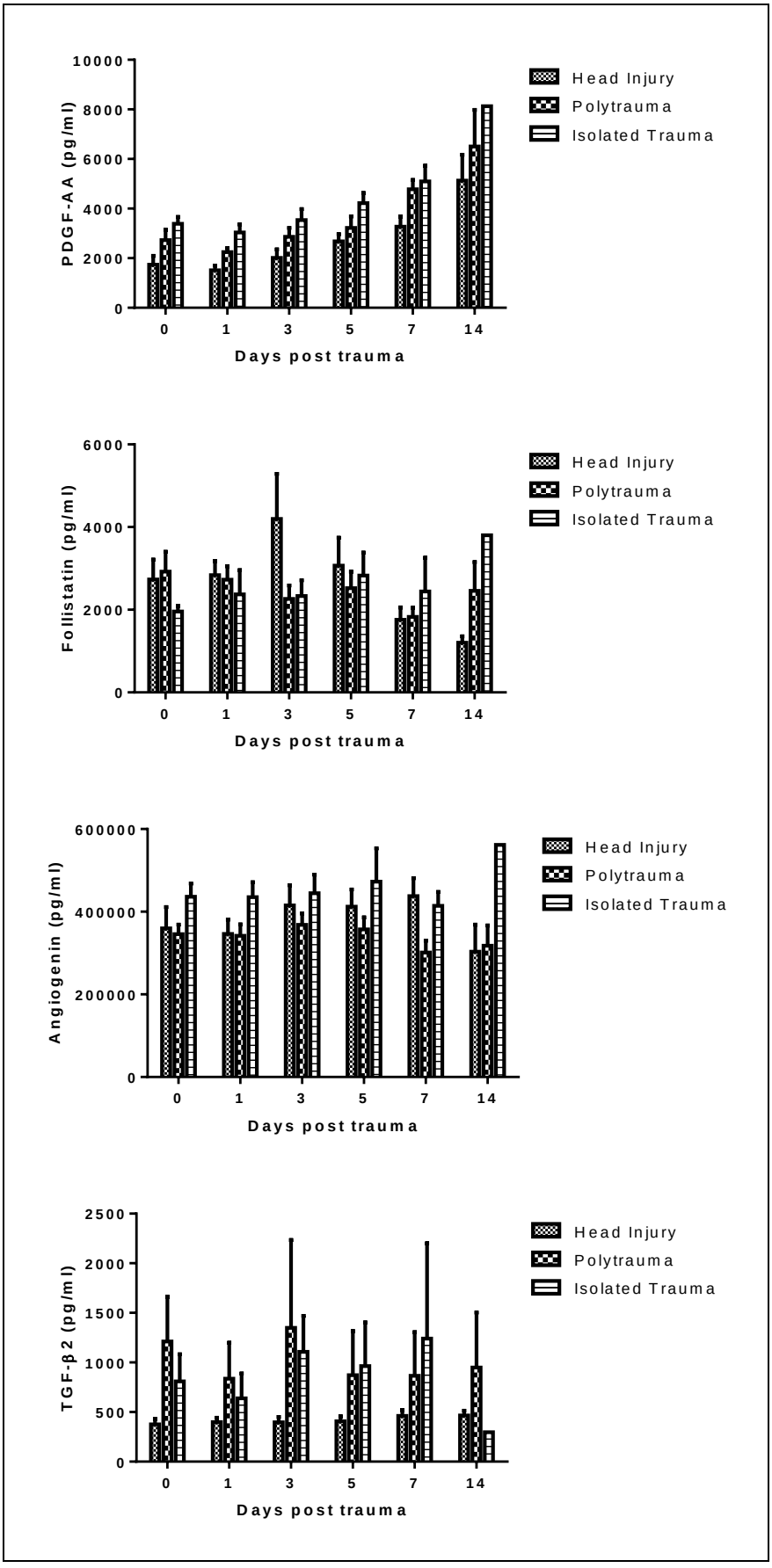

Figure 5.3-8 WCC sub-group: Trends of growth factors over time across trauma groups. Error bars represent one standard deviation

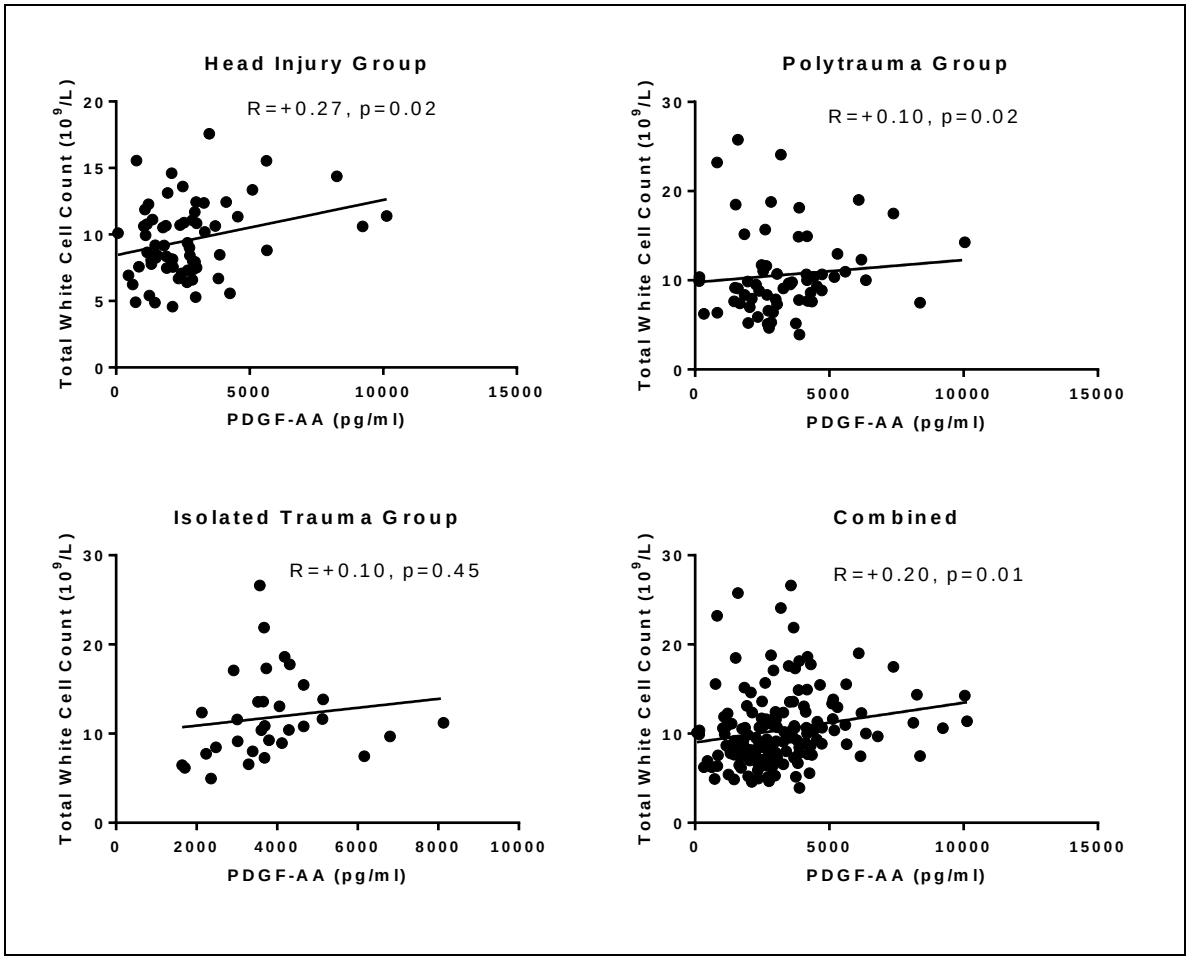

Figure 5.3-9 Relationship between matched WCC and PDGF-AA levels. Weak positive correlations were observed across all trauma groups

Analysis of the molecule PDGF-AA with WCC values (Figure 5.3-9) showed a positive correlation between these 2 parameters, albeit a weak one. Regression analysis revealed R-values ranging between +0.10 to +0.27. This weak association was fairly consistent across all trauma groups, with visual examination of the graphs revealing PDGF-AA values from Isolated Trauma to be least associated with WCC among all 3 groups.

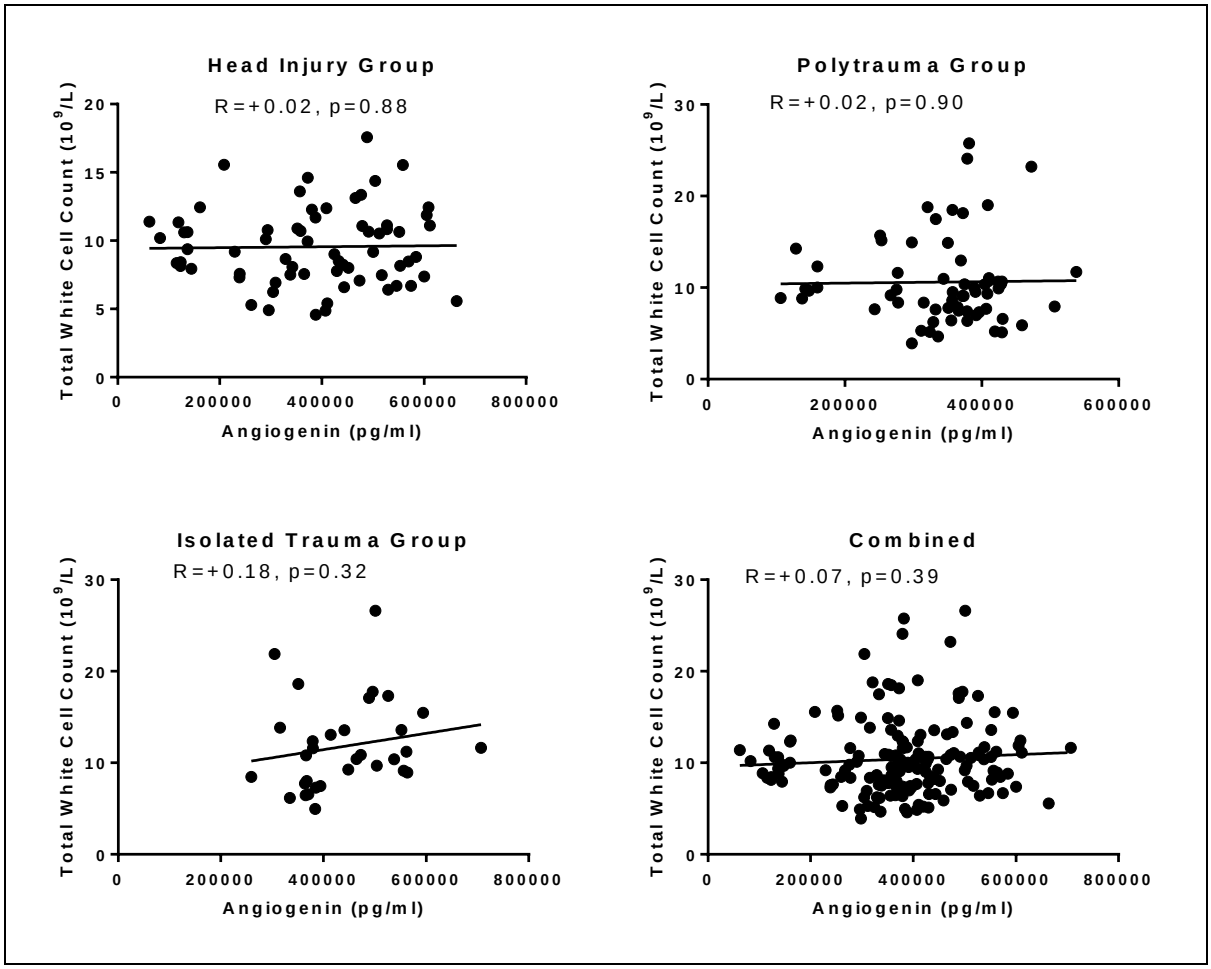

Figure 5.3-10 Relationship between matched WCC and Angiogenin levels. No association was observed between matched WCC and angiogenin values

Regression analysis of angiogenin with WCC (Figure 5.3-10) showed even weaker associations compared to PDGF-AA, with R-values consistently nearing zero (range +0.02 to 0.18). This is not surprising, given the relatively muted dynamics of angiogenin levels over time (Figure 5.3-8).

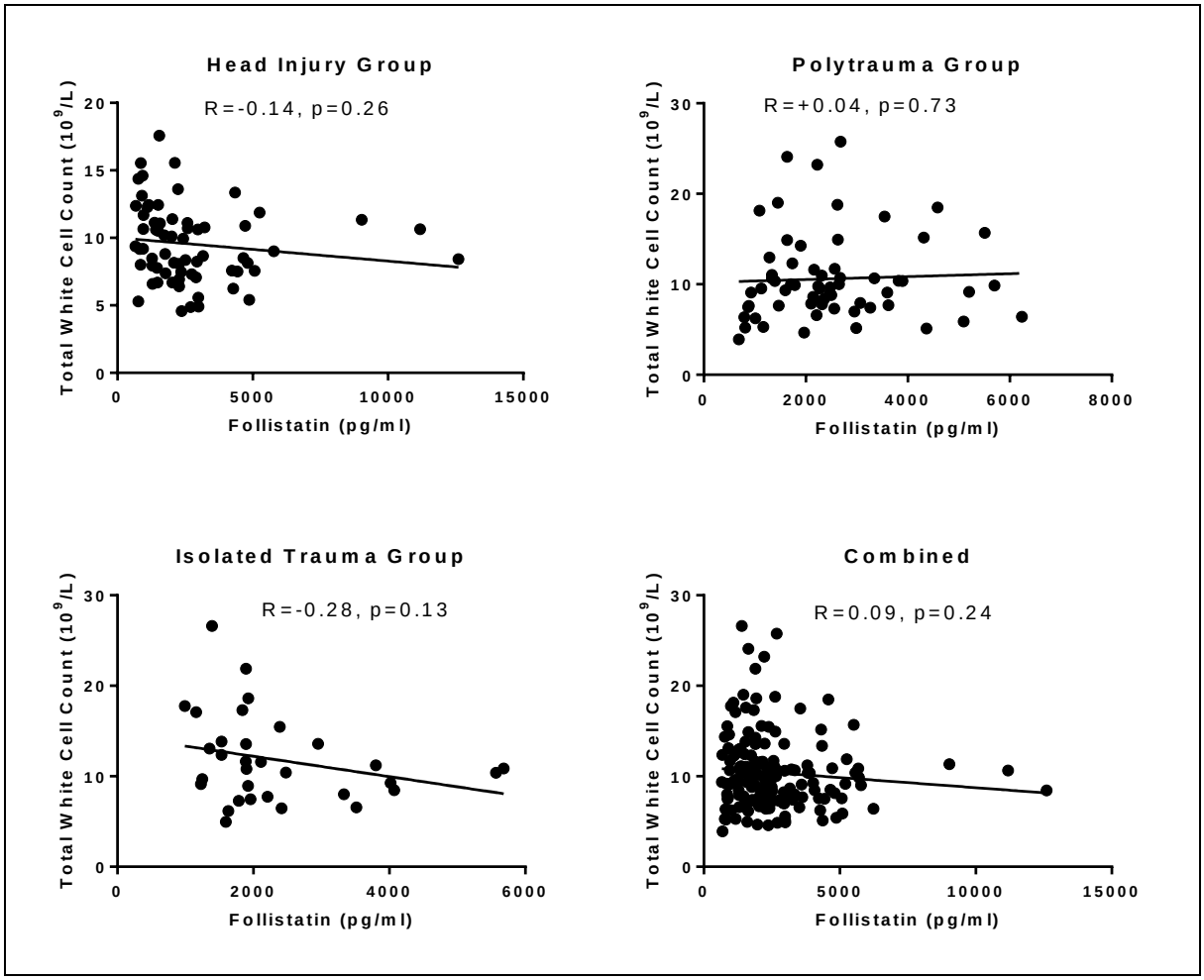

Figure 5.3-11 Relationship between matched WCC and Follistatin levels. No correlations were observed between matched WCC and follistatin levels..

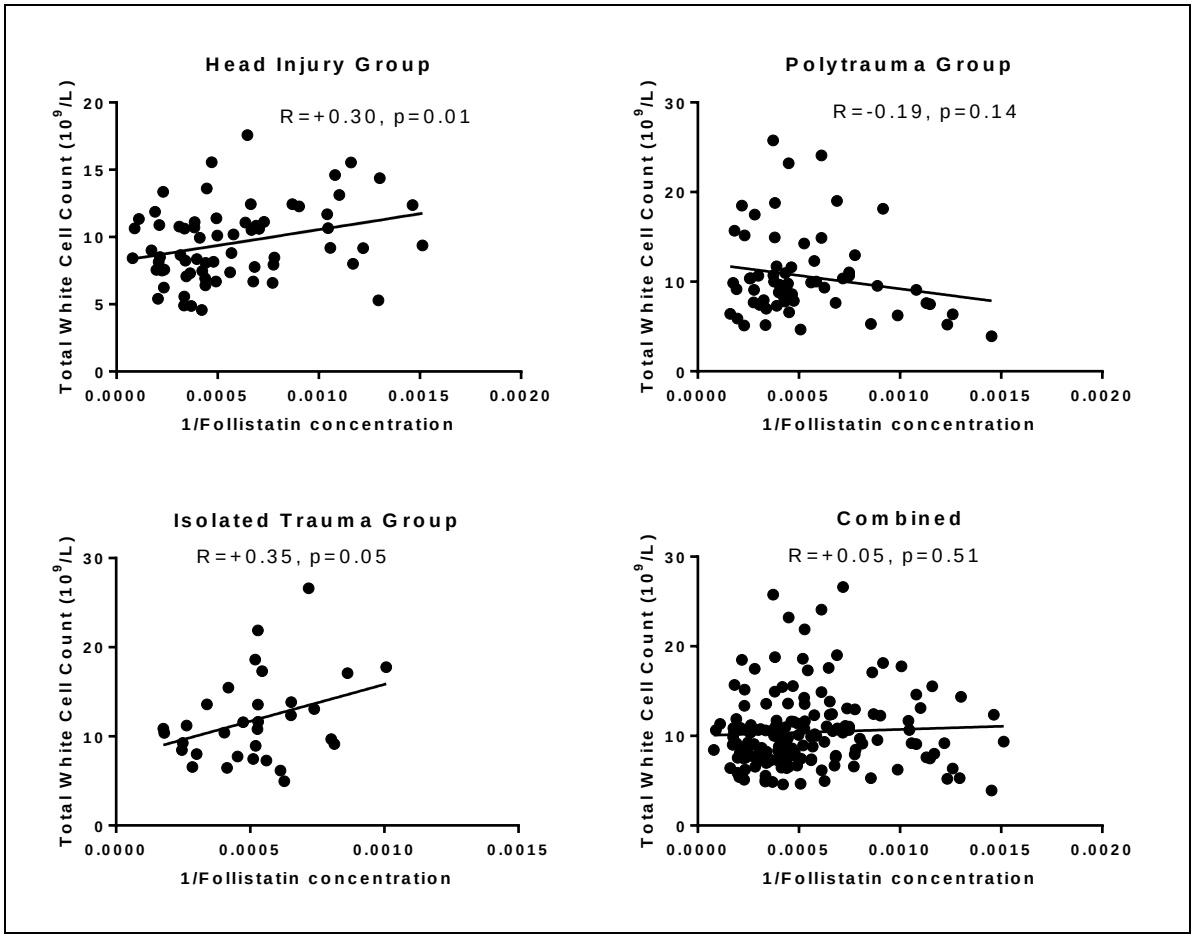

Figure 5.3-12 Inverse relationship between matched WCC and Follistatin levels. Weak inverse relationship observed with Head Injury and Isolated Trauma groups only.

Simple linear regression analysis between follistatin and WCC (Figure 5.3-11) showed little associations (R values between -0.28 and +0.04). However, visual inspections of the scattergrams (Figure 5.3-11) hinted at a possible inverse relationship. Therefore, the values were further analysed for an inverse relationship (Figure 5.3-12). Interestingly, only the Head Injury and Isolated Trauma group revealed a possible inverse relationship between WCC and Follistatin levels, with R values of over 0.30 for both groups (and $p<0.05$ for both groups). Overall, the inverse associations remain relatively weak although better than direct linear relationships.

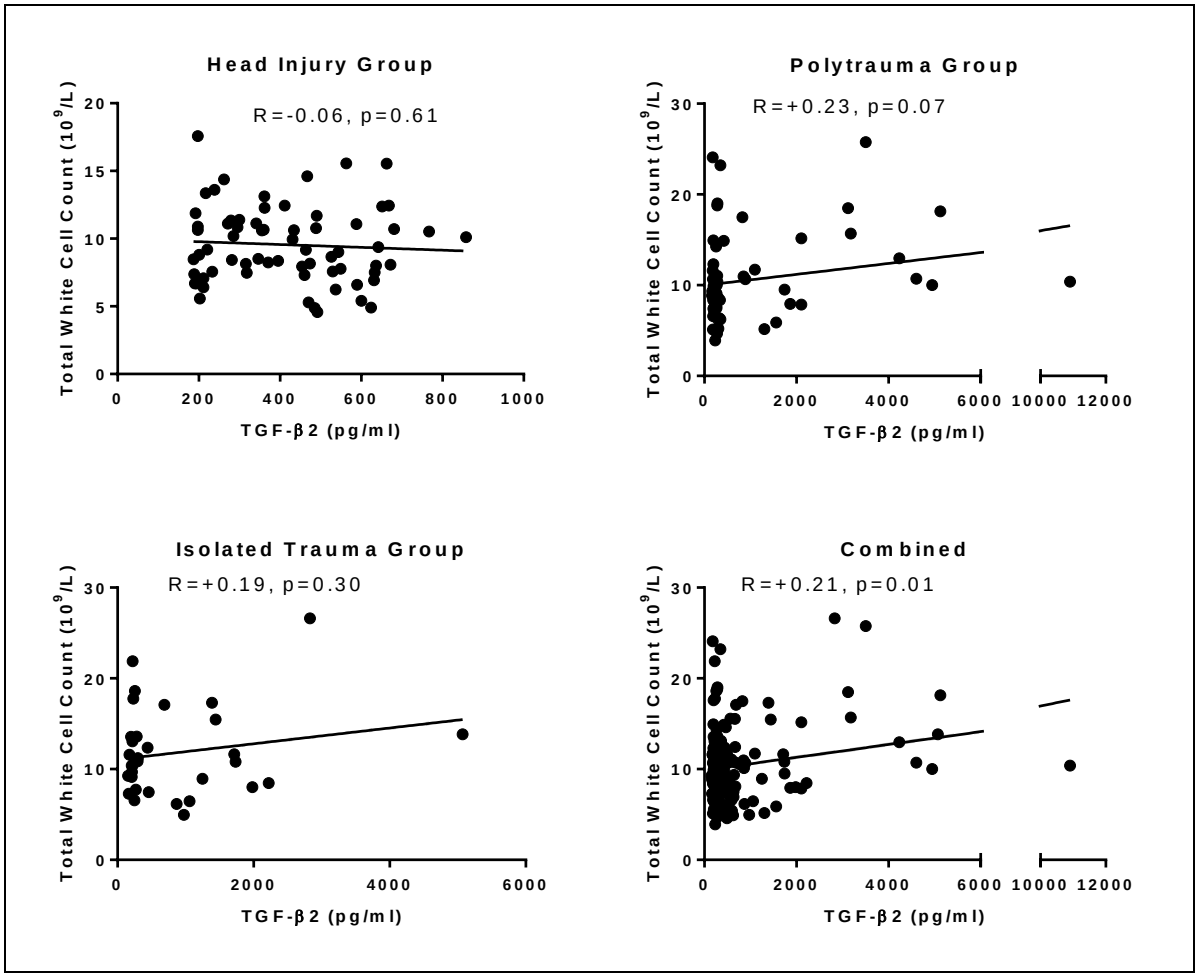

Figure 5.3-13 Relationship between matched WCC and TGF-β2 levels. No associations were found between WCC and TGF-β2.

Finally, similar to analysis of association with CRP above, TGF-β2 (Figure 5.3-6) was overall weakly associated with WCC levels. This is weakest in the Head Injury group (R=-0.06, p=0.61).

In summary, the 4 growth factors measured showed little associations with patient's matched total WCC levels. Table 10.2-2 illustrates the statistical results of regression analysis for all growth factors, across trauma groups against matched WCC values.

Platelets

Finally, time-matched data for platelet as measured in the patient's routine hospital laboratory were analysed against the growth factors. The sub-group of patients who had time matched samples were identical to the ones in the WCC sub-group (Table 5.3-2). Therefore, the summary trends over time across all trauma groups for the growth factors measured for this sub-group would also be identical to the WCC sub-group (Figure 5.3-8).

As can be seen in Figure 5.3-14, levels of platelet progressively rose over time following trauma, with levels up to 4-fold higher (Polytrauma group) by day 14 post trauma. This rise was consistent across all trauma groups.

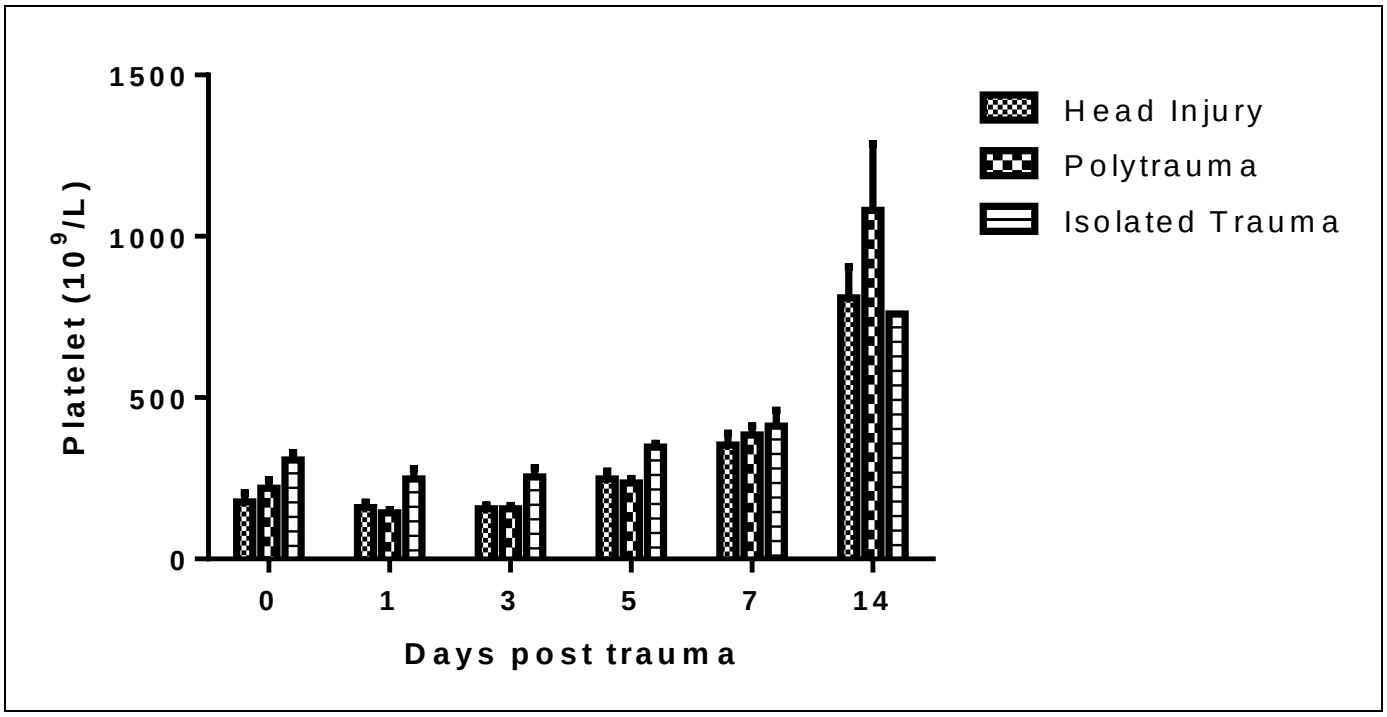

Figure 5.3-14 Mean Platelet values over time across trauma groups. Normal platelet values range 150-400X10^9/L. Error bars represent one standard deviation

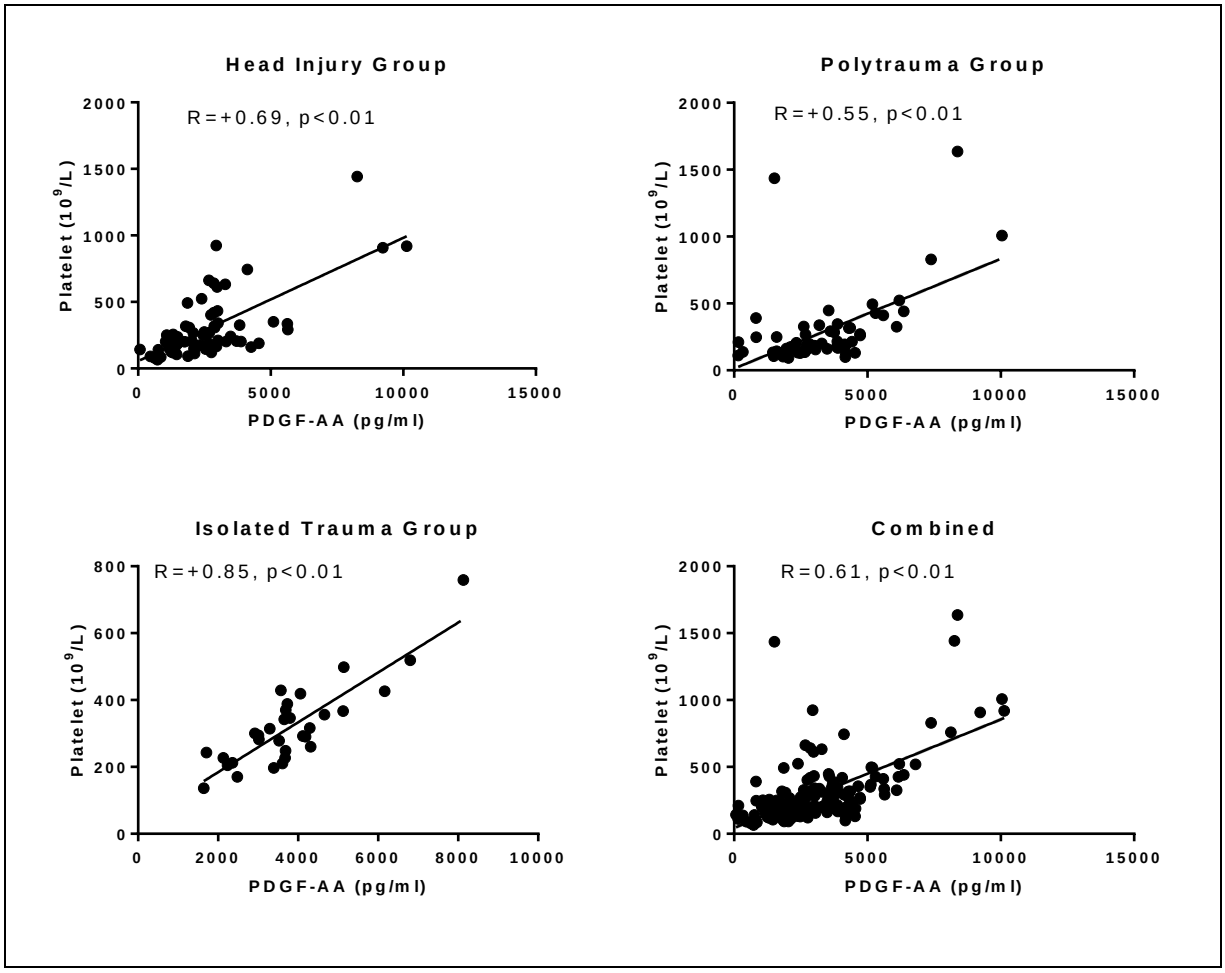

Figure 5.3-15 Relationship between matched Platelets and PDGF-AA levels. Statistically significant association (p<0.01) found between matched samples of platelets and PDGF-AA across all trauma groups.

As can be seen in Figure 5.3-15, PDGF-AA was significantly (p<0.01) correlated with time matched platelets values. This was independent of trauma type/severity, as can been seen by the statistically significant association seen when data from all groups were combined.

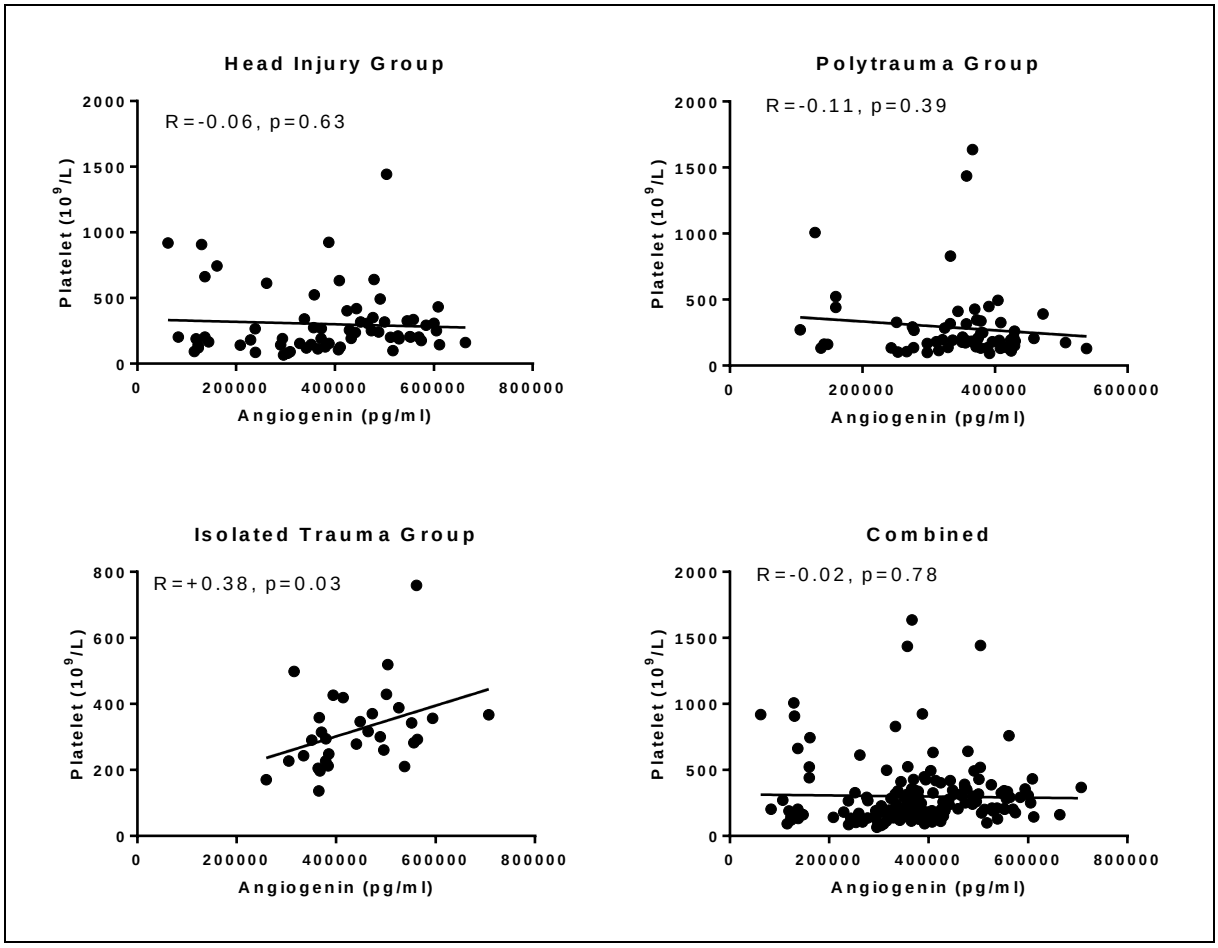

Figure 5.3-16 Relationship between matched Platelets and Angiogenin levels. Weak correlation found between matched platelet and angiogenin levels for the Isolated Trauma Group only.

However, similar to previous analyses with CRP and WCC, angiogenin was only weakly associated with platelet levels Figure 5.3-16). The strongest association was exhibited by the Isolated Trauma group (R=+0.38, p=0.03). In contrast, although both severe trauma groups (Head Injury and Polytrauma) showed a negative association, this was weak (R values -0.06 and -0.11 respectively) and likely to represent an absence of correlations.

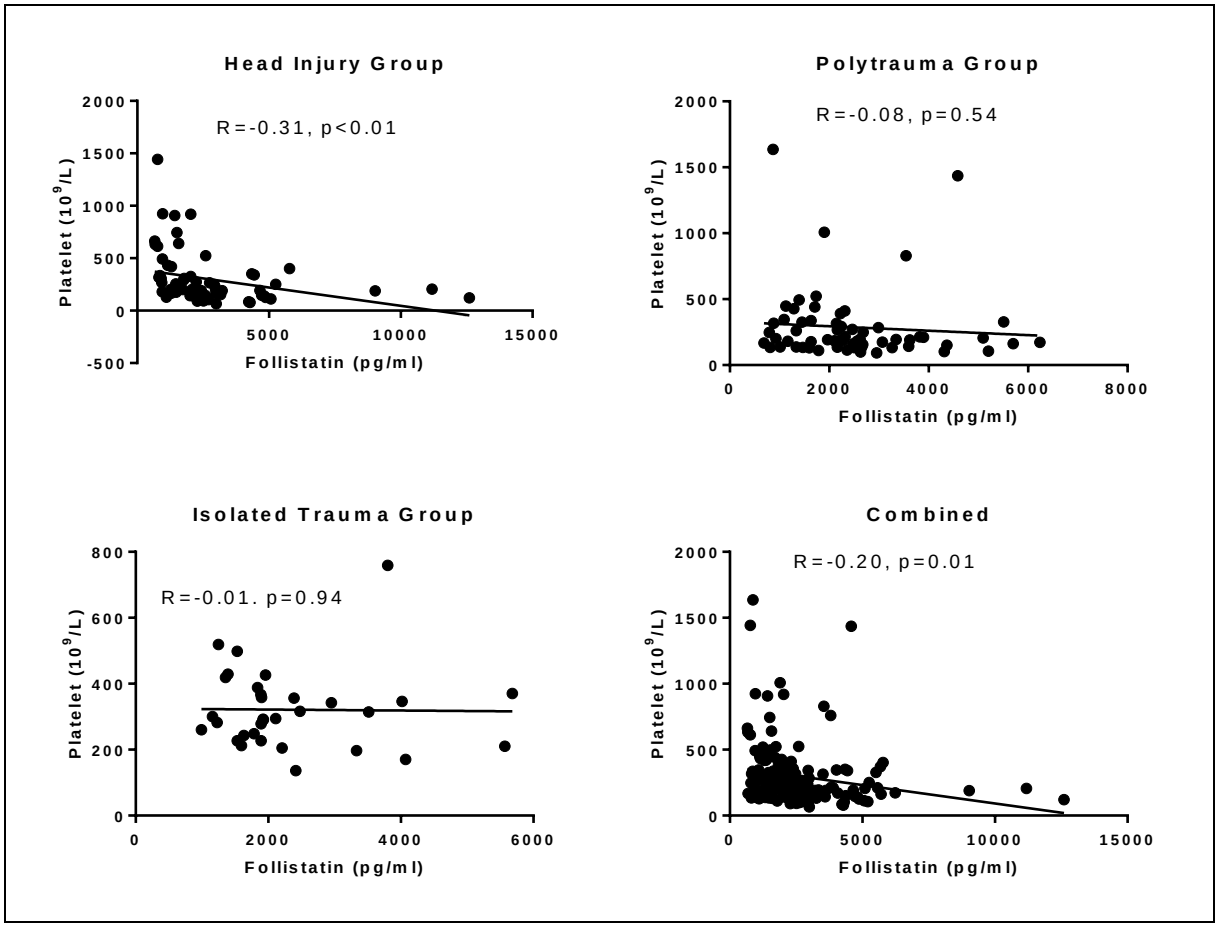

Figure 5.3-17 Relationship between matched Platelets and Follistatin levels. Weak negative correlation found between matched samples of platelet and follistatin in the Head Injury Group only.

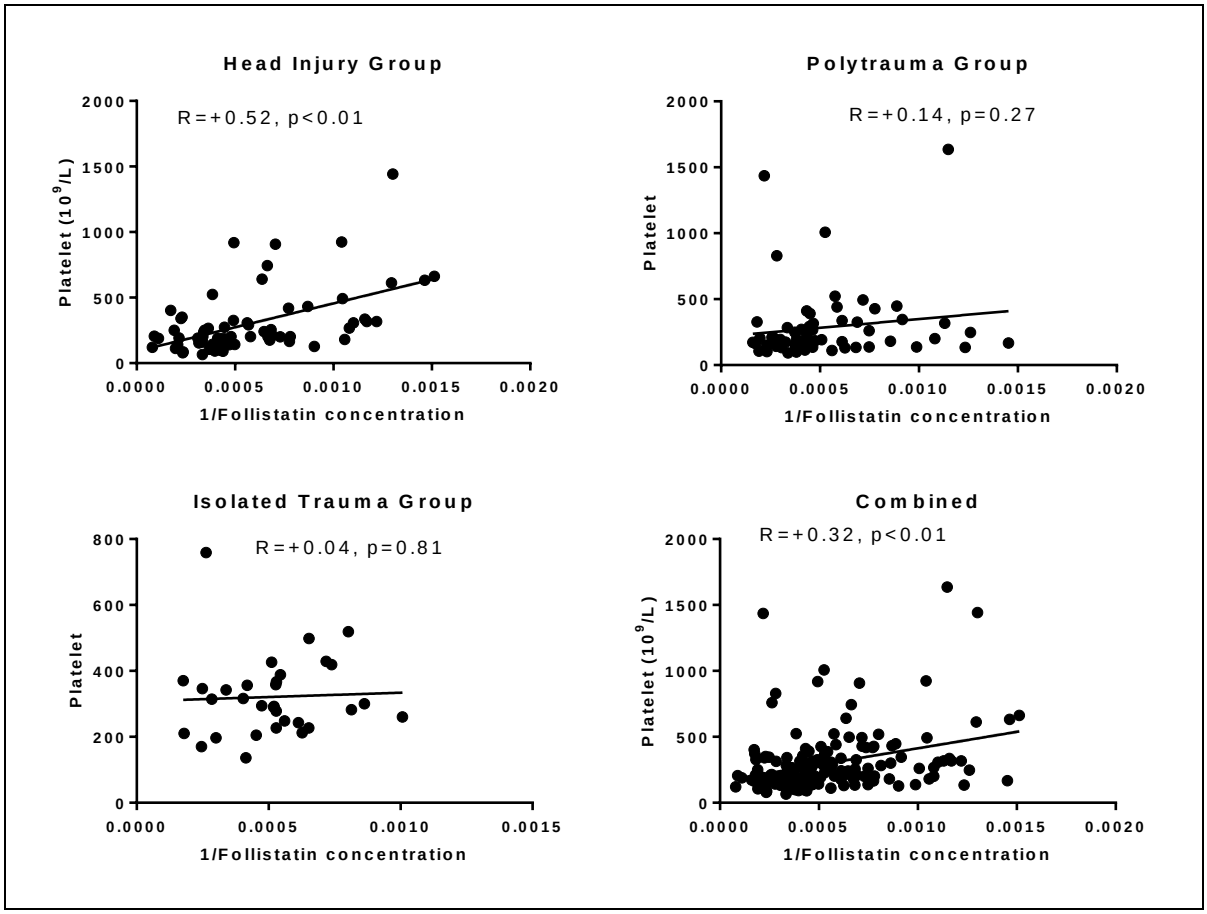

Figure 5.3-18 Inverse relationship between matched Platelets and Follistatin levels. Value for follistatin underwent inverse transformation. Statistically significant relationship found between platelet and inverse follistatin levels in the Head Injury group only.

Analysis of a linear relationship between follistatin and platelet (Figure 5.3-17) revealed relatively weak negative correlations across all trauma groups. However, similar to analyses with WCC, visual inspection of again hinted at a possible inverse relationship, especially in the Head Injury group. An inverse relationship analysis (Figure 5.3-18) showed an improved but still weak relationship within patients from the Head Injury group (R=+0.52, p<0.01), with the Polytrauma group exhibiting slightly weaker association (+0.14, p=0.27) and the Isolated Trauma group the weakest (R=+0.04, p=0.81). However, it appeared overall that follistatin is more likely to be inversely related to platelet levels as seen by the overall improvement in R and p-values on regression analysis.

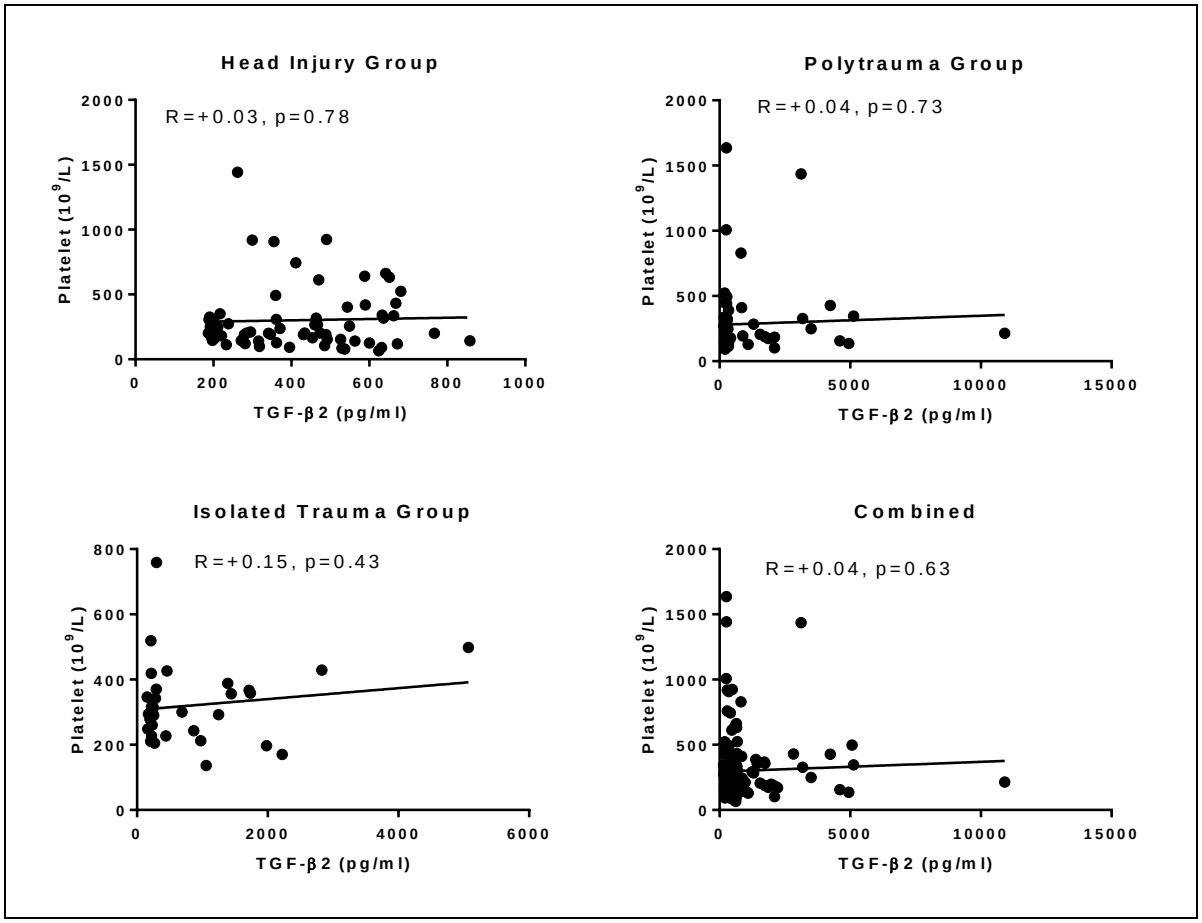

Figure 5.3-19 Relationship between matched Platelets and TGF-β2 levels. No correlation found between matched levels of platelets and TGF-β2.

Finally, regression analysis between TGF-β2 and platelet did not reveal any association, with R values weakly positive (range +0.03 to +0.15). This association appeared consistent with previous association analysis with CRP and WCC. In summary, PDGF-AA was strongly correlated with platelet levels independent of trauma types or severity, and follistatin was inversely correlated with platelet, especially in the Head Injury group of patients. Table 10.2-3 summarises statistical findings following regression analysis across all trauma groups against platelets.

5.3.2. Comparison against early complications (SIRS score)

So far, the relationship between the dynamics of growth factor release and inflammatory response has been explored with regards to levels of different parameters associated with inflammatory response (Section 5.3.1 and 5.3.2) as measured from the peripheral blood. Overall, this did not support the hypothesis that growth factor release was further upregulated by increasing inflammatory response following trauma.

To further analyse this hypothesis, an investigation was carried out on the patients' clinical response (degree of inflammatory response) following trauma. The Systemic Inflammatory Response Syndrome (SIRS) score was used as it represents a measure for a hyper-inflammatory clinical state, and have been predictive of poor outcome such as mortality(1992).

Data representing SIRS score for every matched time point to growth factor measurement were available for 12 Head Injury patients (Table 10.2-4), 14 Polytrauma patients (Table 10.2-5) and 11 Isolated Trauma patients (Table 10.2-6).

For the purpose of analysis, patients were divided into those that had a SIRS score 2 or more at any point during the observed period (herein called the SIRS group), and those that did not (herein called the No SIRS group).

Based on these criteria therefore, 8 out of 12 Head Injury patients, 10 out of 14 Polytrauma patients and 3 out of 11 Isolated Trauma patients would be considered to have developed a clinically significant inflammatory response to their injury. The differences in proportion of patients who developed early SIRS between all groups failed to reach statistical significance (chi-square test, Table 5.3-3).

Table 5.3-3 Comparison of proportion of patients with SIRS within first 24 hours of admission

Trauma Groups	p-value
Head Injury versus Polytrauma	0.79
Head Injury versus Isolated Trauma	0.14
Polytrauma versus Isolated Trauma	0.07

chi-square test, $p<0.05$ is statistically significant

In addition, analyses were separated according to trauma groups for each growth factor, to allow for any observed effect to be due to development of clinical SIRS only, and not the types or number of injuries that the patients sustained.

PDGF-AA

Within the Head Injury group, patients who developed SIRS appeared to have a more suppressed level of PDGF-AA over time, reaching a statistically significant (p=0.03) difference compared non-SIRS patients by Day 14 post injury (Figure 5.3-20). A similar pattern of suppression is observed in patients from the Isolated Trauma group. However, the presence of SIRS appears to exert the opposite effect on patients from the Polytrauma group. The differences observed in both the Polytrauma and Isolated trauma group failed to reach statistical significance across all time points (Mann-Whitney, Table 10.2-7).

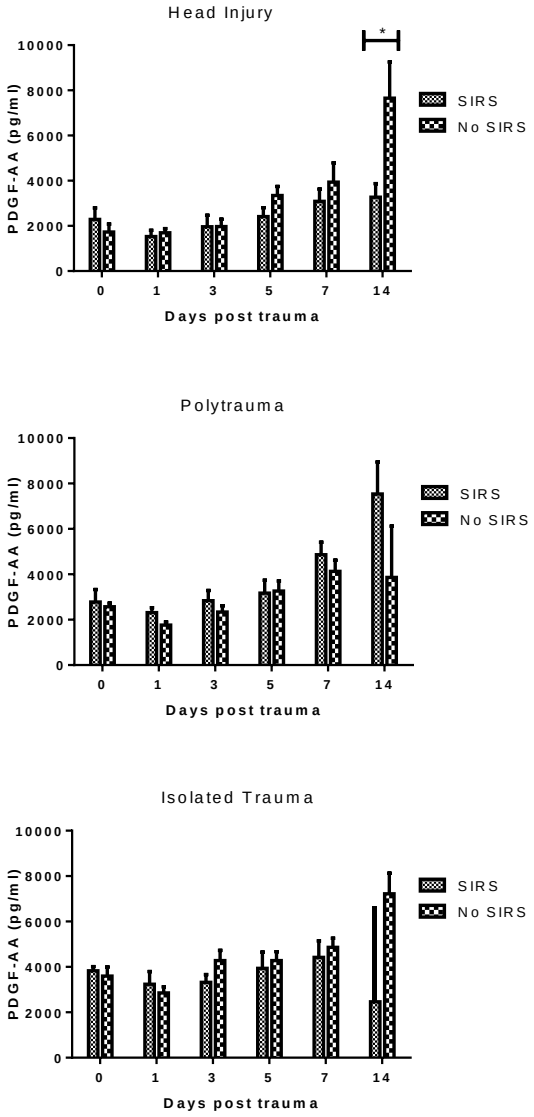

Figure 5.3-20 Comparison of PDGF-AA values between patients who developed SIRs to those who did not. Statistically significant differences were found on day 14 in the Head Injury group between SIRS and no SIRS group of patients. *denotes statistical significance at p<0.05

Angiogenin

Overall, there were no statistically significant differences (p>0.05) in angiogenin levels across all time points and all 3 trauma groups (Mann-Whitney, Table 10.2-8). There were no clear patterns of influence by the additional presence of SIRS on the dynamics of angiogenin over time (Figure 5.3-21).

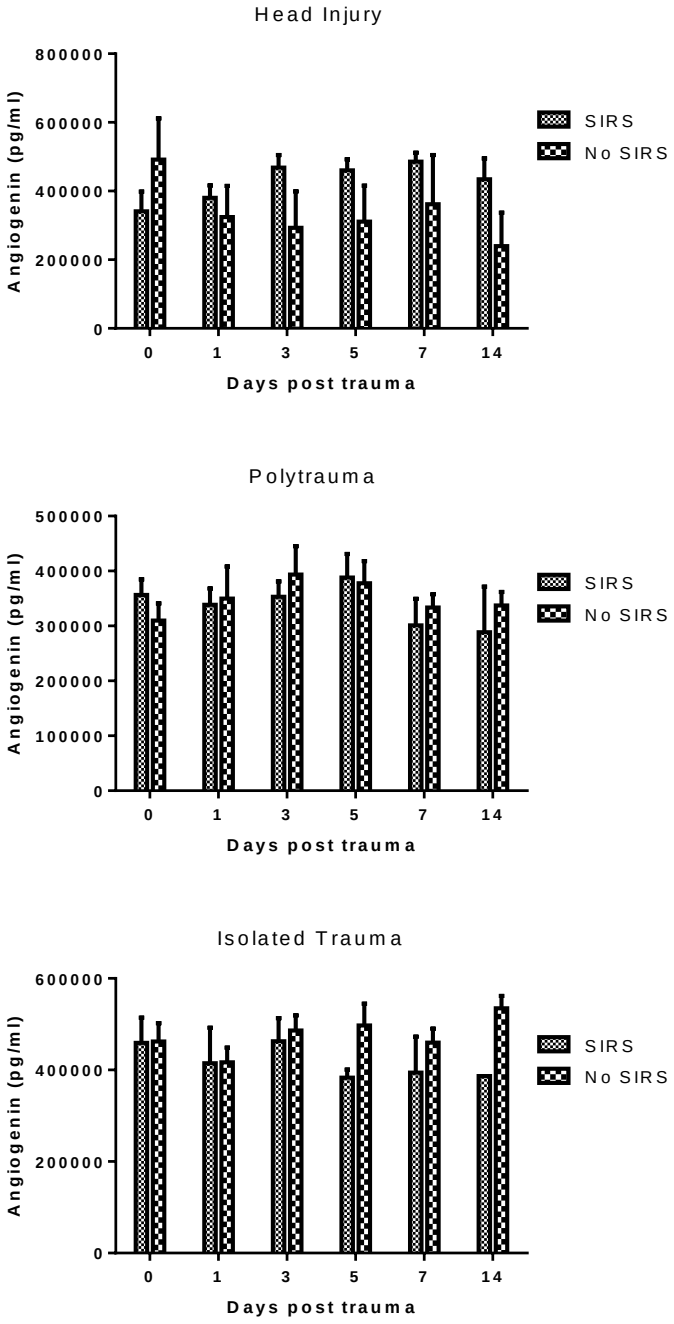

Figure 5.3-21 Comparison of Angiogenin values between patient who developed SIRs to those who did not. No significant differences were found. *denotes statistical significance at p<0.05

Follistatin

The presence of SIRS appears to have a stimulatory effect on levels of follistatin over time within patients from the Polytrauma group, with the higher levels reaching statistical significance ($p<0.01$) on Day 1 post trauma (Figure 5.3-22). The influence on patients from Head Injury and Isolated Trauma is less pronounced. Within the Head Injury group, presence of SIRS appear to have an initial stimulatory effect on the first 48 hours following trauma and a later stimulatory effect on patients from the Isolated Trauma group from Day 5 onwards. However, these observed differences failed to reach statistical significance (Mann-Whitney, Table 10.2-9).

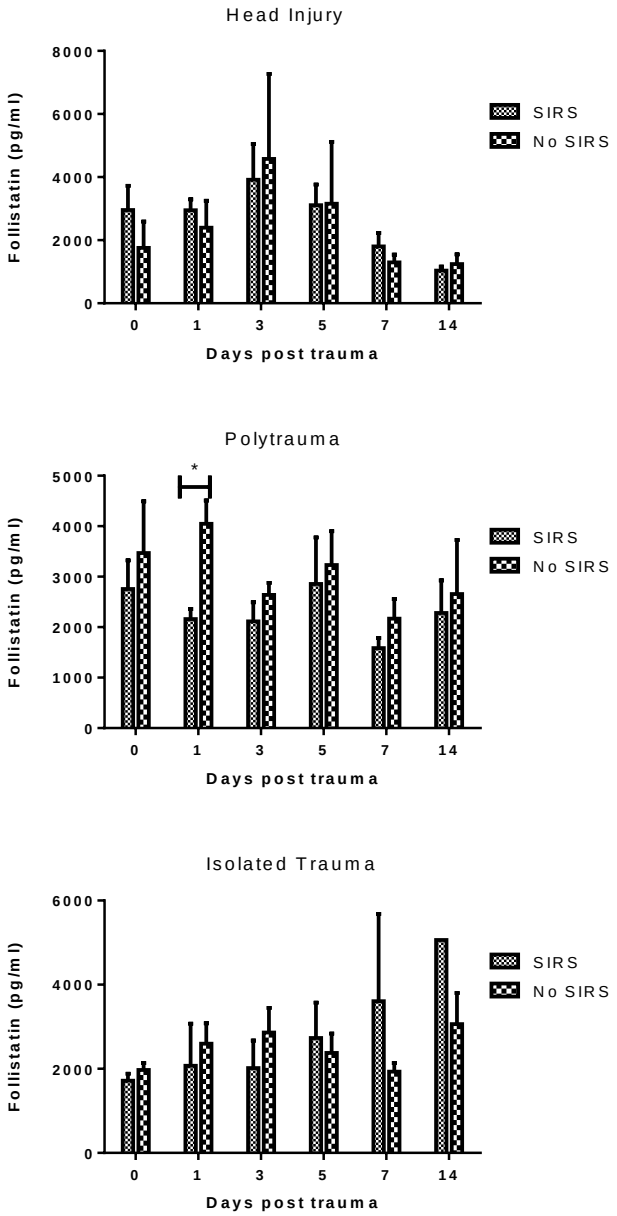

Figure 5.3-22 Comparison of Follistatin values between patient who developed SIRs to those who did not. Statistically significant difference was found on day 1 values within Polytrauma Group between those with SIRs compared to No SIRs. *denotes statistical significance at p<0.05

TGF-β2

Analysis of the effect of SIRS on levels of TGF-β2 was complicated by the high inter-individual variability, which has been previously discussed. Patients with SIRS from the Isolated Trauma group appeared to have an overall higher level compared to those who did not develop SIRS. However, both the Head Injury and Polytrauma group of patients do not exhibit any clear influence from the presence of SIRS on their TGF-β2 levels. None of the observed differences reached statistical significance (Mann-Whitney, Table 10.2-10).

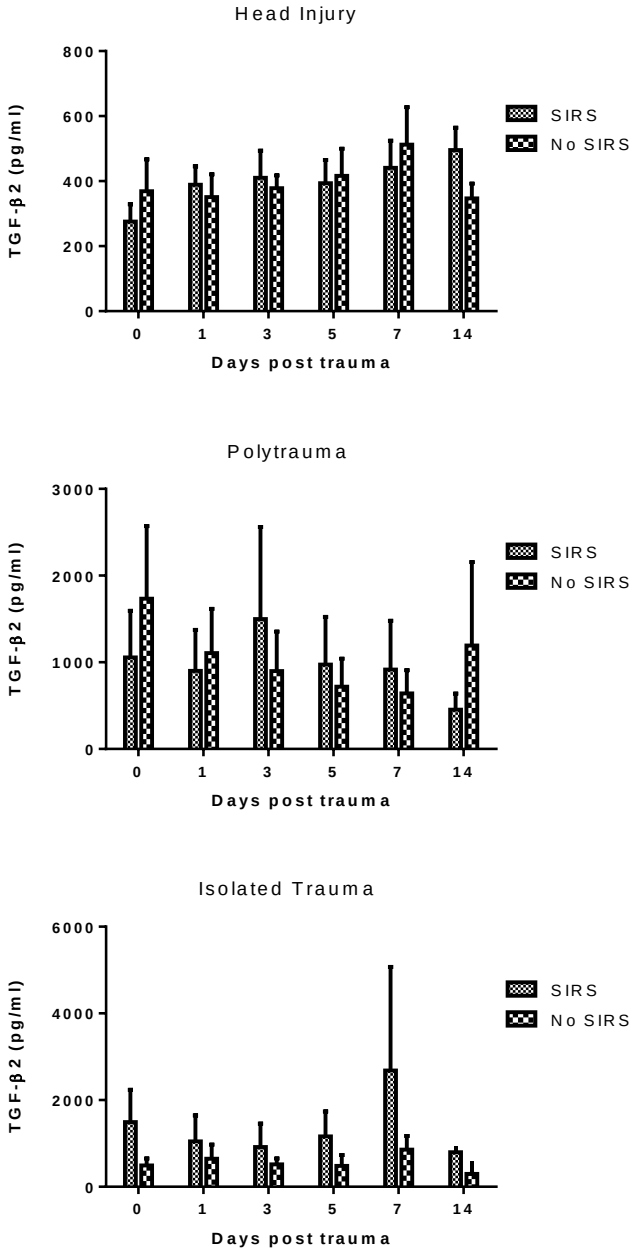

Figure 5.3-23 Comparison of TGF-β2 values between patient who developed SIRs to those who did not. No statistically significant difference found across all trauma group and time invervals. *denotes statistical significance at p<0.05

5.3.3. Comparison against late complications (delayed union, re-operation)

Following discharge from in-patient care, patients who were followed up in Leeds General Infirmary fracture clinic had their fracture healing outcomes recorded. This included the rate of fracture healing as well as requirement for revision surgery relating to the fracture.

In total, fracture healing data were available on 4 patients from the Head Injury Group, 11 patients from the Polytrauma Group, and 10 patients from the Isolated Trauma Group. One patient (BP8) from the Isolated Trauma group was excluded from this analysis as he was recruited to a clinical drug trial (potentially affecting bone metabolism) following discharge from the unit.

The purpose of this analysis was to ascertain whether the early growth factor dynamics (first 14 days post trauma) affected the fracture healing process (problem healers). As can be seen in Table 10.2-11, patients who experienced complications with their fracture healing process sustained either tibia or ulna fractures. To allow a comparative normal healing population, therefore, only patients who sustained long bone diaphyseal fractures (femur, tibia) formed the "control" or normal healing population.

6 patients were considered problem healers (BP4, BP15, BP18, BP3, BP7, BP13) whilst 14 patients made up the normal healing population (BH9, BH17, BP6, BP12, BP20, BP27, BP9, BP8, BP10, BP11, BP14, BP23, BP22, BP24). The median time to heal in the problem healer group was 42 weeks (range 28-84), whereas the median time to heal in the normal healer group was 12 weeks (range 7-20). This difference was statistically significant ($p<0.01$, Mann-Whitney).

The dynamics of both PDGF-AA and angiogenin did not show any difference between the problem and normal healing groups. Both groups and molecules exhibited similar trends of rise and fall in values over time (Figure 5.3-24). This commonality was confirmed with all the comparative analysis between the two healing groups failing to reach statistical significance for both PDGF-AA and angiogenin (Table 10.2-12).

Earlier analysis of general trends of follistatin showed an initial upregulation, with levels dropping over time from trauma. Interestingly, when values were analysed to differentiate the dynamics between problem and normal healers, a clear pattern of upregulation from trauma admission levels, sustained over the observed 2 week period, in the problem healing group. In fact, the median levels of follistatin in the normal healing group dropped nearly 25% from the first 24 hours to day 3 post trauma, whereas the problem healing group's follistatin level rose nearly 2-fold in the same time

period. This difference of median levels at Day 3 post trauma between both groups were statistically significant (p=0.03, Mann-Whitney).

And finally, the molecule TGF-β2 in analysis thus far has always presented difficulty due to its high inter-individual variability. However, despite this issue, when patients were sub-analysed based on their healing outcome, a clear pattern of difference emerged. Patients from the problem healing group showed a clear suppression of levels of TGF-β2 compared to the normal healing group. This difference was significantly different on Days 1 and 3 post trauma (p<0.05, Mann-Whitney).

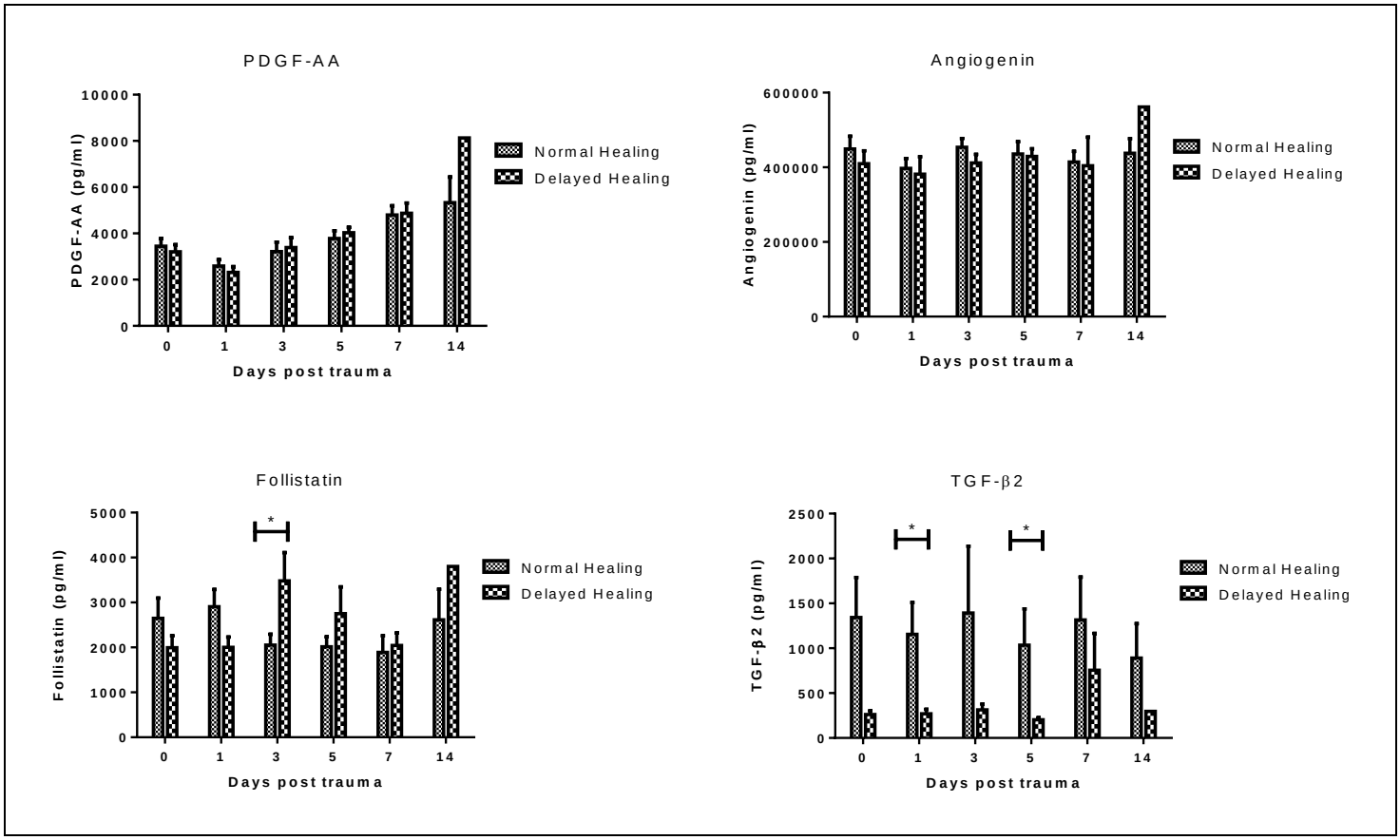

Figure 5.3-24 Comparative analysis of growth factor dynamics between patients with normal healing (n=14) versus delayed healing (n=6). Statistically significant depression of TGF-β2 levels were observed in patients who developed delayed healing compared to normal healing group. *denotes statistically significant difference at p<0.05

5.4. Discussion

5.4.1. Overview

In this chapter, I have attempted to further study the correlations between trauma severity and dynamics of growth factor release. The relationship between trauma severities as determined by the Injury Severity Score has already been discussed in the previous chapter. As trauma is known to elicit an inflammatory response, the correlations between growth factors and parameters from routine hospital blood tests known to be reflective of inflammation (C-reactive protein, total white cell count, platelets) were studied. Then, the patients' acute or early response to trauma, as measured by the Systemic Inflammatory Response Syndrome (SIRS) criteria was studied in correlation with growth factors. Finally, the relationship between the early dynamics of growth factors and fracture healing outcome was analysed.

To allow discussion and synopsis of the analyses, the growth factors are discussed in turn, bringing together all the laboratory and clinical elements

5.4.2. Comparison against hospital blood parameters

C-reactive protein (CRP)

Overall, levels of C-reactive protein were statistically significant both between trauma groups and over time ($p<0.0001$, 2-way ANOVA). It is unsurprising that Polytrauma and Head Injury groups exhibited the highest levels, given the known relationship between CRP and degree of inflammatory response (Black, Kushner et al. 2004).

Total white cell count

In contrast, the levels of total white cell count over time exhibited fewer degrees of changes. Although there was still significant difference over time ($p<0.0001$), the differences between groups failed to reach statistical significance ($p=0.19$, 2-way ANOVA). All groups showed highest levels on admission, reflecting the early role that leucocytes play following an acute injury or trauma.

Platelets

Generally, platelet levels were higher in Isolated Trauma groups ($p<0.0001$) compared to Polytrauma and Head Injury groups. This probably reflects a higher degree of consumption, blood loss or intravenous fluid dilution inherent in patients with more severe trauma. All groups showed an initial drop, before rising over time. This trend is in agreement with current literature(Akca, Haji-Michael et al. 2002). However, by the second week, the significant rise ($p=0.02$, 2-way ANOVA) was observed in levels of platelet in Polytrauma and Head Injury groups reflecting a higher degree of reactive thrombocytosis in these more severely injured groups (Figure 5.3-14).

5.4.3. Validity of SIRS parameter

The criteria for SIRS were used to reflect the systemic inflammatory response of a patient. These were set out based on the seminal work by Bone et al, which shows that patient who fits the criteria for SIRS on the first day of admission to intensive care unit has significantly higher risk of mortality(1992). Elevation of total white cell (leukocytosis) reflects the degree of release of pro-inflammatory cytokines (Keel and Trentz 2005). Conversely, cellular apoptosis could lead to a drop in total white cell (leukopenia) (Keel and Trentz 2005).

An increase in core temperature or fever is due to the presence of mediators called endogenous pyrogens. These include pro-inflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and interferon gamma (IFN-γ). These cytokines induce the production of prostaglandins in the central nervous system, leading to fever. Although the mechanism for hypothermia (<36°C) in SIRS remains unclear, the presence of hypothermia in unwell patients (intensive care unit) is associated with a significantly higher risk of mortality (Clemmer, Fisher et al. 1992). Murine models of SIRS showed that hypothermia was associated with significantly higher levels of IL-6, reflecting a more severe inflammatory response(Remick and Xioa 2006).

As previously discussed local inflammatory response lead to vasodilatation and increased tissue permeability. This causes a drop in blood pressure and a compensatory rise in heart rate to maintain cardiac output(Foex 1999).

And finally, the release of inflammatory mediators may cause endothelial lung injury, resulting in pulmonary oedema. This is then accompanied by a worsening ventilation perfusion mis-match and resultant hypoxaemia. The body compensates by increasing its respiratory rate, which is also reflected by a drop in arterial levels of carbon dioxide (metabolic alkalosis) (Bernard, Artigas et al. 1994).

Individually, these parameters may not necessarily represent the onset of SIRS. For example, a patient admitted acutely following trauma may present with a raised heart and respiratory rate, secondary to pain. Even Professor Bone in his consensus paper cautioned that these guidelines need to be assessed in relation to the overall clinical presentations (1992). However, in the context of trauma and this study, these criteria taken collectively are a simple and quick way to detect unwell patients, and to represent patients with a heightened inflammatory response.

5.4.4. Platelet derived growth factor (PDGF)-AA

PDGF-AA showed a statistically significant ($p<0.01$) direct correlation with platelet count. This was independent of trauma severity and type. In fact, 5 of the patients within this analysed cohort received platelet transfusions during the period of analysis. This implies that the correlation between PDGF-AA with platelet is independent of whether the source of platelet is endogenous or transfused.

PDGF-AA was only weakly correlated with CRP levels and total WCC. The relationship between PDGF-AA and CRP is probably mediated through the cytokine interleukin-6. A positive correlation was found between IL-6 and PDGF-AB (and not –AA) levels in synovial fluid of rheumatoid arthritis patients, but neither affected the growth rate of murine fibroblastic cell lines (Monier, Reme et al. 1994). Although a negative correlation between PDGF-AA and IL-6 levels (both plasma and serum) was found in this study, this was not statistically significant. Additionally, when patients were divided based on their clinical presence of SIRS, no differences were found in the levels of PDGF-AA in the first week following trauma.

Overall, this implies circulating platelet as a source of PDGF-AA in peripheral circulation, a level which is not influenced by trauma severity.

5.4.5. Angiogenin

Overall, the dynamics of angiogenin in the first two weeks following trauma have been rather muted across all trauma groups. The positive correlations found when compared against matched serum levels of IL-6, agrees with a previous study which found that IL-6 directly increases angiogenin protein and mRNA expressions in a murine model (Verselis, Olson et al. 1999). Therefore, the general lack of associations in Head Injury and Polytrauma group, and the negative association in Isolated Trauma between angiogenin and CRP was a surprising finding. This was further confirmed

when angiogenin levels were also not influenced by the additional presence of SIRS in these cohort of patients.

5.4.6. Follistatin

Follistatin is involved following trauma, through its complex relationship with Activin A and the inflammatory cytokines (See Figure 5.4-1). Therefore, the positive correlations between CRP and follistatin levels observed in this study would agree with this relationship. This is in agreement with reported literature on septic patients, where follistatin, activin and CRP levels paralleled each other (Michel, Ebert et al. 2003). Additionally, the strong positive correlations between serum IL-6 and follistatin observed in my study further confirms this relationship, given that CRP production is directly stimulated by IL-6 (Kushner, Jiang et al. 1995).

The lack of relationship with white cell count is in agreement with published literature (Michel, Ebert et al. 2003). Interestingly, despite its association with CRP levels, overall the additional presence of SIRS failed to show any statistically significant difference in follistatin levels. The lack of relationship with WCC (which is a parameter for SIRS diagnosis) may have contributed to this lack of difference.

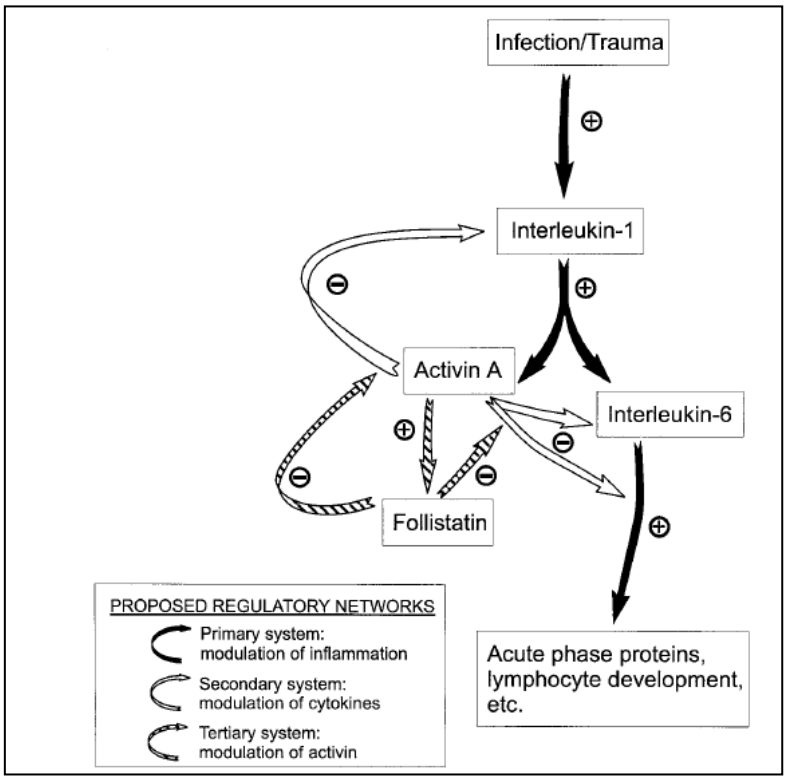

Figure 5.4-1 The interrelationships between activin A, follistatin and the proinflammatory cytokines, IL-1 and IL-6 (From Kretser 1999) (de Kretser, Hedger et al. 1999).

5.4.7. Transforming growth factor (TGF)-β2

Generally, levels of TGF-β2 failed to show any associations with all the measured parameters (CRP, WCC, platelets). When the patient cohorts were divided based on the presence of SIRS, this did not show any statistically significant differences, probably due to the high inter-individual variability previously discussed.

5.4.8. Late complications following trauma (non-union, revision surgery)

Two of the molecules (follistatin, TGF-β2) showed very interesting dynamics when patients were divided based on whether they had normal or problem with their fracture healing.

Patients with normal healing profile showed decreasing trends in follistatin levels, from admission level above healthy control, dropping significantly ($p<0.05$) by the third day post trauma. However, patients in the "problem" healing cohort were observed to exhibit the reverse trend. Not only were the admission levels lower than the healing cohort, this group then showed a rising trend, reaching statistical significance by Day 3 post trauma. A previous work by Dimitriou et al (Dimitriou, Carr et al. 2011), showed that genetic polymorphism for the noggin gene (an inhibitory molecule) was significantly associated with risk of fracture non-union. The authors postulated whether these patients with noggin polymorphism may exhibit higher levels of noggin following fracture, and hence affecting the fracture healing process. Similarly, in this study, the abnormal rise in follistatin level following trauma may either imply an abnormality in the fracture healing process, or these patients may be similarly genetically predisposed to possess a different follistatin expression following trauma.

Patients with "problem" healing showed a clear suppression in levels of TGF-β2 across all time points. This was despite the high inter-individual variability exhibited in the levels of this particular growth factor. A previous study showed a statistically significant decline in TGF-β1 at Week 4 occurring in patients with delayed healing (Zimmermann, Henle et al. 2005), implying the major role played by the TGF-β isoforms in the fracture healing process. This was further supported by another genetic polymorphism study confirming the association of TGF-β codon 10 mutant T and T/C allele to predispose to impairment of fracture healing (Szczesny, Olszewski et al. 2011).

In conclusion, the levels of PDGF-AA were reflected and influenced by levels of platelets only. Angiogenin did not show any association with both measured biochemical parameters (WCC, CRP,

platelets) and clinical parameters (SIRS) of inflammatory response. Finally, early dynamics of follistatin and TGF-β2 may potentially be indicative of fracture healing outcome.

5.4.9. Limitations

This study was limited by the availability of matched hospital laboratory results to the measured time points for growth factor analysis. The hospital laboratory tests were carried out based on clinical needs.

Following discharge from hospital, some patients were lost to follow up, mostly due to them being followed up in other hospitals. A few patients unfortunately also died prior to their healing status being confirmed.

The exact time to fracture healing is almost impossible to determine, unless patients are followed up every day up to the point of fracture healing. However, most fractures would be expected to heal by six months, and together with the additional criteria of revision surgery requirement, the decisions to decide on the cohort of "problem" healing patients would be considered reasonably robust. However, the need to allow a more uniform patient cohort in terms of fracture type, led to analysis of patients in both groups presenting with long bone fractures only. This resulted in a lower number of patients to be analysed.

6. RESULTS: Mesenchymal Stem Cell (MSC) and Trauma

6.1. Introduction

MSCs are multipotent cells capable of differentiating and giving rise to diverse cells such as osteoblasts, chrondrocytes, and adipocytes (Bianco, Cao et al. 2013). They were first identified from BM as colony-forming unit-fibroblasts (CFU-Fs) by Friedenstein (Friedenstein, Gorskaja et al. 1976). Since then, MSCs have been found in the stroma of spleen, thymus (Friedenstein, Gorskaja et al. 1976), trabecular bone (Tuli, Seghatoleslami et al. 2003), cartilage, synovium, periosteum (De Bari, Dell'Accio et al. 2008), adipose tissues (Zhu, Liu et al. 2009), arterial walls (Abedin, Tintut et al. 2004), as well as umbilical cord (Erices, Conget et al. 2000) and fetal circulation (Campagnoli, Roberts et al. 2001). However, it is generally accepted that osteogenic potential and by inference bone repair is best from MSCs obtained from tissue of osseous origin (De Bari, Dell'Accio et al. 2008).

6.1.1. Definition and enumeration of MSCs

The International Society of Cellular Therapy (Dominici, Le Blanc et al. 2006) defines MSCs by three main characteristics:

- adherence to plastic in standard culture conditions
- expression of a set of molecules (CD105, CD73, CD90) with absence of other markers (CD45, CD34, CD14/CD11b, CD79α/CD19, HLA-DR)
- *in vitro* differentiation into osteoblasts, adipocytes, chondroblasts (demonstrated by staining of differentiation cultures) (Pittenger, Mackay et al. 1999; Bianchi, Muraglia et al. 2001).

The ability of MSCs to adhere to plastic and proliferate has been exploited in the colony forming unit fibroblast (CFU-F) assay. Briefly, the culture of BM cells either as direct from aspirate (Galotto, Berisso et al. 1999) or separated by differential gradient centrifugation first (Castro-Malaspina, Gay et al. 1980) has been shown to produce proliferating colonies of fibroblasts. Each colony has been shown to originate from a single colony-forming cell or CFU-F (Castro-Malaspina, Gay et al. 1980) and to have an osteogenic potential (D'Ippolito, Schiller et al. 1999). Normally, MSCs are enumerated based on this assay and counted as CFU-Fs.

6.1.2. MSCs in peripheral circulation

Various sources of MSCs have been previously discussed. However, of considerable interest is the issue of the presence of MSCs in peripheral circulation. Fibroblast-like cells with osteogenic and adipogenic potential, resembling MSCs has been isolated in animal and human peripheral blood (Kuznetsov, Mankani et al. 2001) but in very low numbers. Similar experiments have found a small population of CD34-negative mononuclear cells with MSC-like features (Zvaifler, Marinova-Mutafchieva et al. 2000) with significantly higher amounts seen in peripheral circulation of burn victims(Mansilla, Marin et al. 2006) or following muscular tissue damage (Ramirez, Lucia et al. 2006). However, it must be pointed out that a physiologically relevant pool of circulating MSCs as possible vehicles aimed at fracture repair is extremely contentious. Application of rigorous phenotypic and functional criteria has failed to show evidence for such a population in healthy donors(Jones and McGonagle 2008).

6.1.3. MSCs and trauma

Traditionally, the frequency of these MSCs in the iliac crest bone marrow (ICBM) aspirates is believed to be as low as 30 in a million mononuclear cells (Cuthbert, Boxall et al. 2012). However, recently the Leeds MSC Group (Jones, English et al. 2010) have shown that cells with MSC colony-forming capacity and the surface phenotype are abundant in the trabecular bone niche and incorporated into a three-dimensional network composed of stromal reticular cells and intracellular matrix. Matrix-bound MSCs are however, not released during normal marrow aspiration but can be readily procured using enzymatic release techniques.

Given the known inflammatory response and growth factor release following trauma, it could be speculated that these post-traumatic molecular responses may lead to mobilisation of MSCs from their *in vivo* niches into peripheral circulation.

Although Seebach (Seebach, Henrich et al. 2007) reported a significant difference in ICBM MSC CFU-F numbers between “multiple trauma” and “monofracture” patients, the timing of sample acquisition was unclear and therefore could have influenced their findings. As growth factor in circulation has been shown to be dynamic, it is possible that MSC numbers at different timings following trauma are also dynamic. My study sampled both the bone marrow and peripheral blood at the same time points, and also correlated with levels of growth factors measured at these same time points.

6.2. Methods

6.2.1. Patient Recruitment

Patients were recruited for bone marrow MSC response investigation. Approval from local ethics committee (*Leeds West REC 06/Q1206/127*) was obtained prior to commencement, with all patients providing informed written consent.

To address the role of trauma severity on the MSC dynamics in iliac crest bone marrow and in peripheral circulation, patients recruited into the MSC study were divided into 2 groups, Polytrauma (ISS≥16) and Isolated Trauma (ISS<16).

6.2.2. Sampling time points

Patient ICBM samples can only be obtained when the patient undergoes surgery, as clinically clean and sterile conditions are required during bone marrow aspiration. In addition, due to the expected high inter-individual variations in MSC CFU-F count (Castro-Malaspina, Gay et al. 1980), in order to study the effect of trauma over time on ICBM MSC numbers, at least 2 sampling time points were required, with the first sampling time point (on admission, within 24 hours of injury) acting as baseline value for the individual patients. Hence, for the purpose of recruitment, all patients within the MSC study had to have injuries requiring surgery on admission, followed by another surgery a few days later.

6.2.3. Sample acquisition

Iliac crest bone marrow (ICBM) aspirates were collected on every episode that the patient was in theatre for their operative treatments. Under sterile conditions, ICBM was aspirated from the anterior iliac crest using a trocar (11 Gauge 5" Bevel Tip Match-Ground Introduction Needle, Stryker 306-111) and a 20ml syringe. The trocar/syringe setup was pre-flushed with heparinised saline. Samples obtained were immediately transferred to 5ml EDTA-containing tubes (Vacutainer® BD) to prevent clotting and gently agitated prior to transfer to tissue-culture facility. For each episode of ICBM collection, prior to start of the patient's operative procedure 20ml of peripheral blood (PB) was obtained and collected in 5ml EDTA-containing tubes.

6.2.4. CFU-F assay using ICBM – Direct plating

The assay was performed as described previously by Galotto (Galotto, Berisso et al. 1999). Both ICBM and PB samples were used for direct plating. Nucleated cell count (NCC) enumeration was performed using 3% acetic acid. The counting procedure is described in section 6.2.7. ICBM samples were then seeded volumetrically (300µL per 10-cm diameter Petri dish, in triplicate) in 10ml of attachment media (DMEM/10% FCS/Pen-Strep) and incubated at 37°C, 5% CO2 for 48 hours. Non-adherent cells were then washed off with two 10-ml PBS washes and adherent cells were allowed to grow in non-haemopoietic (NH) Media (Miltenyi Biotec) with twice-weekly half-media changes, for 14 days. On day 14, all media was gently removed from dish, and adherent cells were gently washed with 2 ml PBS. Adherent cells were fixed with 2ml of 3.7% formaldehyde (Fischer Scientific) at RT for 15 minutes. Following removal of formaldehyde, colonies were stained with 2mL 1% Crystal violet (BD Lab) for 2 minutes, washed and air dried. Scoring was performed macroscopically and colony counts over triplicates were calculated to give an average CFU-F score per sample.

6.2.5. CFU-F assay using ICBM – MNC plating

Direct plating is a simple method for the evaluation of CFU-F/ml in a given BM sample but it has potential disadvantages including an occasional formation of clots in which CFU-Fs can be trapped (noticed previously in our laboratory) as well as potential interference from red cells that could inhibit MSC attachment. Hence in this study a parallel evaluation of CFU-F content of ICBM samples was performed based on the isolation and plating of $3X10^6$ mononuclear cells (MNCs) (Castro-Malaspina, Gay et al. 1980) with subsequent calculations of CFU-Fs/ml performed using to the following formula:

CFU-F/ml = CFU-F/10^6 MNCs x MNCs/ml

To perform this assay, sample volumes were first measured prior to 1:1 dilution with PBS. Up to 20mL of diluted samples were then carefully layered onto 20mL of Lymphoprep (Axis-Shield) and underwent centrifugation at 650g at brake rate 0, room temperature (RT) for 25 minutes, to isolate the MNC fraction. The MNC fraction (the interphase) was then collected using a Pasteur pipette, placed into a fresh 50ml Falcon tube and 10ml of PBS was next added to perform washes aimed to remove the residual Lymphoprep. Cells were washed by centrifugation at 400g at brake rate 9, RT for 5 minutes, after which the Lymphoprep/PBS mixture was removed by decanting, leaving the

MNC pellet intact. The pellet was broken by gentle tapping and re-suspended in appropriate volume of PBS (2-10ml), depending on the size of the pellet.

MNC count was next performed using trypan blue exclusion. Counting was performed as described in Section 6.2.7. The MNCs were then seeded in triplicate 10-cm diameter Petri dishes in 10ml of attachment media ($3x10^6$ MNC/dish) and incubated at 37°C, 5% CO_2 for 48 hours. Non-adherent cells were removed with two 10-ml PBS washes and adherent cells were allowed to grow in NH Media with twice-weekly half-media changes, for 14 days, after which CFU-F staining and enumeration was performed as described above (Section 6.2.7).

6.2.6. CFU-F assay using PB – Direct and MNC plating

Methods described above for Direct and MNC plating of ICBM were similarly applied to PB obtained at the same time as ICBM sampling. 20ml of PB was obtained from antecubital vein of the patient and collected in 5ml EDTA-containing vacutainer tubes. The seeding density for Direct plating is similar, volumetrically at 300µL per dish in duplicate. The seeding density for MNC plating is however, higher at $1x10^7$ MNC/dish in duplicate, as circulating MSCs are exceptionally rare in man (Kuznetsov, Mankani et al. 2001) and therefore a higher seeding density would allow the detection (as CFU-F colonies) of such a rare population.

6.2.7. Cell counting

NCC was performed to identify the density of nucleated cells in PB and ICBM sample. As the cell suspension contains red blood cells, 3% acetic acid was initially used to lyse red blood cells. MNC counts were performed using trypan blue. The purpose of trypan blue is to distinguish non-viable cells (which would absorb the dye) and live cells. For counting 10µL of diluted cell suspension is pipetted onto the haemocytometer chamber. The haemocytometer was first prepared by positioning the moist cover slip onto the haemocytometer and ensured to be properly fitted by the presence of Newton's rings. The number of viable cells would then be counted over $1mm^2$ grid (Figure 6.2-1) under a light microscope. The number of cells per ml of sample would then be calculated with the formula: MNC or NCC/ml= number of viable cells in $1mm^2$ grid X dilution X 10^4.

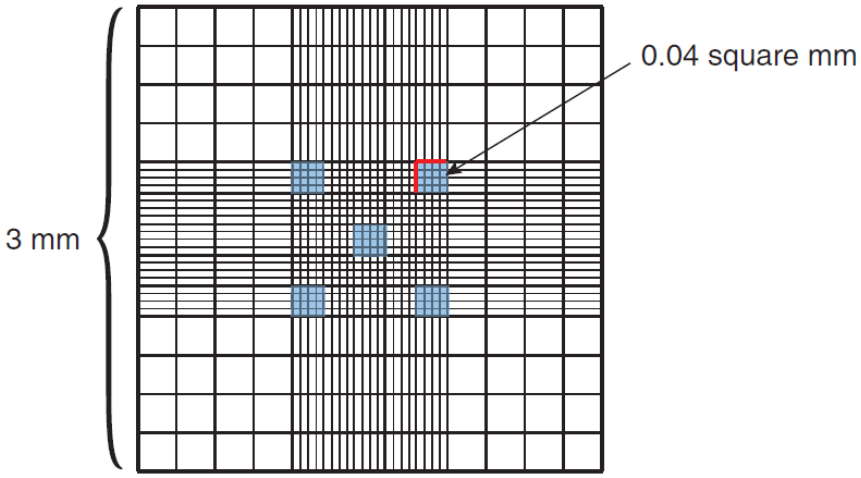

Figure 6.2-1 Schematic of a hemocytometer counting grid. The grid measures 3 mm on each side and is divided into nine large squares (From Fey 2007) (Fey, Kowal et al. 2007)

6.2.8. CFU-F assay – Surface area

The resultant CFU-F colony sizes vary within the same plates, and therefore, larger colony sizes, although still considered a single colony, may represent a higher proliferative (growth) potential of the MSC in culture. For this, dishes were scanned using an Epson 3590 digital scanner and digital images were analyzed using NIS elements BR 2.20 imaging software (Nikon, Tokyo, Japan), giving a readout of total colony area per dish. This would then allow the calculation of average surface area per colony to be made.

6.2.9. Statistics

Statistical analysis was carried out using PASW Statistics (SPSS) version 17.0.2 and graphing performed using Graph Pad Prism version 4.00 for Windows (San Diego, California, USA). As Gaussian distribution could not be assumed given the small sample size, non-parametric tests were carried out. Mann-Whitney test was used to compare differences between two independent samples, in cases of three or more groups. Wilcoxon signed-rank test was used to compare two dependent samples (same patient, two different time points). Chi-square test was used for comparison of nominal data (for example comparing sex distribution between groups). Coefficient of variation was used to assess the variability of results. Statistical significance is assumed at $p<0.05$.

6.3. Results

6.3.1. Patient Recruitment

In total, 17 patients were recruited into the Polytrauma group (Table 6.3-1) and 8 patients were recruited into the Isolated Trauma group (Table 6.3-2). In addition, 3 healthy control patients (Table 6.3-3), undergoing elective pelvic surgery were recruited.

Table 6.3-1 MSC Study: Polytrauma patients (n=17)

PATIENT ID	SEX	AGE (years)	ISS	NCC/ML (10^6) OF ICBM SAMPLE ON ADMISSION**	MNC/ML (10^6) OF ICBM SAMPLE ON ADMISSION**	SAMPLING TIME POINTS (days)***
BT001	M	27	25	n/a	16.7	0, 5
BT005	F	25	27	20.7	18.7	0, 1, 21
BT010*	M	21	50	18.0	11.4	0, 2, 22
BT015	M	50	24	18.0	9.0	0, 2
BT016	M	21	24	23.7	2.2	0, 4, 25
BT017	F	45	38	19.6	7.4	0, 32
BT018	M	54	24	19.4	5.9	0, 5
BT019	F	40	34	20	16	0, 15
BT020*	M	41	29	19.9	20.4	0, 2
BT021	M	27	27	18.9	7.8	0, 4, 12
BT025	M	37	22	20	14.35	0, 2, 4
BT027*	M	49	34	18.2	6.6	0, 5
BT028	M	44	22	19.2	10.1	0, 2, 5
BT029*	M	22	34	13	7.2	0, 3
BT031	F	23	27	26.5	16.7	0, 3, 11
BT033	M	49	25	20.0	7.5	0, 2, 8
BT034	M	44	24	48.4	20.3	0, 8, 12
	13M/4F	Median 40 Range 21-54	Median 27 Range 22-50	Median 19.8 Range 13-48.4	Median 10.1 Range 2.2-20.4	

*denotes presence of head injury

**Method for the assessment of NCC/ml and MNC/ml is described in Section 6.2.7

***As addressed further in Section 6.2.2

Table 6.3-2 MSC Study: Isolated Trauma patients (n=8)

PATIENT ID	SEX	AGE (years)	ISS	NCC/ML (10^6) OF ICBM SAMPLE ON ADMISSION*	MNC/ML (10^6) OF ICBM SAMPLE ON ADMISSION*	SAMPLING TIME POINTS (days)**
BT004	M	33	9	n/a	22.2	0, 2, 16
BT006	M	54	9	8.7	15.6	0, 3
BT007	M	21	4	20.0	14.2	0, 3
BT008	M	64	4	14.7	9.5	0, 8
BT009	M	22	8	18.5	10.6	0, 12, 16
BT011	M	45	4	22.2	14.0	0, 9
BT013	M	51	4	14.1	17.1	0, 3
BT014	F	33	9	21.1	14.2	0, 7
	7M/1F	Median 39 Range 21-64	Median 6 Range 4-9	Median 18.5 Range 8.7-22.2	Median 14.2 Range 9.5-22.2	

*Method for the assessment of NCC/ml and MNC/ml is described in Section 6.2.7

**As addressed further in Section 6.2.2

Table 6.3-3 MSC Study: Healthy Control (Non-trauma) patients (n=3)

PATIENT ID	SEX	AGE	NCC/ML (10^6) OF ICBM SAMPLE ON ADMISSION*	MNC/ML (10^6) OF ICBM SAMPLE ON ADMISSION*
BT023	F	52	17.8	8.4
BT024	F	19	16	12.3
BT026	M	40	20	3.5
	1M/2F	Median 40 Range 19-52	Median 17.8 Range 16-20	Median 8.4 Range 3.5-12.3

*Method for the assessment of NCC/ml and MNC/ml is described in Section 6.2.7

Within both the Polytrauma and Isolated Trauma group, as seen in Table 6.3-1 and Table 6.3-2, there was a male dominance, similar to the findings in the Growth Factor Study. Both trauma groups were matched in terms of age (p=0.50, Mann-Whitney) and sex ratio (p=1.00, chi-square).

Although there were more females in the Healthy Control group, this difference did not reach statistical significance when compared with both the Polytrauma Group (p=0.20, chi-square) and Isolated Trauma Group (p=0.15, chi-square). Similarly, there were no statistically significant differences in age between the Healthy Control and Polytrauma (p=0.92, Mann-Whitney) and Isolated Trauma (p=0.68, Mann-Whitney) patients.

The median NCC/ml in both the Polytrauma and Isolated Trauma groups were similar (p=0.37, Mann-Whitney. Similarly, the MNC/ml counts between Polytrauma and Simple trauma group did not show a significant difference (p=0.22, Mann-Whitney).

6.3.2. Validation of CFU-F assay

The CFU-F assay is a long (2 weeks in duration) functional assay, which was performed in triplicates. Intra-donor variation was first assessed to evaluate the coefficient of variations (CV) between the triplicates (Table 6.3-4 and Table 6.3-5). CV is defined as the ratio of the standard deviation (SD) to the mean, which is then multiplied by 100 to allow expression as a percentage.

Table 6.3-4 CFU-F assay validation using MNC Plating (n= 28 patients)

GROUP	PT ID	CFU-F (1)*	CFU-F (2)*	CFU-F (3)*	MEAN	SD	CV (%)
POLYTRAUMA	BT001	14	10	12	12.0	2.0	16.7
	BT005	1	2	2	1.7	0.6	34.6
	BT010	19	17	16	17.3	1.5	8.8
	BT015	17	22	18	19.0	2.6	13.9
	BT016	22	16	20	19.3	3.1	15.8
	BT017	13	12	10	11.7	1.5	13.1
	BT018	64	45	52	53.7	9.6	17.9
	BT019	198	190	150	179.3	25.7	14.3
	BT020	80	66	56	67.3	12.1	17.9
	BT021	102	96	102	100.0	3.5	3.5
	BT025	73	50	69	64.0	12.3	19.2
	BT027	27	33	37	32.3	5.0	15.6
	BT028	18	18	25	20.3	4.0	19.9
	BT029	16	10	8	11.3	4.2	36.7
	BT031	26	28	27	27.0	1.0	3.7
	BT033	14	21	20	18.3	3.8	20.7
	BT034	41	42	12	31.7	17.0	53.8
ISOLATED TRAUMA	BT004	26	26	26	26.0	0.0	0.0
	BT006	0	0	1	0.3	0.6	173.2
	BT007	51	50	55	52.0	2.6	5.1
	BT008	56	35	33	41.3	12.7	30.8
	BT009	50	53	43	48.7	5.1	10.5
	BT011	16	20	15	17.0	2.6	15.6
	BT013	2	5	3	3.3	1.5	45.8
	BT014	49	54	60	54.3	5.5	10.1
CONTROL	BT023	56	47	47	50.0	5.2	10.4
	BT024	39	41	51	43.7	6.4	14.7
	BT026	25	29	30	28.0	2.6	9.4
						MEAN CV (%)	23.3

***Results are shown as colonies/dish**

Table 6.3-5 CFU-F assay validation using Direct Plating (n= 25 patients)

GROUP	PT ID	CFU-F (1)*	CFU-F (2)*	CFU-F (3)*	MEAN	SD	CV (%)
POLYTRAUMA	BT010	44	34	43	40.3	5.5	13.7
	BT015	14	17	19	16.7	2.5	15.1
	BT016	63	52	58	57.7	5.5	9.6
	BT017	10	12	15	12.3	2.5	20.4
	BT018	37	54	62	51.0	12.8	25.0
	BT019	145	175	234	184.7	45.3	24.5
	BT020	138	149	134	140.3	7.8	5.5
	BT021	78	75	104	85.7	15.9	18.6
	BT025	135	126	134	131.7	4.9	3.7
	BT027	43	36	47	42.0	5.6	13.3
	BT028	42	49	53	48.0	5.6	11.6
	BT029	14	13	16	14.3	1.5	10.7
	BT031	65	67	61	64.3	3.1	4.7
	BT033	34	28	39	33.7	5.5	16.4
	BT034	41	42	43	42.0	1.0	2.4
ISOLATED TRAUMA	BT006	0	2	2	1.3	1.2	86.6
	BT007	140	142	139	140.3	1.5	1.1
	BT008	60	67	60	62.3	4.0	6.5
	BT009	69	53	71	64.3	9.9	15.3
	BT011	54	56	52	54.0	2.0	3.7
	BT013	1	3	2	2.0	1.0	50.0
	BT014	57	38	47	47.3	9.5	20.1
CONTROL	BT023	134	117	52	101.0	43.3	42.8
	BT024	18	11	9	12.7	4.7	37.3
	BT026	36	48	38	40.7	6.4	15.8
						MEAN CV (%)	19.0

***Results are shown as colonies/dish**

The average mean CVs of 23.3% (MNC plating) and 19% (Direct plating) were comparable with the data obtained by others in the laboratory and deemed acceptable for the evaluation of differences in CFU-Fs between different time-points following injury in the same patient. Highest CVs were observed for the patients with lowest numbers of colonies/dish, as expected (BT006, BT013, BT005).

Direct plating appeared to be slightly less variable, with lower CVs (19%) compared to MNC plating (23.3%). But these differences were small and not statistically significant (p=0.52, Mann-Whitney), indicating that both direct plating and MNC plating techniques were equally acceptable for the purpose of this study.

6.3.3. Validation of CFU-F colony area

To account for the observations that the sizes of CFU-F colonies differ in size and thus may reflect different cellular proliferative potential in culture, the CFU-F colonies were calculated for total stained area per dish. Coefficient of variations was calculated to investigate intra-donor differences in terms of stained colony area per dish for both MNC (Table 6.3-6) and Direct (Table 6.3-7) plating methods.

Table 6.3-6 CFU-F total area validation using MNC Plating (n=27 patients)

GROUP	PT ID	Area (1)*	Area (2)*	Area (3)*	MEAN	SD	CV (%)
POLYTRAUMA	BT001	0.23	0.22	0.22	0.22	0.01	2.59
	BT005	2.13	1.86	1.57	1.85	0.28	15.11
	BT010	5.87	3.93	4.46	4.75	1.00	21.09
	BT015	13.33	11.31	12.41	12.35	1.01	8.19
	BT016	8.54	14.43	8.61	10.53	3.38	32.11
	BT017	14.56	14.56	12.35	13.82	1.28	9.23
	BT018	19.48	13.79	11.13	14.80	4.27	28.82
	BT019	63.32	60.57	63.73	62.54	1.72	2.75
	BT020	8.90	10.81	7.79	9.17	1.53	16.66
	BT021	36.00	32.73	36.38	35.04	2.01	5.73
	BT025	3.73	4.83	6.14	4.90	1.21	24.62
	BT027	3.78	4.23	1.45	3.15	1.49	47.32
	BT028	33.61	32.97	24.64	30.41	5.00	16.46
	BT029	37.78	14.41	22.63	24.94	11.86	47.53
	BT031	28.12	33.09	21.28	27.50	5.93	21.56
	BT033	42.33	47.91	43.54	44.59	2.94	6.58
	BT034	24.17	18.19	20.11	20.82	3.05	14.66
ISOLATED TRAUMA	BT004	8.72	12.90	9.18	10.27	2.29	22.33
	BT007	3.64	3.27	3.21	3.37	0.23	6.90
	BT008	14.65	19.98	23.29	19.31	4.36	22.58
	BT009	7.85	12.09	14.21	11.38	3.24	28.45
	BT011	7.19	6.09	6.30	6.53	0.58	8.95
	BT013	5.78	6.31	6.05	6.05	0.27	4.38
	BT014	22.58	30.38	20.93	24.63	5.05	20.49
CONTROL	BT023	5.05	4.76	6.39	5.40	0.87	16.10
	BT024	1.31	1.02	0.97	1.10	0.18	16.69
	BT026	2.87	3.67	3.57	3.37	0.44	12.93
						MEAN CV (%)	17.81

*Results are shown as total colony area per dish (cm^2)

Table 6.3-7 CFU-F total area validation using Direct Plating (n=25 patients)

GROUP	PT ID	CFU-F (1)*	CFU-F (2)*	CFU-F (3)*	MEAN	SD	CV (%)
POLYTRAUMA	BT010	21.95	16.34	25.61	21.30	4.67	21.92
	BT015	9.17	11.03	9.28	9.83	1.04	10.62
	BT016	8.54	14.43	8.61	10.53	3.38	32.11
	BT017	8.40	7.43	6.99	7.61	0.72	9.48
	BT018	24.00	21.82	30.73	25.52	4.64	18.20
	BT019	59.83	65.09	70.22	65.05	5.20	7.99
	BT020	45.69	48.47	43.72	45.96	2.39	5.19
	BT021	27.06	40.14	28.61	31.94	7.15	22.38
	BT025	44.98	52.06	44.77	47.27	4.15	8.78
	BT027	5.02	2.54	4.32	3.96	1.28	32.29
	BT028	62.98	57.45	65.33	61.92	4.05	6.53
	BT029	7.88	6.44	6.75	7.02	0.76	10.79
	BT031	17.86	18.72	14.72	17.10	2.11	12.31
	BT033	24.18	46.91	28.85	33.31	12.00	36.03
	BT034	17.55	11.78	13.28	14.20	2.99	21.08
ISOLATED TRAUMA	BT006	3.99	3.34	4.22	3.85	0.46	11.85
	BT007	64.53	70.26	64.31	66.37	3.37	5.08
	BT008	39.28	33.54	29.93	34.25	4.72	13.77
	BT009	12.50	43.73	46.10	34.11	18.75	54.98
	BT011	18.46	16.00	15.89	16.78	1.45	8.66
	BT013	0.19	0.19	0.26	0.21	0.04	18.94
	BT014	12.53	16.79	13.27	14.20	2.28	16.03
CONTROL	BT023	2.36	3.88	6.92	4.39	2.32	52.93
	BT024	26.84	4.46	-	15.65	15.83	101.12
	BT026	6.83	6.77	5.77	6.46	0.60	9.22
						MEAN CV (%)	21.93

*Results are shown as total colony area per dish (cm^2)

In the assay of total colony area per dish, the mean CV of 17% (MNC Plating) and 22% (Direct Plating) were comparable with the analysis for CV of CFU-F assays. This method may provide additional insight into not just colony (MSC) numbers, but its proliferative potential.

6.3.4. Relationship between CFU-F MNC and Direct plating Methods

As stated above ICBM aspirates obtained were plated to both the MNC and Direct method. Matched samples were analysed for relationship between both MNC and Direct methods.

Analysis of the relationship between matched samples showed statistically significant ($p<0.05$) correlations between CFU-F values of MNC and Direct Plating methods when data were normalized to CFU-F per ml of ICBM. As can be seen in Figure 6.3-1, this was independent of trauma severity (both Polytrauma and Isolated Trauma groups were significantly associated). The weak relationship observed in the Control group may be due to sampling error, as only 3 time points (patients) were available.

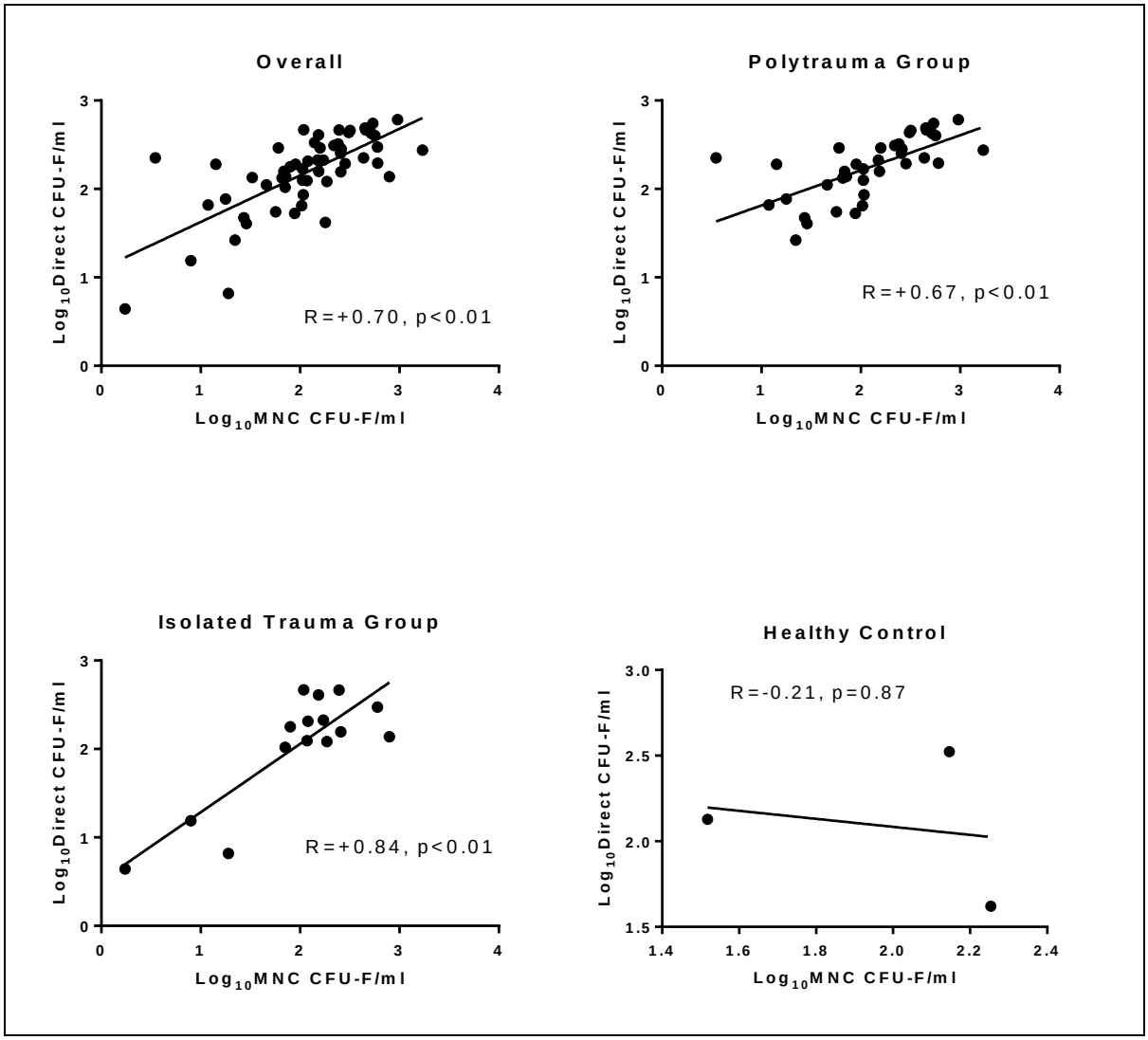

Figure 6.3-1 Relationship between CFU-F MNC and Direct Plating Methods. Matched samples were statistically correlated between MNC and Direct methods across both trauma groups. Data was expressed in logarithmic scales, and statistically significant correlations was found across both Polytrauma and Isolated Trauma

However, this relationship was not similarly seen when the samples were analysed in terms of CFU-F Area. Samples were normalized to average surface area per colony for this analysis (Table 10.3-1).

As can be seen in Figure 6.3-2, there were no correlations between the average CFU-F colony size between MNC and Direct plating methods.

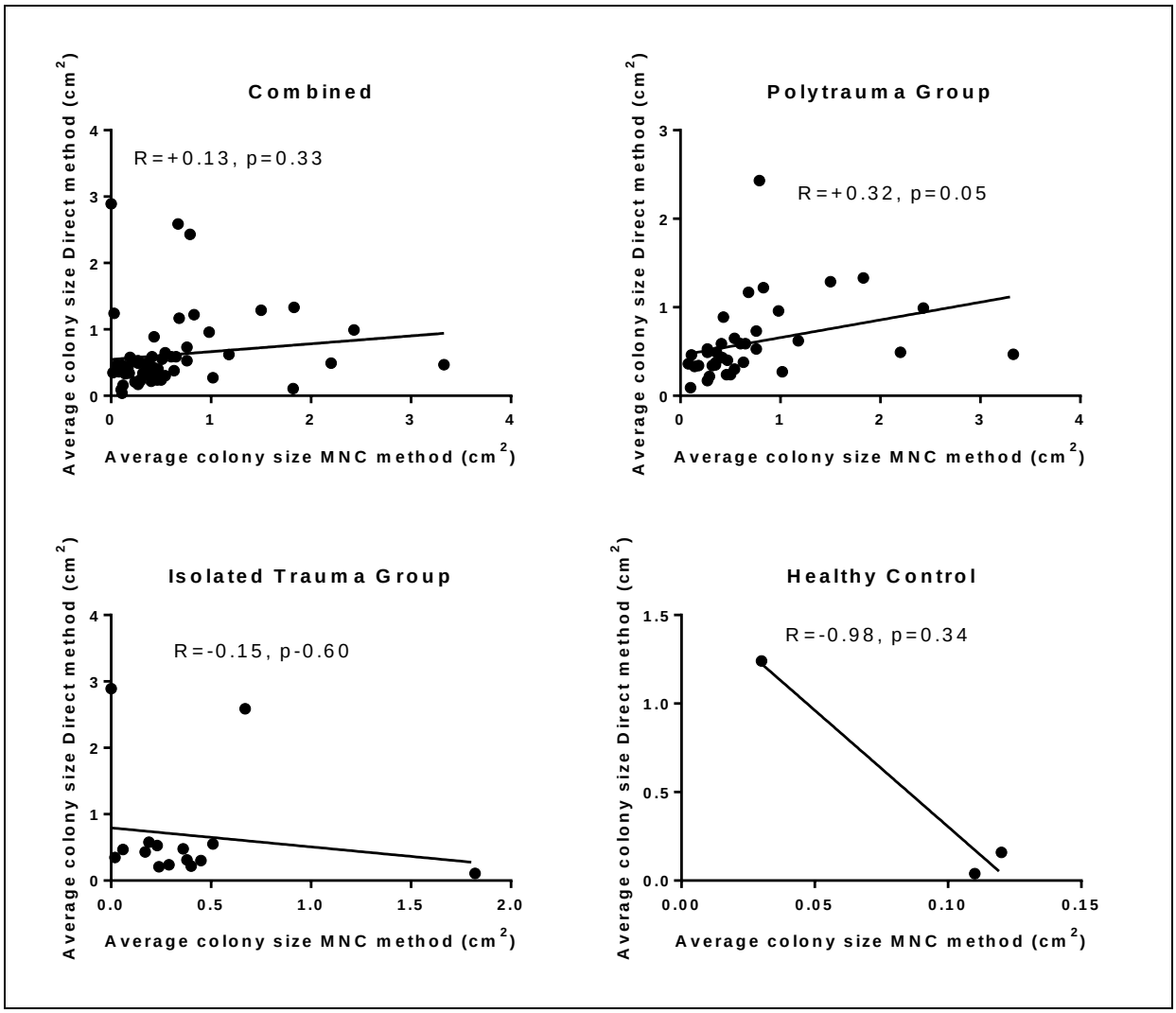

Figure 6.3-2 Relationship of average CFU-F colony size between MNC and Direct Plating Methods. There were no correlations between matched samples across all trauma groups.

However, within the same method, when the relationships between the number of CFU-F colonies and CFU-F surface area were analysed, they showed overall significant ($p<0.05$) associations independent of trauma severity (Polytrauma and Isolated Trauma). This was true for both the MNC (Figure 6.3-3) and Direct (Figure 6.3-4) Plating methods.

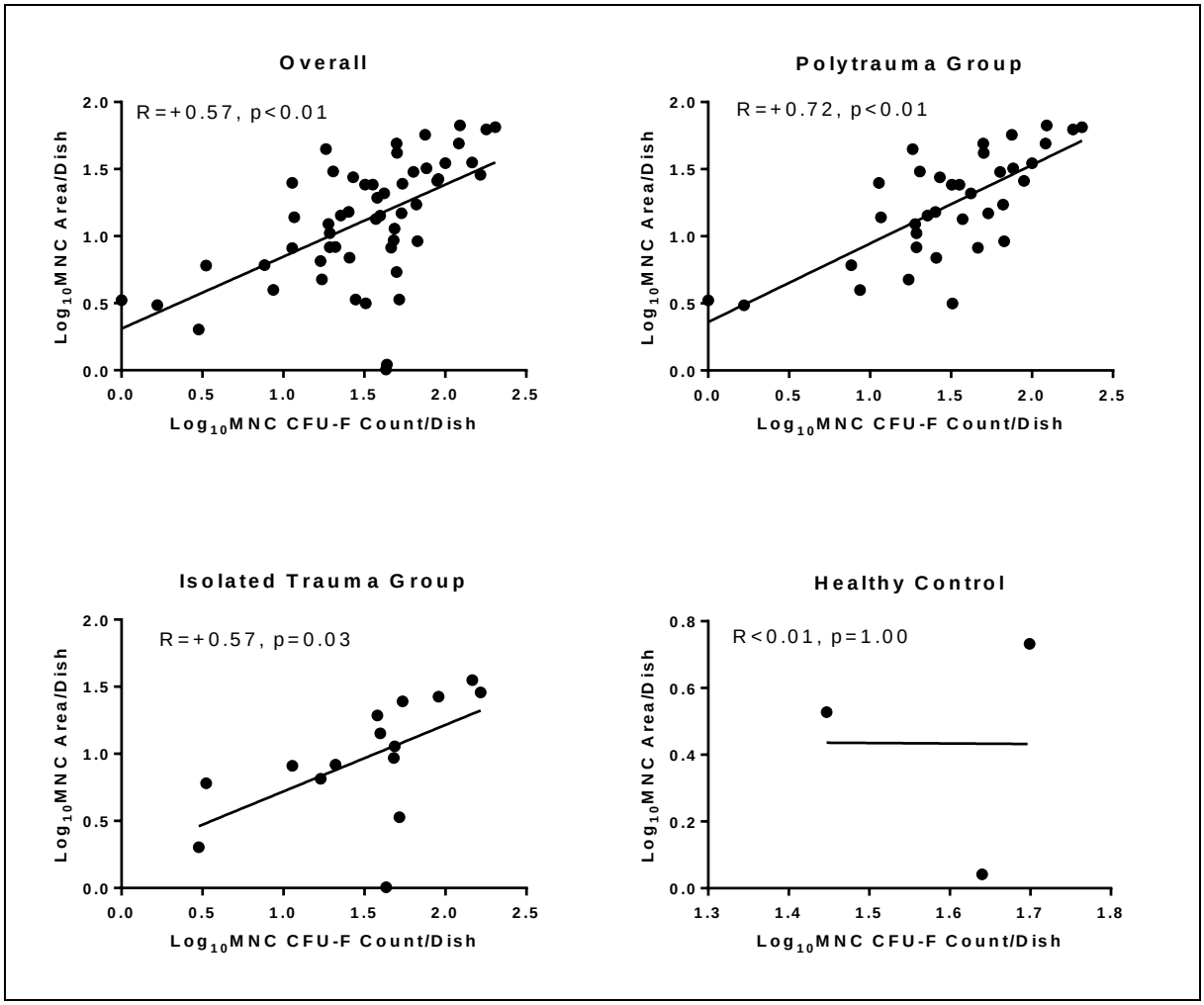

Figure 6.3-3 Relationship between CFU-F Count and Area per Dish using MNC Method. Matched samples were statistically correlated between CFU-F and area per dish. Data was expressed in logarithmic scales, and statistically significant correlations was found across both Polytrauma and Isolated Trauma.

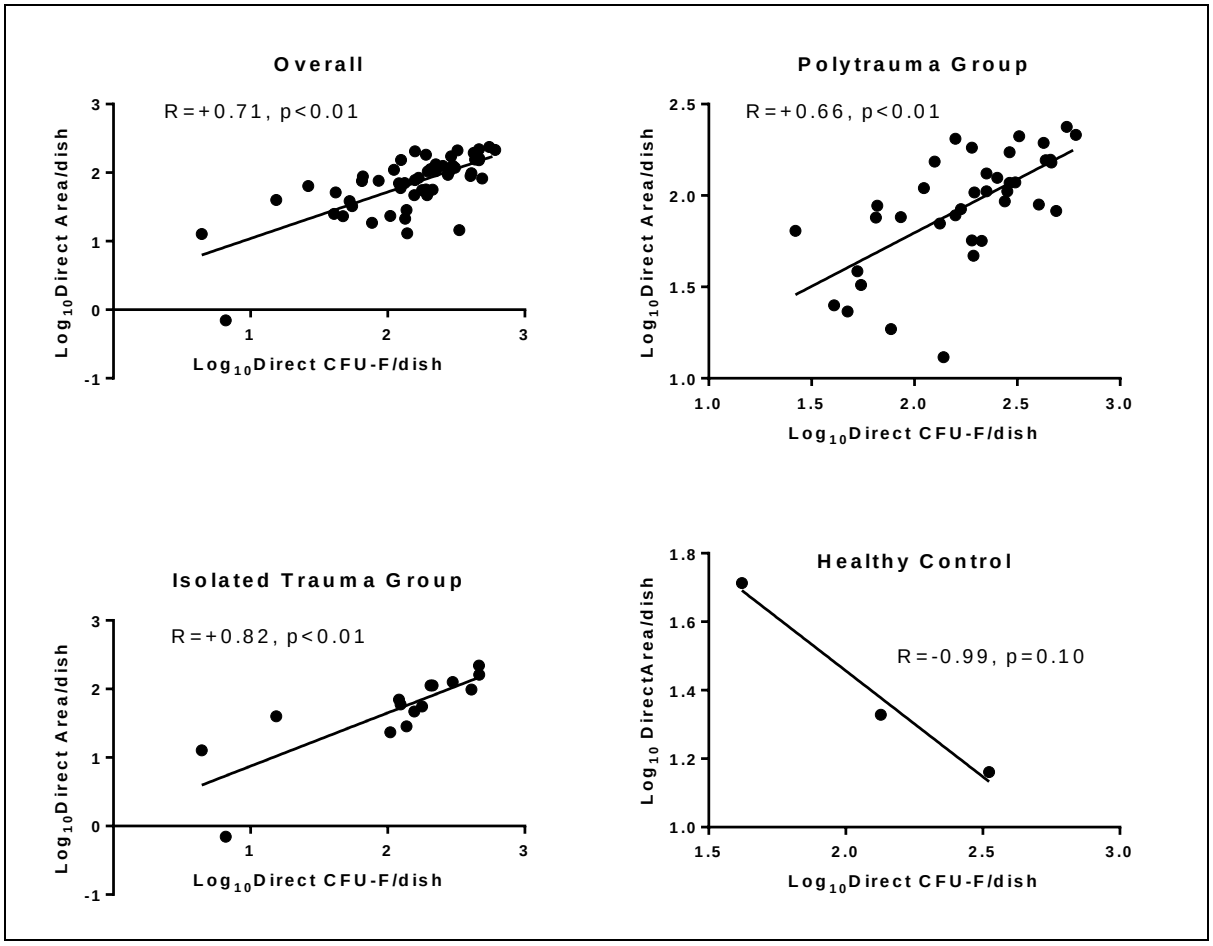

Figure 6.3-4 Relationship between CFU-F Count and Area per dish by Direct Plating Method. Matched samples were statistically correlated between CFU-F and area per dish. Data was expressed in logarithmic scales, and statistically significant correlations was found across both Polytrauma and Isolated Trauma.

Finally, the relationship over time between CFU-F count, CFU-F area and average colony size within the same patient were explored. Representative examples were chosen from patients recruited from the Polytrauma Group (Figure 6.3-5) and Isolated Trauma Group (Figure 6.3-6). In both examples (BT019 and BT009), across both MNC and Direct plating methods, it was observed that the CFU-F count per dish and total CFU-F area changes proportionate to each other. This was not surprising, as more colonies would result in more total surface area. However, when compared with average colony size, the changes are less variable and did not change proportionately to colony count changes. This implied that the rate of proliferation (average colony size) was independent of MSC concentration (in ICBM) and most likely was intrinsically determined from the patient himself.

Taken together, these findings implied that both total surface areas per dish could be used as a surrogate of CFU-F colony numbers. However, given the findings of unequal average colony sizes between both MNC and Direct plating methods (as shown in Figure 6.3-2) this method (total surface

areas per dish) is unlikely to be accurate enough to reflect changes in the number of MSCs in ICBM over time. Therefore, to address the hypothesis of systemic upregulation of MSCs over time following trauma, analyses will be carried out with the CFU-F count method only.

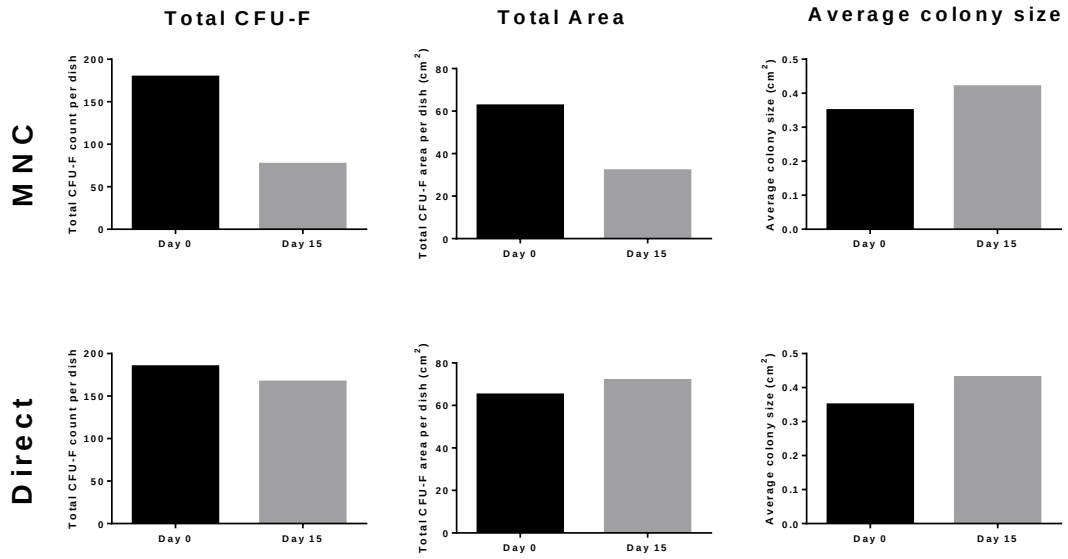

Figure 6.3-5 Representative figure from Polytrauma Group (BT019) showing relationship between CFU-F count, CFU-F area and average colony size over time (Day 0 and Day 16). Top row represents data from MNC Plating and bottom row represents data from Direct plating methods respectively

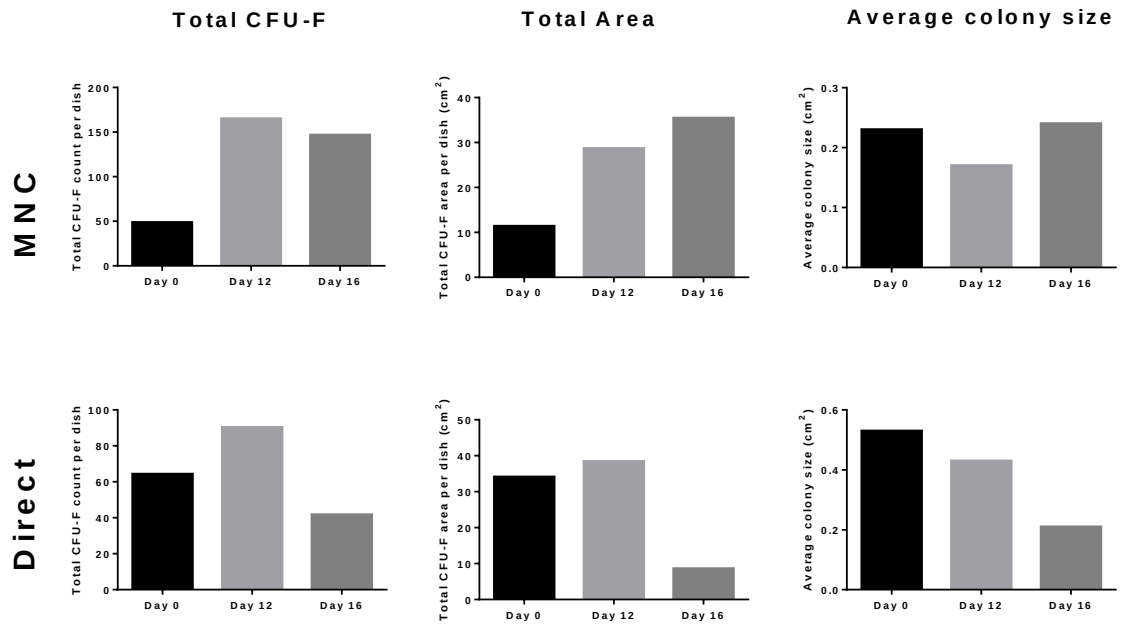

Figure 6.3-6 Representative figure from Polytrauma Group (BT009) showing relationship between CFU-F count, CFU-F area and average colony size over time (Days 0, 12, and 16). Top row represents data from MNC Plating and bottom row represents data from Direct plating methods respectively

6.3.5. Dynamics of CFU-F over time

As previously shown in Section 6.3.2, there is high inter-individual variability in baseline ICBM CFU-F count. This was further confirmed by the 3 Healthy Control donors CFU-F counts for both the MNC (range 33-180) and Direct (range 42-333) Plating Methods. Therefore, to further investigate the effect of trauma on MSC ICBM dynamics, all patients were analysed based on their CFU-F changes from baseline (Day 0) to the next time points of BM aspiration.

As can be seen in Figure 6.3-7, within the Isolated Trauma group of patients, half the patients (BT006, BT008, BT009, BT013) showed a rising trend in CFU-F count from admission levels. The other half within the group (BT004, BT007, BT011, BT014) showed either a falling trend or minimal changes from baseline. For the 4 patients with rising CFU-F levels, the change in CFUF/ml from first time point to second ranged between 1.2 to 10-fold (median 4.6) with the MNC plating technique, and between 1.4 to 18-fold increase (median 2.8) for the Direct plating technique. Within the sub-group that showed decreasing CFU-F/ml levels, the changes ranged between 15% to 89% (median 45%) with the MNC plating technique and between 59 to 80% (median 70%) with the Direct plating technique. Trends (either increasing or decreasing) were similar with both plating techniques. Taking into account intra-patient CVs of between 21 and 28%, any changes around 1.3 fold could be purely due to technical limitations.

Similarly, Figure 6.3-8 and Figure 6.3-9 showed the changes in CFU-F/ml in Polytrauma sub-group of patients displaying an increasing trend over time, whereas Figure 6.3-10 shows the changes in CFU-F/ml in the Polytrauma sub-group of patients displaying either a decreasing or minimal changes from baseline over time.

Within the rising sub-group, the maximum increase observed was 40-fold (BT016) with the MNC plating technique and 15-fold (BT017) with the Direct plating technique. The maximum decrease seen in MNC plating was 31% of Day0 value (BT015) and 90% for Direct plating (BT019). There was less agreement between the two different techniques in this group, as patient BT015 showed a decreasing trend (0.3-fold) in MNC plating, and increasing trend in direct plating (1.4-fold). However, taking into consideration the intra-patient variability shown in Sections 0 and 6.2.5, decreasing trends observed within this sub-group could be caused simply by technical limitations.

Overall, these data indicated that Polytrauma groups of patients appeared to show more pronounced changes in CFU-F dynamics compared to the Isolated Trauma groups. It is noteworthy, that patients showing more pronounced increasing trends had lower baseline (Day 0) CFU-F counts compared to patients with a decreasing trend, which was observed for both Simple Trauma and Polytrauma groups.

CFU-F in Peripheral Blood

All the PB samples plated for CFU-F with both MNC and Direct plating failed to reveal any colonies. A total of 100 [n=25 patients, plated with two techniques (MNC and Direct), in duplicates] dishes were set-up in total. Representative dishes are shown in the next section.

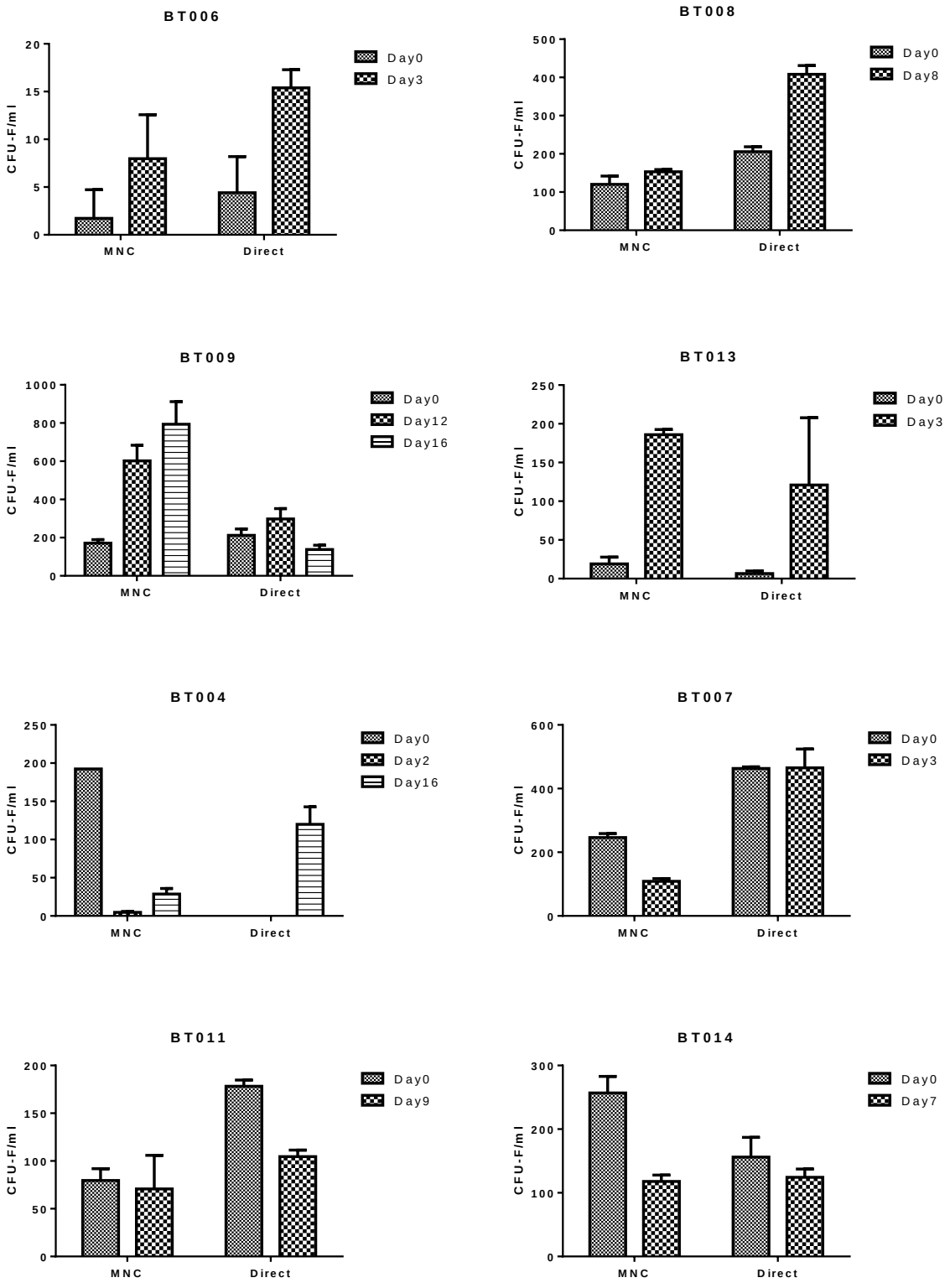

Figure 6.3-7 CFU-F/ml changes over time in Isolated Trauma Group of patients. MNC refers to MNC plating technique, Direct refers to Direct plating technique. Samples are mean of dishes plated in triplicates. Section with gap (y=0) implies no colonies were found.

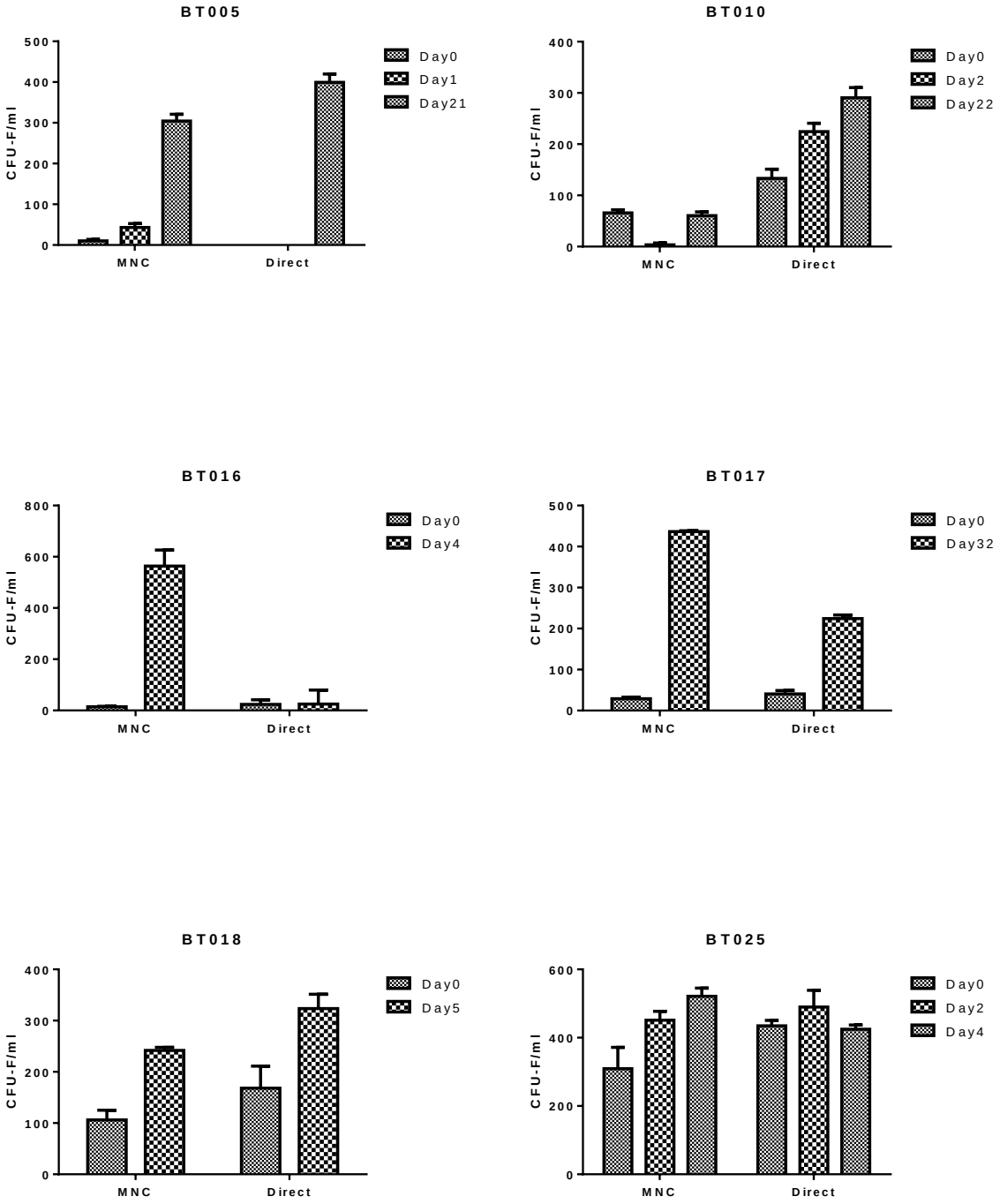

Figure 6.3-8 CFU-F/ml changes over time in Polytrauma Trauma Group of patients (Rising Trend Sub-group 1). MNC refers to MNC plating technique, Direct refers to Direct plating technique. Samples are mean of dishes plated in triplicates. Section with gap (y=0) implies no colonies were found.

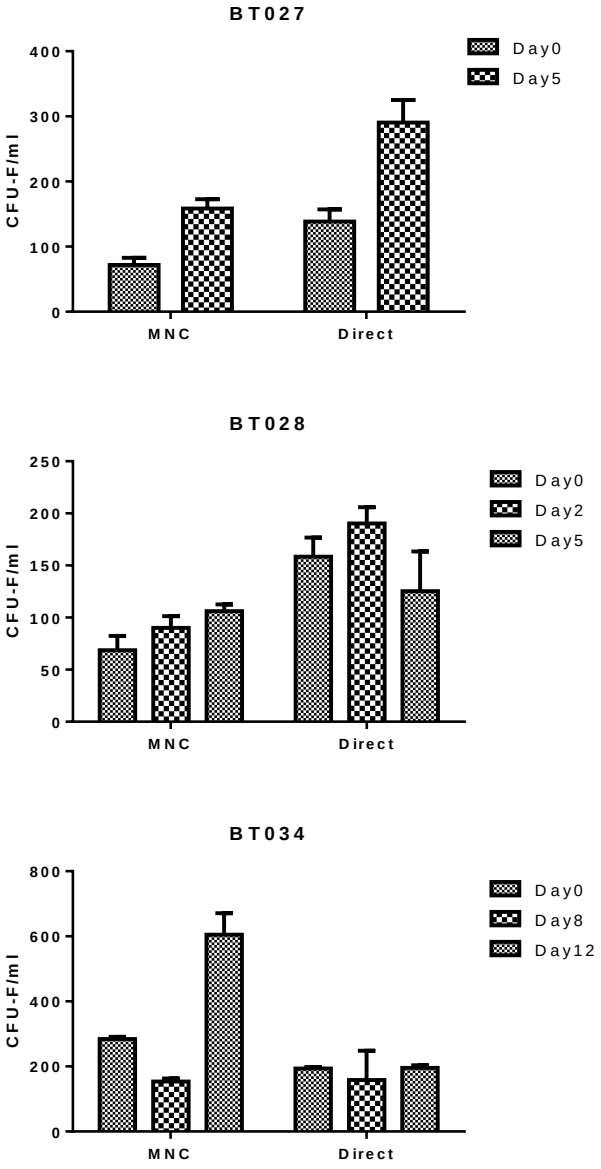

Figure 6.3-9 CFU-F/ml changes over time in Polytrauma Trauma Group of patients (Rising Trend Sub-group 2). MNC refers to MNC plating technique, Direct refers to Direct plating technique. Samples are mean of dishes plated in triplicates. Section with gap (y=0) implies no colonies were found.

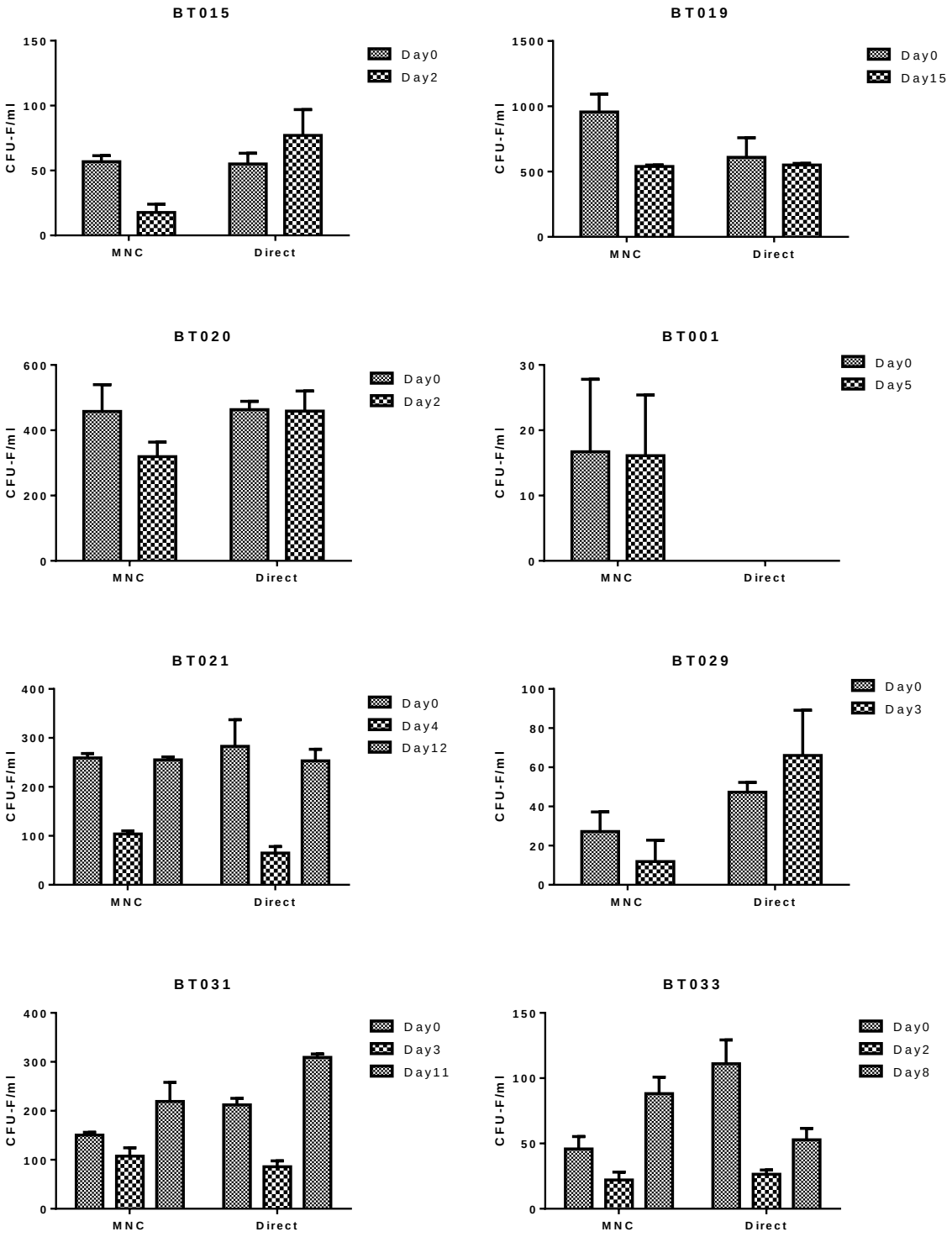

Figure 6.3-10 CFU-F/ml changes over time in Polytrauma Trauma Group of patients (Falling Trends). MNC refers to MNC plating technique, Direct refers to Direct plating technique. Samples are mean of dishes plated in triplicates. Section with gap (y=0) implies no colonies were found.

Representative dishes in the Isolated Trauma (Figure 6.3-11) and Polytrauma (Figure 6.3-12) groups are shown.

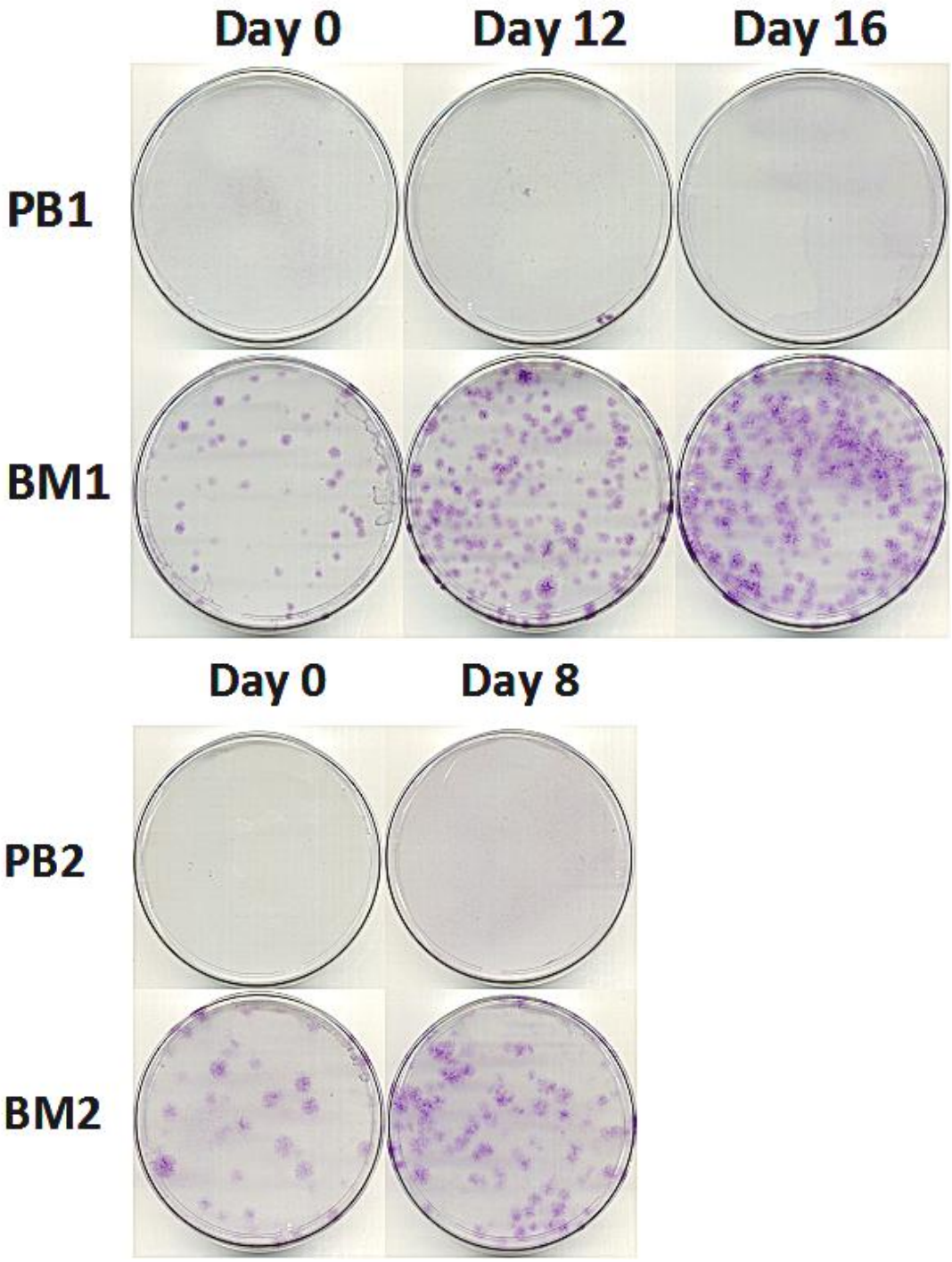

Figure 6.3-11 Patient 1 (BT009) (top) samples taken on Day0 (admission) and days 12 and 16 post-injury. Patient 2 (BT008) (bottom) sample taken on Day0 (admission) and day 8 post-injury. For BM sample, there is progressive increase in CFU-F count over time. No CFU-F colonies were found in PB samples

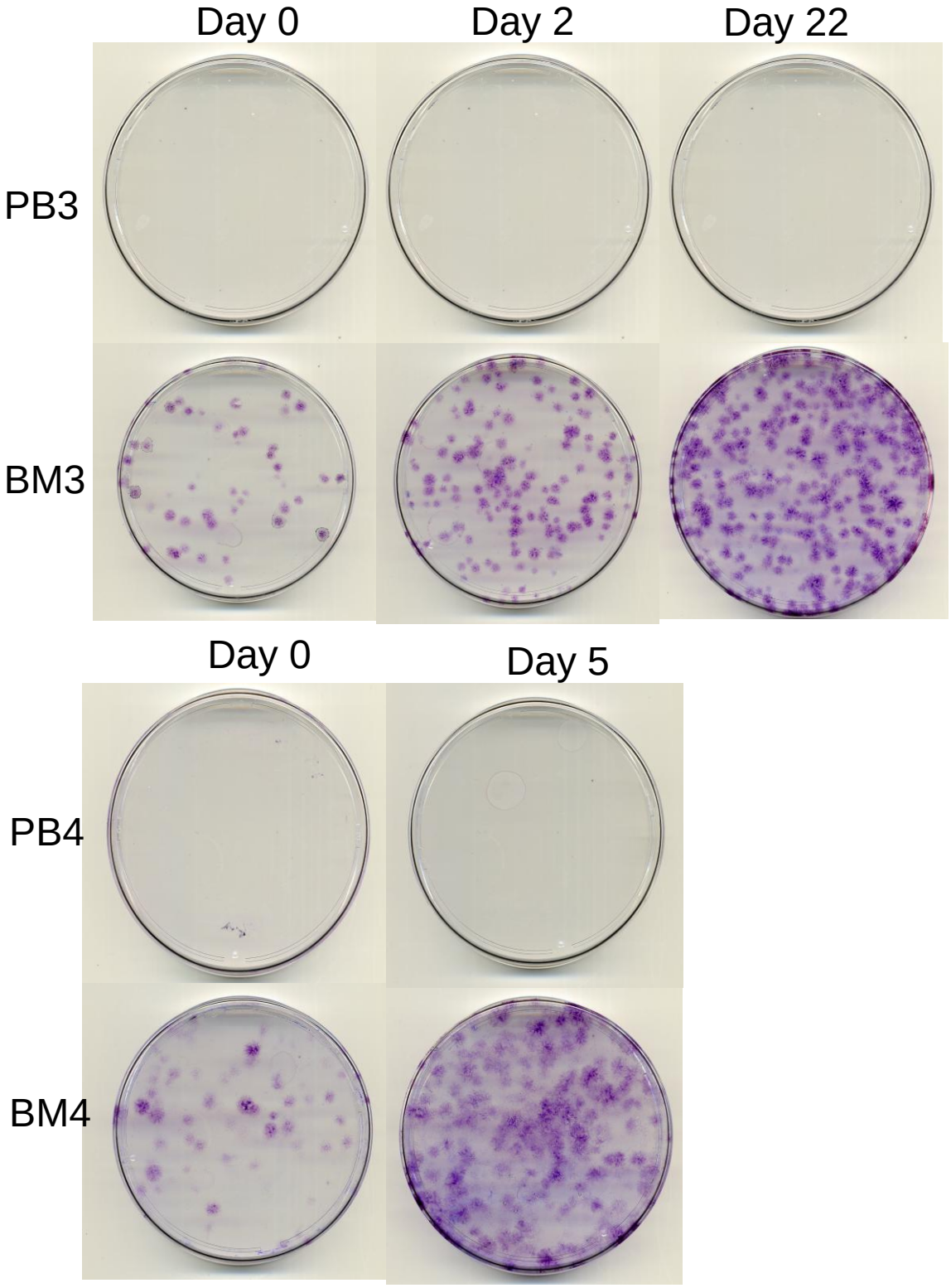

Figure 6.3-12 Patient 3 (BT010) (top) samples taken on Day0 (admission) and days 2 and 25 post-injury. Patient 4 (BT018) (bottom) sample taken on Day0 (admission) and day 5 post-injury. For BM sample, there is progressive increase in CFU-F count over time. No CFU-F colonies were found in PB samples

6.3.6. CFU-F dynamics and clinical course

It is evident from the previous analyses that the MSC numbers in BM aspirate, as measured by the CFU-F assays, do not show any differences in trends between trauma groups, and within trauma groups no single trend was observed. It is likely that the dynamic changes in CFU-F over time observed in this study are secondary to other stimulus apart from trauma severity.

Within the trauma group, the immediate post-traumatic (first 2 weeks) sequelae may differ between patients. As discussed in the previous chapter, following trauma some patients develop complications, such as the Systemic Inflammatory Response Syndrome (SIRS). Given the known cytokine storm that accompanies SIRS (Yiu, Graham et al. 2012), it is possible that the MSC CFU-F dynamics are influenced by the presence of SIRS.

Patients recruited into the MSC study were analysed for presence of SIRS, based on established criteria of core temperature, respiratory rate, heart rate and total white cell counts (1992). Further details regarding SIRS have already been discussed in the previous chapter. Data for determination of SIRS were available on 16 Polytrauma patients (Table 6.3-8) and 7 Isolated Trauma Patients (Table 6.3-9).

Overall, within the first 2 weeks following trauma, 11 out of 16 patients within the Polytrauma Group developed a SIRS score of 2 or more. In contrast, only 2 out of 8 patients within the Isolated Trauma Group developed SIRS, a difference which nearly reached statistical significance (p=0.08, Chi-square test).

When analysed against CFU-F trends, within the Polytrauma Group, 6 patients with SIRS had a falling trend, compared to 5 rising. Two patients without SIRS had a falling trend and 3 showed a rising. Against our expectation, the CFU-F dynamics in the Polytrauma group was not determined by presence of SIRS (p=1.00, Chi-square test).

Within the Isolated Trauma group, half of patients with SIRS had a rising trend, and half of patients without SIRS had a falling trend, and therefore similar to the Polytrauma group, the CFU-F dynamics was not determined by presence of SIRS (p=1.00, Chi-square test).

Table 6.3-8 SIRS Score in first 14 days post trauma in Polytrauma Group (n=16)

SIRS?	Patient ID	Admission	Day 1	Day 3	Day 5	Day 7	Day 14
Yes	BT001	2	0	0	2	3	2
Yes	BT005	1	0	1	3	2	2
No	BT010	1	0	1	1	1	1
Yes	BT015	2	1	0	0	0	
Yes	BT016	0	1	0	0	3	2
Yes	BT017	2	1	1	1		
No	BT018	1	0	0	0	1	1
Yes	BT019	2	1	0	3	2	1
Yes	BT020	2	0	0	0	3	1
No	BT021	1	1	0	0	0	
Yes	BT025	2	1	0	0		
Yes	BT027	2	0	1	2	2	
Yes	BT029	1	1	0	1	2	
Yes	BT031	1	1	3	3	3	2
No	BT033	1	0	0	0	0	
No	BT034	1	0	1	0	0	0

Table 6.3-9 SIRS Score in first 14 days post trauma in Isolated Trauma Group (n=8)

SIRS?	Patient ID	Admission	Day 1	Day 3	Day 5	Day 7	Day 14
Yes	BT004	0	1	0	2	1	1
No	BT006	1	0	0	0	0	
Yes	BT008	2	1	0	0	0	1
No	BT009	0	0	0	0	0	0
No	BT011	0	0	0	0	0	0
No	BT013	1	0	0	0	0	
No	BT014	1	0	0	0	0	

Table 6.3-10 Correlation between presence of SIRS and CFU-F Trends in Polytrauma Patients

Patient ID	SIRS?	CFU-F Trends*
BT001	Yes	Declining
BT005	Yes	Rising
BT010	No	Rising
BT015	Yes	Declining
BT016	Yes	Rising
BT017	Yes	Rising
BT018	No	Rising
BT019	Yes	Declining
BT020	Yes	Declining
BT021	No	Declining
BT025	Yes	Rising
BT027	Yes	Rising
BT029	Yes	Declining
BT031	Yes	Declining
BT033	No	Declining
BT034	No	Rising

***Trends were based on both MNC and Direct Plating**

Table 6.3-11 Correlation between presence of SIRS and CFU-F trends in Isolated Trauma Patients

Patient ID	SIRS?	CFU-F Trends*
BT004	Yes	Declining
BT006	No	Rising
BT008	Yes	Rising
BT009	No	Rising
BT011	No	Declining
BT013	No	Rising
BT014	No	Declining

***Trends were based on both MNC and Direct Plating**

Finally, similar to analysis carried out with Growth Factor dynamics, the relationship between CFU-F trends and fracture healing outcome was explored. Data regarding fracture healing was available on 13 patients from the Polytrauma Group and 7 patients from the Isolated Trauma Group (Table 6.3-12). There were 3 patients who had delayed or non-union of their fractures, in both the Polytrauma and Isolated Trauma Groups respectively.

Within the Polytrauma Group, when the fracture healing status was analysed against CFU-F trends, 2 of the "problem healers" had declining trends, with the remaining patient showing a rising trend. Normal healers from the Polytrauma Group had 6 rising and 4 declining CFU-F trends. Overall, early trends in CFU-F were not significantly associated with fracture healing outcome in this group ($p=0.56$, chi-square test).

Similar analysis within the Isolated Trauma group revealed 2 rising and 1 declining in the "problem healers" and half rising and half declining in the normal healers. Again, the CFU-F trends were not correlated with fracture healing outcome in this group ($p=1.00$, chi-square test).

Table 6.3-12 Correlation between Fracture Healing Status and CFU-F Trends

GROUP	**Patient ID**	**Fracture location**	**Healing status**	**CFU-F Trends***
POLYTRAUMA	BT001	Pelvic	Normal	Declining
	BT005	Femur	Normal	Rising
	BT010	Femur	Normal	Rising
	BT015	Ulna	Non-union	Declining
	BT016	Pelvic	Normal	Rising
	BT017	Ulna	Non-union	Rising
	BT018	Pelvic	Normal	Rising
	BT019	Femur	Normal	Declining
	BT021	Tibia	Normal	Declining
	BT028	Femur	Normal	Rising
	BT029	Tibia	Delayed union	Declining
	BT033	Elbow	Normal	Declining
	BT034	Ankle	Normal	Rising
ISOLATED TRAUMA	BT004	Tibia	Delayed union	Declining
	BT006	Tibia	Delayed union	Rising
	BT008	Tibia	Normal	Rising
	BT009	Ankle	Normal	Rising
	BT011	Tibia	Normal	Declining
	BT013	Tibia	Non-union	Rising
	BT014	Tibia	Normal	Declining

***Trends were based on both MNC and Direct Plating,**

**** Healing status: Delayed healing (>6 months), Non-union (Requiring surgical re-interventions)**

6.4. Discussion

6.4.1. Review of culture technique used

In this section, I analysed the effect of trauma severity on MSC numbers in ICBM and their changes over time. I used several validated techniques (Castro-Malaspina, Gay et al. 1980; Galotto, Berisso et al. 1999) for these investigations (CFU-F) assay and evaluated their utility for the purpose of this investigation. The CFU-F count in healthy adults using the Direct plating technique can vary from 50 to 2300 CFU-F/ml (Galotto, Berisso et al. 1999; Cox, McGonagle et al. 2011) whereas using the MNC plating technique, colonies counts were affected by seeding density (Castro-Malaspina, Gay et al. 1980; Van Landuyt, Jones et al. 2010). All the CFU-F data in this study were on average within the published reference range (Van Landuyt, Jones et al. 2010; Cox, Boxall et al. 2011).

6.4.2. Review of bone marrow aspiration technique

It has been previously reported in our laboratory that the method of BM aspiration may affect CFU-F MSC count (Cuthbert, Boxall et al. 2012). The volume of bone marrow aspirate affects the cellular content obtained (Batinic, Marusic et al. 1990; Muschler, Boehm et al. 1997). In fact, the Leeds MSC Group recently showed that the first 5ml of ICBM aspiration volume had far higher density of MSC/CFU-F compared to the next 15ml combined (Cuthbert, Boxall et al. 2012). All patients recruited into this CFU-F study underwent their bone marrow aspiration by me. For all patient sampling time points, I consistently used the same technique in terms of surgeon (myself only), aspirate location (anterior iliac crest), tools (Stryker 306-111, 11-gauge, bevel tipped trocar), volume of aspirate (20ml) and draw method (single draw to fill full 20ml syringe). With measures taken, it is likely that the CFU-F dynamics observed are genuine rather than due to inadvertent inconsistencies during bone marrow aspiration.

6.4.3. Relationship between CFU-F Count and CFU-F Area

I then attempted to analyse the issue of CFU-F colony sizes. Each CFU-F colony represents a progeny of single MSC, which proliferates forming a typical fibroblast concentric pattern (Castro-Malaspina, Gay et al. 1980). Therefore, the bigger the colony size, the higher the rate of division and proliferation, given the fixed time (14 days) between seeding and staining. Although both MNC and Direct plating methods showed associations between CFU-F count and total surface area per dish (See Figure 6.3-3 and Figure 6.3-4), the variability in average colony areas, and the variability in

colony sizes per dish implies that the CFU-F total area method cannot directly replace CFU-F count to assess MSC numbers arising from ICBM plating.

6.4.4. Relationship between CFU-F MNC and Direct plating

Iliac crest bone marrow aspirate obtained were plated using both the MNC and Direct methods. The Direct method's potential advantage was that it better represented the original BM sample obtained and the volumetric based method was simpler and less likely to incur errors. However, this method meant that the presence of other cellular phenotypes in abundance such as red blood cells may cause an "overcrowding" of the dish in culture, resulting in failure of adherence of some MSC to the plate and hence failure to form CFU-F colonies. Furthermore, a fibrin layer occasionally forms within the first 24 hours of culture, which may lead "MSC losses" secondary to adherence to that layer instead of the plastic surface. Additionally, ICBM MSCs can also be trapped in clots (Centeno, Busse et al. 2008), which sometimes form on the dish following direct plating.

On the other hand, MNC plating appealed as the culture method is based on cellular density seeding. Additionally, as by and large only the mononuclear cells remain during seeding, leading to a better availability of surface area for MSC adhesion. However, the CFU-F count has been shown to be inversely related to the seeding density (Veyrat-Masson, Boiret-Dupre et al. 2007), and therefore valid comparisons can only be made between samples with identical seeding densities. Additionally, the MNC method requires more processing and some MSCs are likely to be lost during the Lymphoprep separation process.

To my best knowledge, this is the first study to assess the relationship between MNC (as described by Castro-Malaspina(Castro-Malaspina, Gay et al. 1980)) and Direct Plating Method (as described by Galotto (Galotto, Berisso et al. 1999)) for the assessment of MSC numbers in ICBM aspirate.

6.4.5. Systemic stimulation of MSC by trauma and relationship with SIRS

There were no clear trends on the effect of trauma over BM MSC over time. Interestingly, patients with higher baseline (Day 0) levels of MSCs appeared more likely to have a decreasing trend over time. Presence of Isolated Trauma appeared to upregulate MSC (CFU-F count) up to 18-fold over a period from 3 to 8 days post-trauma in some patients, but in others the CFU-F count dropped to a median of 45% Day 0 value over similar periods of time. Within the Polytrauma group, patients with increasing trend showed an increase up to 40-fold over a period from 4 to 32 days post injury.

Patients with a decreasing trend in the polytrauma group has relatively higher Day 0 CFU-F count (450-900 CFU-F/ml) compared to patients with an increasing trend (14-100), thus possibly reflecting an already upregulated MSC state in the former group.

Although Seebach et al (Seebach, Henrich et al. 2007) reported a higher CFU-F ICBM MSC from their "Multiple trauma" cohort, their comparative "Monofracture" cohort all presented with pelvic fractures as well, despite using ISS≥16 as criteria for inclusion into their Multiple Trauma cohort. There are no other similar studies previously published. My study has shown that MSC in bone marrow is dynamic and changes over time following trauma, and therefore the timing of bone marrow aspirate may affect CFU-F MSC numbers. It remains unclear whether the time of sampling in Seebach's work is consistent between both trauma groups.

My study also failed to find any associations between MSC/CFU-F trends and the presence of SIRS. This in agreement with previously reported lack of associations between bone marrow CFU-F numbers and levels of the pro-inflammatory cytokine interleukin-6 as well as total white cell count in peripheral circulation (Seebach, Henrich et al. 2007).

It is therefore likely, that the MSC dynamics may be influenced by cytokines, other than inflammatory ones, including anabolic growth factors such as platelet derived growth factor, epidermal growth factors and basic fibroblast growth factor(Short, Brouard et al. 2003).

6.4.6. MSC in peripheral circulation

No CFU-F colonies were isolated from all the peripheral blood samples obtained in this study. Animal models have previously found that MSCs are present in peripheral circulation (Kuznetsov, Mankani et al. 2001; Rochefort, Delorme et al. 2006), with ability to mobilise (Duan, Yang et al. 2006; Yoon, Park et al. 2010) and migrate to sites of injury from remote bone marrow cavity (Shirley, Marsh et al. 2005). Similarly, human studies have suggested the presence of MSCs in peripheral circulation in healthy controls (Zvaifler, Marinova-Mutafchieva et al. 2000; Kuznetsov, Mankani et al. 2001; Khosla and Eghbali-Fatourechi 2006; Mansilla, Marin et al. 2006). However, criteria used by these authors do not fully conform to the criteria of MSCs as set out by the ISCT definition (Dominici, Le Blanc et al. 2006). The use of a single cell surface marker alone (Zvaifler, Marinova-Mutafchieva et al. 2000; Khosla and Eghbali-Fatourechi 2006) , or properties of plastic adherence and osteogenecity without confirming the trilineage potentiality of these cells (Zvaifler, Marinova-Mutafchieva et al. 2000; Kuznetsov, Mankani et al. 2001) may be inadequately rigorous. By application of scrupulous functional and phenotypic standards, our group previously failed to prove the existence of MSC in

peripheral circulation despite having a total sample volume of over 500ml (multiple patients) (Jones and McGonagle 2008).

Interestingly, a previous study found the presence of MSCs in peripheral circulation in about half their lower limb fracture patients (Alm, Koivu et al. 2010). However, all their positive samples were obtained soon after surgery. This differs from my study, where peripheral blood samples (for CFU-F) were obtained immediately prior to their surgery. The positive findings in Alm et al (Alm, Koivu et al. 2010) could be due to physical release or translocation of MSCs during surgery (similar to fat embolus) rather than as a result of systemic mobilisation of MSCs following trauma.

6.4.7. Limitations

In order to investigate the temporal dynamics of MSC following trauma, suitable patients need to have operative procedures at least twice during the same admission. Ideally, for the CFU-F study, all samples would be taken on fixed time points from injury, such as on admission and day 5 following trauma. However, ICBM samples can only be obtained when the patients go to theatre for their planned operative procedures. As most trauma cases require either one or no operative procedures, this very much limits the pool of suitable patients to recruit. In addition, identical time points for BM sample collection were very hard to obtain as timing for the patient's operative procedures was dictated by clinical needs. This made comparison of data within the same group (Isolated trauma or Polytrauma) difficult as the BM samples were taken at different time points to each other.

7. RESULTS: Relationship between MSC and Growth factors dynamics following Trauma

7.1. Introduction

Thus far, this study has shown that within the same patient, following trauma, bone marrow MSC colony-forming unit fibroblast (CFU-F) numbers tend to dynamically change over time. Attempts to correlate these MSC changes to trauma severity and presence of acute complications (SIRS) did not reveal particularly strong relationships.

As mentioned in previous chapters, following fracture, there is an inflammatory response locally, with aggregation of leucocytes and other inflammatory cell types (Bolander 1992). These trigger release of further cascades of growth factors and cytokines (Bolander 1992). Some of these molecules "escape" into the peripheral circulation and get rapidly diluted explaining why some of these growth factors in peripheral circulation occur in areas distant from the fracture site (Zimmermann, Henle et al. 2005).

This study has also shown that circulating levels of growth factors such as platelet derived growth factor-AA (PDGF-AA), angiogenin, follistatin and transforming growth factor beta 2 (TGF-β2) are affected by trauma severity/types. In turn, follistatin and TGF-β2 impact on the fracture healing outcome (delayed healing). As MSCs are precursors of osteoblasts (Justesen, Stenderup et al. 2002; Bielby, Jones et al. 2007; Chagastelles, Nardi et al. 2010), and therefore integral in the fracture healing process, it would be reasonable to infer that at least PDGF-AA and TGF-β2 could be responsible for the observed systemic bone marrow MSC changes following trauma.

Some of the growth factor measured has already been shown to exhibit *in vitro* effects on MSCs. Although PDGF-BB has been reported to have both a proliferative and osteogenic stimulatory effect on MSC in *in vitro* culture (Pountos, Georgouli et al. 2010), other studies reported that stimulation of the PDGF β receptor site induces MSC proliferation (Kumar, Salimath et al. 2010) and may inhibit osteogenic differentiation (Tokunaga, Oya et al. 2008). Co-culture of MSC with TGF-β in different concentrations upregulated the expression of several extracellular matrix proteins and promotes MSC proliferation (Zhao, Li et al. 2011). The effect of follistatin on MSCs has not previously been reported, although Activin A (which is inhibited by follistatin) was shown to play a pivotal role in osteogenic differentiation of MSCs (Djouad, Jackson et al. 2010) suggesting that follistatin may be inhibitory. It has been suggested that the initial inflammatory response on the fracture site creates

an avascular region (Bolander 1992), and therefore, angiogenin's ability to improve MSC survival in hypoxic conditions (Liu, Bai et al. 2008) may be pivotal in the fracture healing process.

Therefore, the aim of this chapter is study the influence that growth factors in peripheral circulation may exert on bone marrow MSCs. Given the previously documented influence that PDGF and TGF-β exerts on MSC, the relationship between PDGF-AA and TGF-β2 with MSC will be studied in more details. As this study did not explore the osteogenic potential of MSC (in relationship to follistatin) or hypoxic survivability (in relationship to angiogenin), detailed analysis for these 2 molecules will not be carried out.

7.2. Methods

7.2.1. Patient Selection

In order to further understand the changes in bone marrow MSC CFU-F over time, and whether these changes could be influenced by PDGF-AA, angiogenin, follistatin and TGF-β2 in peripheral circulation, a group of patients was chosen to participate in both the Growth Factor and MSC studies, allowing direct correlations to be made between cytokine release in peripheral blood (Growth Factor study) and MSC dynamics (MSC study). Patients were only included within this group if samples for both Growth Factor and MSC study were time-matched (in terms of time post injury). The characteristics of patients (n=15) participating in both Growth Factor and MSC Study are shown in Table 7.2-1.

Table 7.2-1 Patients participating in both Growth Factor and MSC Study (n=15)

	MSC ID	Growth Factor ID	Sex	Age	ISS
ISOLATED TRAUMA	BT006	BP7	M	53	9
	BT007	BP8	M	21	9
	BT008	BP9	M	63	9
	BT013	BP13	M	51	4
	BT014	BP14	F	32	9
			4M/1F	Median 51 Range 21-63	Median 9 Range 4-9
POLYTRAUMA	*BT005	BP6	F	25	27
	BT015	BP15	M	49	24
	*BT016	BP16	M	21	24
	BT017	BP18	F	45	38
	BT018	BP19	M	53	24
	BT019	BP20	F	41	34
	BT020	BH18	M	41	29

	*BT021	BP27	M	28	27
	*BT025	BP31	M	38	27
	BT027	BH21	M	49	34
			7M/3F	Median 41 Range 21-49	Median 27 Range 24-38

*denotes patients with 3 matched time-points, all other patients have 2 matched time-points

7.2.2. Growth Factor and MSC Assays

Details of both the method for both Growth Factor and MSC assays have been previously described in Sections 4.2.4 and 6.2. Briefly, growth factors PDGF-AA, angiogenin, follistatin and TGF-β2 were analyzed using the ELISA method. MSC assays are carried out based on the CFU-F colony counts using both MNC and Direct Plating methods.

Serum samples identical to those already measured for growth factors PDGF-AA, angiogenin, follistatin and TGF-β2, were additionally analyzed for levels of PDGF-BB. This was carried out with a commercially available kit from R&D Systems, employing the quantitative sandwich enzyme immunoassay technique, similarly to those measured for the other growth factors factors PDGF-AA, angiogenin, follistatin and TGF-β2.

In brief, a monoclonal antibody specific for PDGF-BB has been pre-coated onto a micro plate. Standards or samples (pre-diluted 20 fold) were then pipetted into the wells where molecules of interest were bound by relevant immobilized antibodies, by incubating for 2 hours at room temperature. After washing away any unbound substances (4 washes), a further enzyme-linked polyclonal antibody specific for the study molecule (Conjugate) was then added to the well, and incubated a further 2 hours at room temperature. Following a further 4-wash cycle to remove any unbound antibody-Conjugate, a Colour reagent (Substrate solution) was then added to the wells and colour would then develop in proportion to the amount of study molecule bound. The colour development occurs at room temperature over 30 minutes before being stopped by adding sulphuric acid before the colour intensity is read by Plate Reader. The methods of using Plate Readers and interpretation of data have been previously described in Section 4.2.4.5.

Given the high inter-individual variations in baseline (Day 0) values for both Growth Factors and MSC studies, subsequent analyses were carried out based on changes from baseline, with baseline values normalized to 100%, and all further time points represented as percentage change from baseline.

The differences in Growth Factor values between Polytrauma and Isolated Trauma patients have already been previously described in Section 4.3.4 and showed a more pronounced suppression of PDGF-AA and angiogenin and elevation of follistatin in the first 48 hours post injury within the Polytrauma group compared to Isolated Trauma. There was no clear difference in TGF-β2 dynamics between these two trauma groups. Therefore, the inter-individual differences between both trauma groups would have been taken into consideration, allowing for collation of data from all the patients within this cohort, to investigate the relationship between growth factor and MSC dynamics. In other words, for this section of analysis, patients in both trauma groups are analysed together.

Finally, as previously discussed in Chapter 5, platelets are a potential source of growth factors, including PDGF-AA, PDGF-BB and TGF-β2. The statistically significant ($p<0.01$) relationship between circulating levels of PDGF-AA and platelet has already been previously shown (Section 5.3.1), whereas TGF-β2 did not exhibit a relationship with circulating platelet levels. Therefore, in addition to analysis of the relationship between the measured growth factors and MSC/CFU-F, further analysis was carried out to determine whether circulating platelets level influences MSC/CFU-F numbers.

7.2.3. Statistics

Statistical analysis was carried out using PASW Statistics (SPSS) version 17.0.2 and graphing performed using Graph Pad Prism version 4.00 for Windows (San Diego, California, USA). As Gaussian distribution could not be assumed given the small sample size, non-parametric tests were carried out. Spearman's rank correlation coefficient was used to test relationship between two variables (for example correlations between changes in cytokine levels in circulation with changes in CFU-F counts). Statistical significance is assumed at $p<0.05$.

7.3. Results

In order to further understand the relationship between serum concentrations of the measured growth factors (PDGF-AA, follistatin, angiogenin and TGF-β2) and the bone marrow (BM) MSC/CFU-F dynamics, the growth factors were analysed against time matched BM MSC/CFU-F (both MNC and Direct Plating methods) (Figure 7.3-1).

7.3.1. Relationship between Growth Factor and BM MSC

As can be seen in Figure 7.3-1, no correlations were found between all the growth factors and CFU-F (Direct Method). However, when the growth factors were analysed against CFU-F (MNC Method), correlations were observed with 2 growth factors. Firstly, follistatin was negatively correlated ($R=-0.41$, $p=0.02$) with CFU-F. Secondly, PDGF-AA showed a strong statistically significant positive correlation ($R=+0.55$, $p<0.01$).

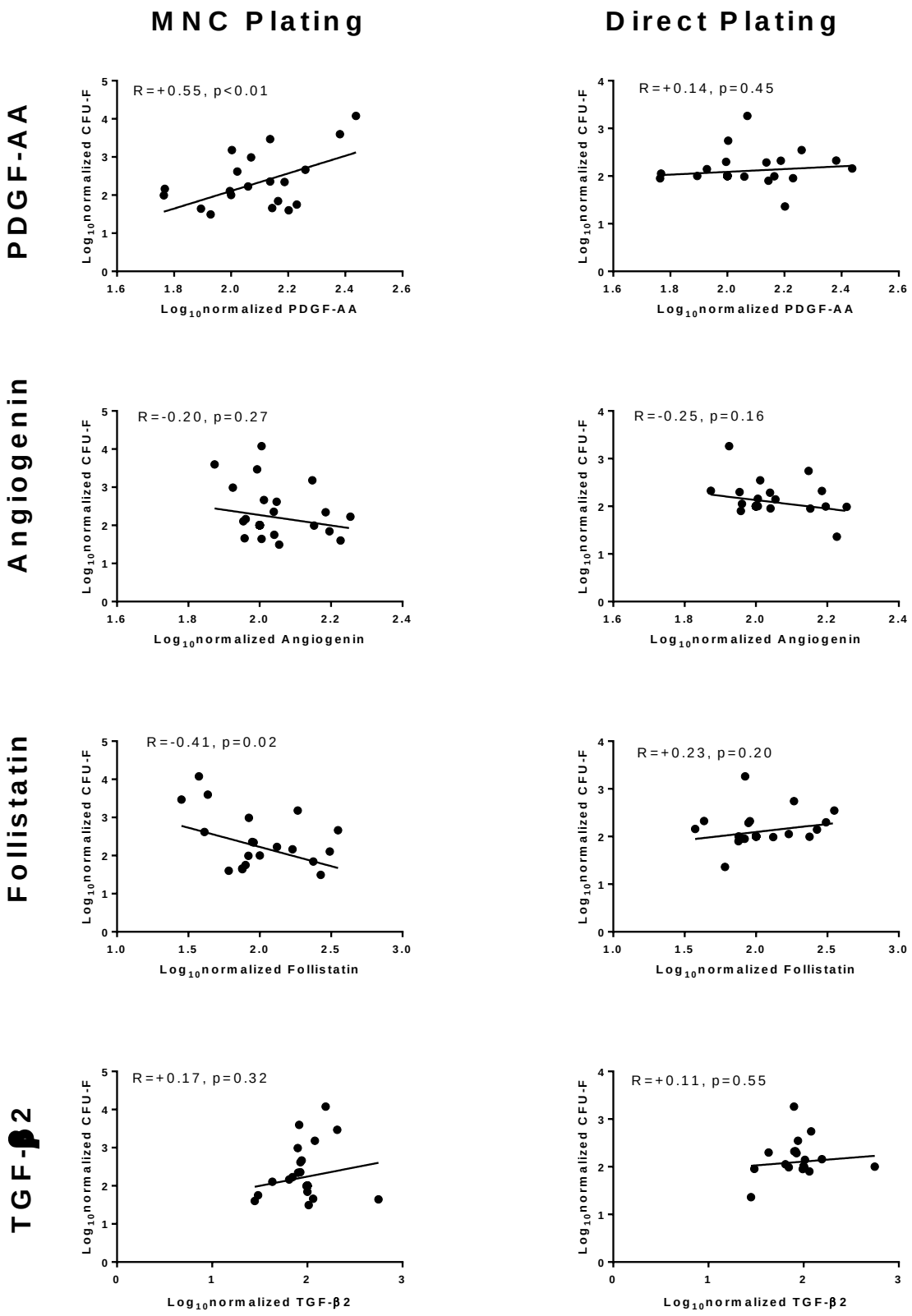

Figure 7.3-1 Relationship between Growth Factor and CFU-F. Values for both CFU-F and growth factors are normalized to Day0=100 and then transformed by applying the logarithmic scale. There was a statistically significant relationship between time matched PDGF-AA levels and CFU-F count via MNC method.

7.3.2. Relationship between Platelets, PDGF-AA and BM MSC

It has been observed that the amount of PDGF-AA isoform in peripheral circulation had the strongest systemic effect on bone marrow MSC (Figure 7.3-1). In order to further investigate the underlying causes of this relationship between PDGF-AA and bone marrow MSC, further investigations were carried out.

Firstly, the positive relationship between circulating levels of PDGF-AA and platelets have already been previously shown (Section 5.3.1, implicating platelets as the source of PDGF-AA (reproduced here as Figure 7.3-2-A). This statistically significant relationship was maintained when analysed by data derived from the sub-group of patients participating in the Growth Factor/MSC-CFU-F analysis (Figure 7.3-2-B).

However, platelets, as a source of cytokine storage, also release the PDGF-BB isoform and other growth factors such as TGF-β2 on activation (McNicol and Israels 2008). PDGF-BB isoform is one of the 3 common PDGF isoforms (the third one being –AB) and are able to bind to both the PDGF receptor-α and β types (Heidaran, Pierce et al. 1991). The relationship between circulating levels of PDGF-BB and platelets (Figure 7.3-2-B) is less significant (R=+0.55) compared to the PDGF-AA/platelets association (R=+0.68). However, an investigation of the relationship between both the –AA and –BB isoforms (Figure 7.3-2-D) illustrated a statistically significant correlation (R=+0.61, $p<0.05$) between both isoforms.

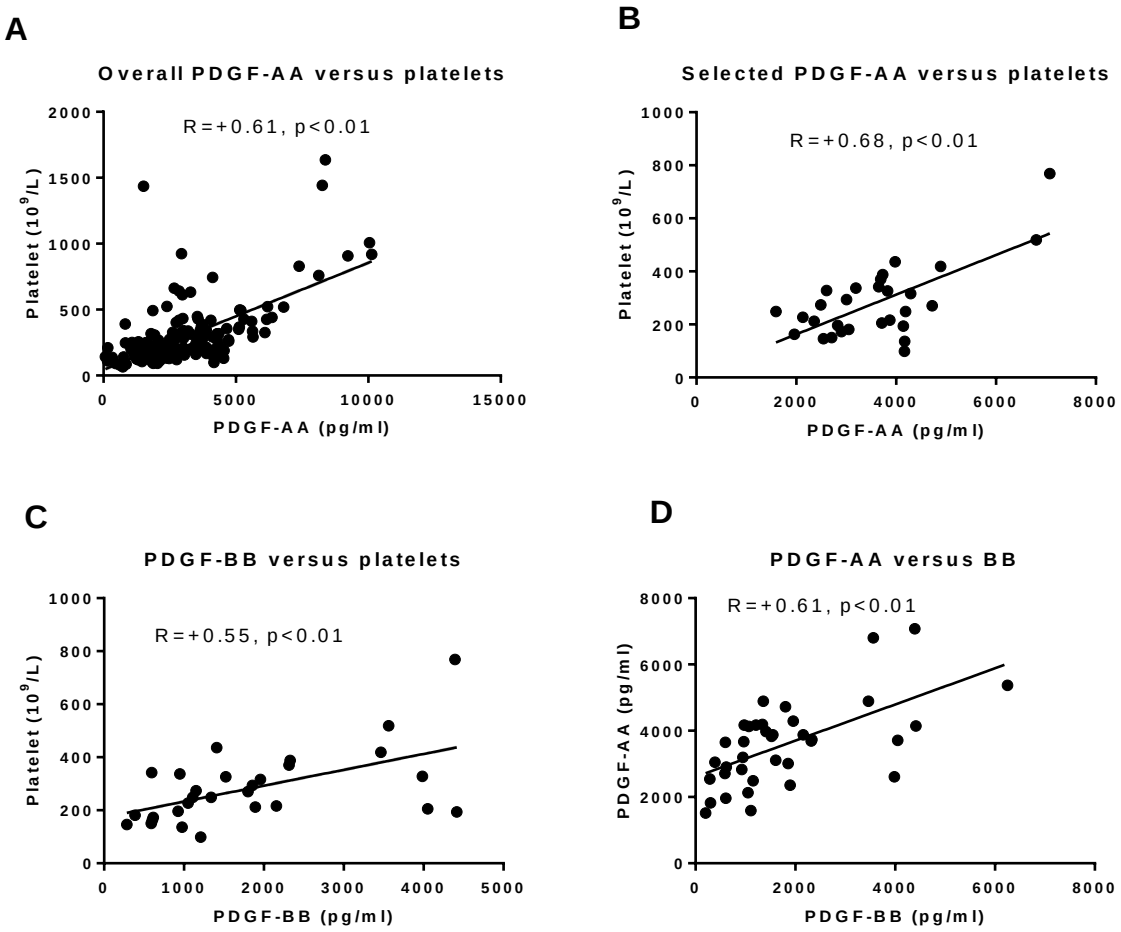

Figure 7.3-2 Relationship between platelets, PDGF-AA and PDGF-BB.

(A) Represents the relationship between matched time points for levels of platelets and PDGF-AA in peripheral circulation for all patients recruited in this study. (B) Represents the sub-group of 15 patients who have matched growth factor and CFU-F time points and hence represents the relationship between matched time points for levels of platelets and PDGF-AA in peripheral circulation in this sub-group. (C) Represents the relationship between matched time points between platelets and PDGF-BB in peripheral circulation in the same 15 patient cohort as in B. (D) represents the relationship between matched samples for levels of PDGF-AA and PDGF-BB in the same sub-group of patients as in B.

However, not only has the PDGF-BB isoform which have been shown to be most potent in in inducing collagen synthesis and alkaline phosphatase activity in osteoblastic cells of murine origin (Pfeilschifter, Krempien et al. 1992), it is also isoform which has been shown to affect MSC proliferation (Pountos, Georgouli et al. 2010). Therefore, given the relationship with PDGF-AA and platelets seen in Figure 7.3-2, the relationship between PDGF-BB and MSC/CFU-F were next investigated.

As expected, given the relationships shown above, an investigation on the relationship between PDGF-BB and CFU-F (MNC Plating) (Figure 7.3-3) showed a similar positive correlation between these two parameters (R=+0.38, p=0.03). However, this relationship was weaker compared to PDGF-AA, implying that it may be the –AA isoform which was responsible for the observed relationships. The relationship between PDGF-BB and CFU-F (Direct Plating) remains similarly weak.

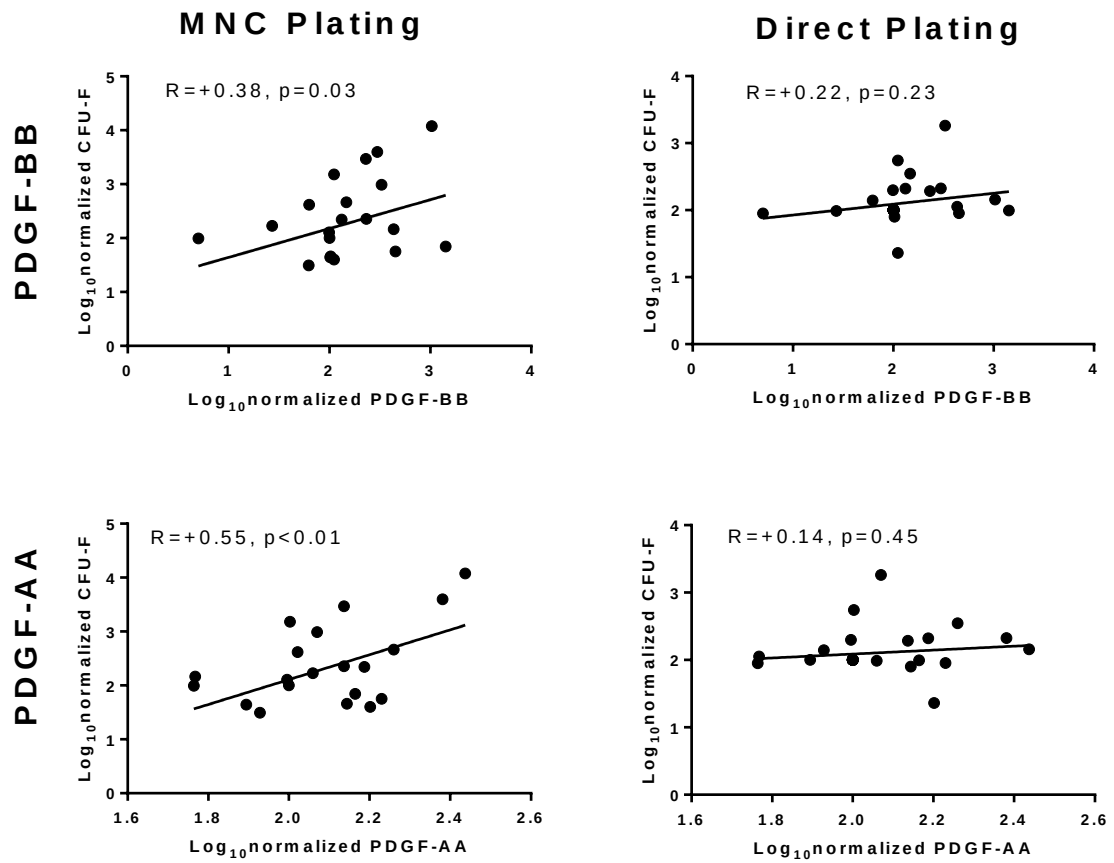

Figure 7.3-3 Relationship between PDGF-AA, PDGF-BB and CFU-F. Values for CFU-F, PDGF-AA and –BB are normalized to Day0=100 and then transformed by applying the logarithmic scale. There was a statistically significant correlation between PDGF-AA and CFU-F (MNC plating).

Having analysed the influence of PDGF-AA and –BB peripheral circulation on ICBM CFU-F, I next analysed the influence of both PDGF isoforms on cellular proliferation. Given identical *in vitro* culture conditions, any changes in the average CFU-F colony size (and hence rate of proliferation) over time in the same patient, would therefore be derived from *in vivo* changes in that patient. Interestingly, as seen in Figure 7.3-4, changes in average colony size over time was not influenced by changes in levels of PDGF-AA and –BB. This implies that circulating PDGF-AA and –BB does not exert an influence on *in vitro* cellular MSC proliferation. The duration the ICBM MSCs were exposed to *in vitro* conditions (14 days) may have diminished the *in vivo* influence on the proliferative rate of these cells.

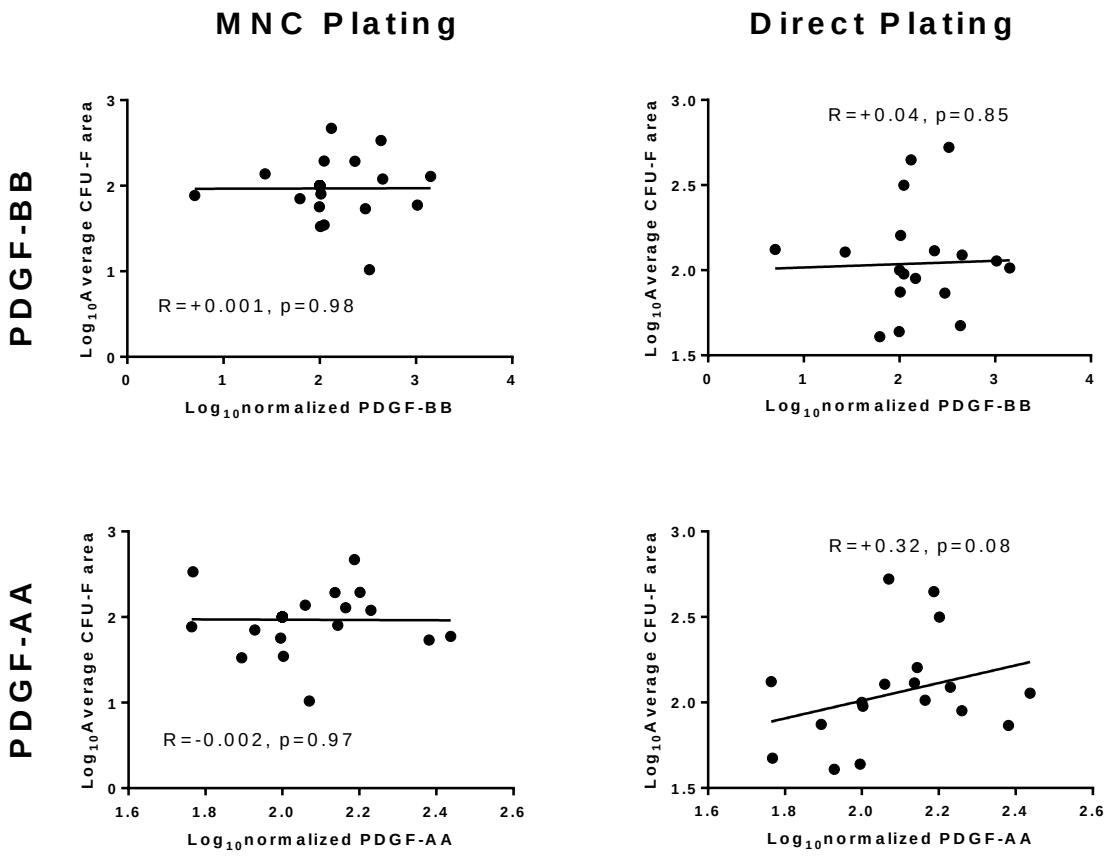

Figure 7.3-4 Relationship between Average CFU-F size and PDGF-AA/BB. Values for both CFU-F and PDGF-AA/BB are normalized to Day0=100 and then transformed by applying the logarithmic scale. No correlation found between CFU-F area and PDGF-AA/-BB levels.

Finally, given the association between platelets and PDGF-AA/BB levels shown in Figure 7.3-2, the influence of platelets levels on CFU-F levels were next investigated.

As can be seen in Figure 7.3-5, there is a positive relationship between changes in platelet levels and CFU-F (MNC Plating) from baseline levels, but is weaker compared to the relationship between PDGF-AA and CFU-F (MNC Plating). Similar to analysis with PDGF-AA and -BB, the relationship between platelets and CFU-F (Direct plating) is weak.

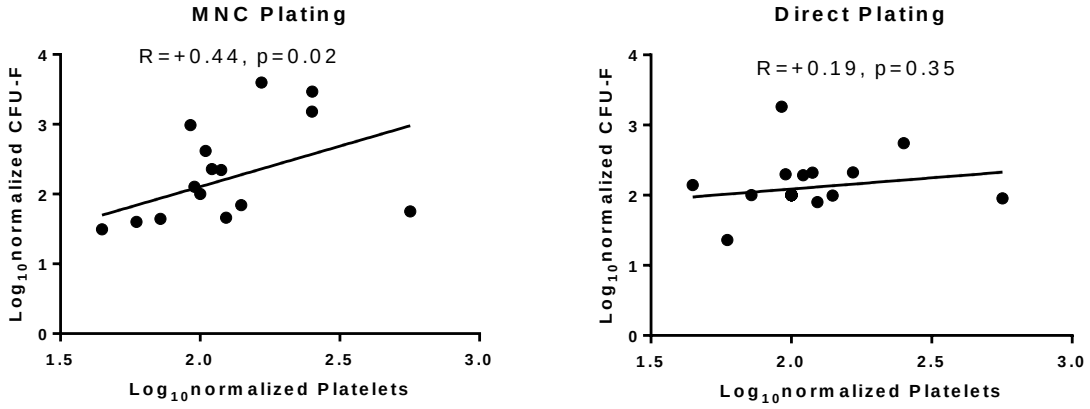

Figure 7.3-5 Relationship between Platelets and CFU-F. Values for both CFU-F and platelets are normalized to Day0=100 and then transformed by applying the logarithmic scale. Statistically significant correlation found between matched platelets and CFU-F (MNC) levels.

7.4. Discussion

7.4.1. Influence of PDGF-AA on MSC dynamics

Histological studies of callus formation site revealed a presence of PDGF-AA expression in all stages of fracture healing, and predominating in the early (reactive) and late (remodelling) phase of fracture healing, with the –BB isoform being dominant in between(Andrew, Hoyland et al. 1995). Most of the current literature relates to the stimulatory effect of PDGF-BB isoform on MSC proliferation *in vitro*(Pountos, Georgouli et al. 2010), accelerated fracture healing in rat (Lee, Park et al. 2000; Hollinger, Onikepe et al. 2008; Moore, Ehrlich et al. 2009) and rabbit (Nash, Howlett et al. 1994) models and improved osseous closure as part of synthetic bone scaffold in human periodontal defect (Nevins, Giannobile et al. 2005). In this study, there appears to be a strong correlation between changes in CFU-F levels in MNC plating with changes in both PDGF-AA and -BB levels in peripheral circulation following fracture/trauma. Although the associations with the –AA isoform is more statistically significant, given the wealth of literature on the effect of the –BB isoform on MSC stimulation and bone healing, it is highly likely that both isoforms play a role in the observed relationship. The relationship with Direct plating however was not significant. This could be due to the inclusion of whole bone marrow constituent during the plating process, including platelets (compared to mononuclear cells only during MNC plating). This constituent may contain additional cytokines which exert an influence on MSC in culture (such as increased proliferation), resulting in an artificially inflated CFU-F count and hence MSC enumeration. As a result, MNC plating in comparison provided a “cleaner” readout, and better representing MSC enumeration from the ICBM aspirate.

Unfortunately, only a single patient (BP31/BT025) received platelet transfusion between sampling time points. This was insufficient to carry out an analysis of whether patients who have received platelet transfusion could exhibit similar effects on MSC dynamics compared to patients with endogenous platelets fluctuations only.

7.4.2. MSC-pericytes-PDGF relationship

It remains unclear the mechanism underpinning the changes in MSC CFU-F over time following trauma. MSCs within the trabecular bone have been shown to be far more abundant in numbers compared to those obtained on bone marrow aspiration (Sakaguchi, Sekiya et al. 2004; Jones, English et al. 2010). The increased/decreased numbers of MSC on bone marrow aspiration may be due to a change in MSC proliferation (total number) or increased availability (mobilisation).

Recently, there have been suggestions that cell lining the blood vessels (pericyte) may be a source of MSC (Crisan, Yap et al. 2008; Crisan, Chen et al. 2009; Caplan and Correa 2011). Based on this model, PDGF-BB has been suggested to play a key role in mobilizing MSC from its pericyte origin (Caplan and Correa 2011). To my knowledge, this is the first study to show the *in vivo* relationship between PDGF-AA/BB in peripheral circulation and its systemic effect on bone marrow MSCs. If the pericyte hypothesis is true, then the changes in BM MSC CFU-F are due to increased mobilisation rather than proliferation.

7.4.3. Influence of Follistatin on MSC Dynamics

The influence of serum follistatin on MSC dynamics is likely related to its close inhibitory relationship with activin A (de Kretser, Hedger et al. 1999). Activin A has already been shown to affect MSC proliferation (Stewart, Guan et al. 2010) and osteogenic differentiation (Djouad, Jackson et al. 2010). Therefore, being an inhibitory molecule, it would be in keeping with current literature that follistatin has a negative correlation with bone marrow MSC CFU-F changes.

7.4.4. Future work arising

The most interesting finding within the chapter is the association found between PDGF-AA/BB/platelets and MSCs. This finding however does not prove causation. There would be potential to further this work, firstly to prove causation in an animal model. Preferably, due to the known short half-life of PDGF, it is likely that this model would involve the transfusion of platelets and the comparison of its effect on bone marrow MSCs pre- and post-transfusion.

7.4.5. Limitations

This study did not conclusively show whether the influence of PDGF on MSC BM dynamics was due to increased mobilization from its cellular niche (increased availability) or increased cellular proliferation. Further investigations would need to be carried out to unravel this question. This may involve concomitant trephine core biopsy with bone marrow aspiration, and immunohisto-staining of MSC cells to show changes in numbers in its native bony environment.

Secondly, the observation of the negative effect follistatin has on MSC dynamics, can be extrapolated to a positive relationship between serum activin and MSC changes. The serum levels of activin have not been measured within this study.

8. Conclusion and Future Work

8.1. Growth Factors and Trauma

In my first results chapter, I assessed the early dynamics of growth factors (PDGF-AA, angiogenin, TGF-β2, follistatin) over time following trauma, across 4 different groups of patients (Head Injury, Polytrauma, Isolated Trauma, Healthy Control).

The growth factors were chosen, as they are known to play an important role in the fracture healing process (Dimitriou, Tsiridis et al. 2005). PDGF is a major mitogen for MSCs and strongly induces the mitogenic and migratory response of MSCs (Mehrotra, Krane et al. 2004) with the PDGF-AA isoform playing a crucial role in osteoblast replication (Yang, Chen et al. 2000). Angiogenin facilitates the process of endothelial cell invasion and angiogenesis (Hu, Riordan et al. 1994), creating the obligatory pre-condition of healthy tissue perfusion in the fracture healing environment. TGF-β2 is secreted by osteoblasts and osteoclasts (Robey, Young et al. 1987) and have been shown to significantly enhance new bone formation in animal models(Mackie and Trechsel 1990). Finally, follistatin has been shown to have inhibitory effects on various other molecules of the BMP family (Fainsod, Deissler et al. 1997; Otsuka, Moore et al. 2001; Amthor, Christ et al. 2002).

The Isolated Trauma group was selected to understand the influence of trauma on the temporal dynamics of growth factors. The Polytrauma Group additionally helped to understand the influence of further injuries and fractures, compared to controls. The Head Injury Group provided supplementary information, on the additional effects of traumatic brain injury on multiply injured patients (Polytrauma Group). Finally, the Healthy Control group was aged and sex matched to all 3 trauma groups, to provide baseline (pre-trauma) levels of these growth factors.

My main finding from this analysis was the suppression of anabolic growth factors (PDGF-AA, angiogenin) initially by day 1 compared to control group of patients and upregulated in the inhibitory growth factor (follistatin). This effect was observed to be greater in the more severely injured group (Head Injury and Polytrauma). In other words, the more severe the trauma, the more pronounced the suppression/upregulation.

There have been other studies published on the dynamics of growth factors following fractures, these studies were limited to patients with single fractures only (Weiss, Zimmermann et al. 2009; Pountos, Georgouli et al. 2013) . However, to my best knowledge, my study is the first to compare the effects of the growth factor across trauma groups with different severity (Isolated Trauma versus

Polytrauma Groups) as well as the additional effect of the presence of traumatic brain injury (Head Injury Group).

I then attempted to understand the mechanism or reason behind the initial suppressions of these growth factors, which appeared to worsen with increasing trauma severity. One possible factor explored was the effect of intravenous fluid dilution, as patients admitted following trauma often receive intravenous fluid as part of their resuscitation. Moreover, patients with multiple injuries (hence Polytrauma/Head Injury groups) often require higher volume of fluid resuscitations. Although I found a significantly higher volume of intravenous fluid transfusion in Polytrauma/Head Injury patients compared to Isolated Trauma, when analysed against the levels of all 4 growth factors, I failed to find any significant associations between volumes of fluid transfusion to concentration of growth factors.

Hence, I believe that the observed initial suppression of anabolic growth factors is genuine suppression. Firstly, as described above, no correlations with fluid dilutions were found. Secondly, identical/matched samples from these patients were observed to have an upregulation of follistatin, with both suppression (PDGF-AA, angiogenin) and upregulation (follistatin) trends returning towards baseline (Healthy Control) levels by 1 week post trauma. Thirdly, the *in vivo* half-lives of the molecules measured are relatively short [PDGF-AA (2 minutes) (Cianciolo, Stefoni et al. 1999), follistatin (4 minutes) (Kogure, Zhang et al. 1996), TGF-β2 (2 minutes) (Kaminska, Wesolowska et al. 2005), angiogenin (12 hours) (Hatzi, Bassaglia et al. 2000)]. This implies that the concentrations of these growth factors detected represented what was being actively synthesised and released into the peripheral circulation at that moment in time. The mechanisms behind this observed dynamics remain to be explored.

8.2. Growth Factor and Inflammatory Response

My next step to try to address the observed growth factor dynamics was based on the observations that the degree of trauma severity (Isolated versus Polytrauma/Head Injury) exerted differential influence on the levels of these growth factors. Previous studies have reported on the association between the severity of trauma and levels of inflammatory response cytokines in peripheral circulation (Giannoudis, Harwood et al. 2008; Giannoudis, Mallina et al. 2010). Therefore, the differences in growth factor dynamics were explored in relation to degree of inflammatory response. To my knowledge, this association has not been previously explored.

I selected parameters available from routine hospital laboratory investigations that were known to be reflective of inflammatory state (white cell count, C-reactive protein, platelets). Overall, no

correlations were found between the inflammatory states of the patients (as represented by levels of white cell count, C-reactive protein, platelets). The exception was the incidental finding of correlation between levels of platelets and PDGF-AA. This was not surprising, given that the alpha-granules of platelets were a major storage site of cytokines including PDGF-AA (McNicol and Israels 2008).

As the analysis of biochemical parameters representing inflammatory state of the patients did not address the observed differences between trauma groups, I then analysed the dynamics of growth factors against clinical presence of inflammation. Systemic Inflammatory Response Syndrome (SIRS) is a recognized clinical phenomenon representing a severe degree of inflammatory response. Patients in all groups were divided into either the ones who fulfilled criteria for SIRS against those that did not. Similarly, no significant differences were found in the growth factor levels over time when compared to the presence or absence of SIRS.

Finally, I explored the potential translational significance to clinical practice of my growth factor dynamics observations. I correlated the levels of growth factors into patients who had normal healing response compared to patients who had delayed or fracture non-union. It appeared that levels of follistatin non-union group showed an increasing trend in the first 3 days post injury, compared to a decreasing trend in the normal healing group. However, the differences between levels on matched time points between these 2 groups of patients in isolation did not reach statistical significance overall. Therefore application of the concept of increasing follistatin trends in the first 3 days post trauma as a predictive stratification of fracture non-union may be possible. However, if the trends when applied to future patients were less noticeable, then interpretation (into high or low risk of fracture non-union) can be tricky.

Thus far, interpretation of TGF-β2 dynamics has been fraught with difficulty, due to the high inter-individual variations in levels both in trauma as well as healthy control patients. Yet, when analysed against patients with normal versus “non-union” fracture healing, a clear and significant difference emerged. Patients who developed fracture non-union had consistently low levels of TGF-β2 in the first week following injury, and this difference was statistically significant. Bearing in mind the high inter-individual difference in baseline (non-trauma) levels, I would speculate that it is the patients who have inherently low levels of TGF-β2, who then continued to express low levels following trauma, and eventually developing fracture non-union. A different isoform of this molecule (TGF-β1) have also similarly been reported to be depressed in patients with fracture non-union, but only detectable at week 4 post injury (Zimmermann, Henle et al. 2005). Therefore, TGF-β2 would be a better candidate molecule for application as an early predictive tool of fracture non-union. Ideally, I would like to measure the levels of TGF-β2 in patients pre- and post-fracture and confirms their

correlation with fracture non-union. However, this would require mobilisation of significant resources on a large (national) scale basis in order to obtain pre-fracture blood samples, then matching with a very small proportion or patients who eventually sustained a fracture, and fracture non-union. Similar efforts have been carried out for the investigation of bone turnover markers as predictors of fracture risk in the elderly with some success (Ivaska, Gerdhem et al. 2010).

To conclude, the degree of inflammatory response (biochemical and clinical) does not correlate with growth factor dynamics, despite differences observed between patients with different trauma severity. TGF-β2 may be a candidate molecule for early prediction of fracture non-union.

8.3. MSCs and Trauma

Thus far, I have explored the relationship between circulating growth factors and trauma. I have shown that the levels of growth factors following trauma is dynamic and changes over time. Following fracture, MSCs play a central role in the fracture healing process, as it is the progenitor cells that will differentiate amongst others to osteoblast, which is responsible for bone formation(De Bari, Dell'Accio et al. 2008). Additionally, MSCs have been shown to respond to different cytokines *in vitro*, including inflammatory cytokines (Crop, Baan et al. 2010; Herrmann, Weil et al. 2011), and growth factors (Pountos, Georgouli et al. 2010). This *in vitro* relationship has never been explored in humans *in vivo*. Therefore, I wanted to assess the systemic effect that trauma (and circulating growth factors) exerts on MSCs.

For this analysis, I obtained samples from patients' iliac crest bone marrow (ICBM) as well as peripheral blood. ICBM aspirate can be easily obtained, and is well described as a source of osteoprogenitor cells and bone graft material (Hernigou, Poignard et al. 2005). Animal models have shown that following injury, MSCs may be released into the peripheral circulation, and homing in to the fracture site (Shirley, Marsh et al. 2005; Yoon, Park et al. 2010). Therefore, I wanted to assess for the presence and levels of MSCs in peripheral blood following trauma.

The classical method of colony forming fibroblast unit (CFU-F) was used for the purpose of MSC enumeration. I carried out two different previously published techniques in parallel, Direct plating (Galotto, Berisso et al. 1999) and Mononuclear Cell (MNC) Plating (Castro-Malaspina, Gay et al. 1980). Overall, I did not find one technique to be superior to the other. Hence the Direct Plating method appeared favourable as it is technically simpler, and therefore less opportunity for technical errors to occur. However, the Direct plating method involved the additional presence of bone marrow constituents (cells, serum) compared to MNC Plating method, and may influence MSC

culture *in vitro*. This was clearly shown by the lack of association between matched PDGF-AA and MSC enumeration by the Direct Plating method, compared to MNC Plating method.

No MSC were found in peripheral blood samples following trauma, implying that MSCs are not mobilized into the peripheral circulation. Rather, the source of MSCs on the fracture healing site is locally derived (from surrounding bone marrow for example).

I found that ICBM MSCs is not static, and changes dynamically over time, and this were proven by both Direct and MNC Plating methods. However, the trend of upregulation or downregulation in MSC numbers over time appeared variable, and was independent of trauma severity. Additionally, the presence of SIRS did not exert any influence on this trend. Similarly, fracture healing outcome was also not associated with any early systemic trends in ICBM MSC numbers.

Given the above findings, I then sought to analyse whether the trends in MSC dynamics could be linked to the dynamics of circulating growth factors. To my knowledge, this is the first study to demonstrate that ICBM MSC numbers is influenced by changes in levels of PDGF and platelets. Therapeutically, this could lead to potential application of platelets transfusion to boost MSC numbers prior to ICBM harvest.

However, as MSCs are not mobilized into the peripheral circulation, the changes in MSC numbers observed could either be due to increased proliferation or local migration within the trabecular bone niche. To address this, I would inoculate identical batches of MSC *in vitro* with patient serum (known previously measured quantities of PDGFs), and observed the proliferative effect on MSCs. I could also set up migration assays with similar inoculation of the same patient serum, and compare the effect on MSC migration versus MSC proliferation. I would be keen to test the hypothesis that platelet transfusion could cause a systemic stimulation of bone marrow MSCs, initially with an animal model, and if proven true, potentially escalate towards human clinical trials.

8.4. Summary

In summary, this thesis showed that trauma severity directly influences the dynamics of growth factors in the peripheral circulation following injury. However, trauma severity does not influence the dynamics of bone marrow MSCs. Instead, it is shown to be associated by changes in levels of platelet/PDGF-AA/BB. Finally, trauma does not result in mobilization of MSCs into the peripheral circulation.

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10. Appendix

10.1. Growth factor dynamics following trauma

Table 10.1-1 Inter-individual variations in PDGF-AA (pg/ml)

	DONOR ID	Day 0	Day 1	Day 3	Day 5	Day 7	Day 14
HEAD INJURY	BH1		1152.2	1365.1	1929.6	1860.2	1968.3
	BH3		1107.4	1509.7	2748.5	2398.6	2854.3
	BH4	852.7	621.4				
	BH6	2081.1	1322.8	2038.2	2868.6	2852.3	2983.2
	BH8		1164.8	1248.0	1485.4	1798.9	2667.5
	BH9		697.9	859.6	1315.5	1751.1	2997.6
	BH10	466.1	729.9	1455.7	2114.3	2681.6	4120.5
	BH11	1038.0	763.7	68.9	3014.0	3285.0	2942.2
	BH13		1464.3	1882.6	2954.1	3324.4	9228.0
	BH16		2113.4	2761.6	4553.5		10133.2
	BH17	1371.5	1892.0	1215.4	3008.5	5625.9	8258.8
	BH18	2133.3	2544.6	3713.2	1077.4	5107.4	6173.9
	BH20	3491.2	2338.0	4259.2	3882.7	5642.8	
	BH21	2491.9	2453.2	2656.6	3830.1	2887.0	
	MEAN	1740.7	1454.7	1925.7	2675.6	3267.9	4938.9
	SD	995.8	689.1	1159.9	1054.9	1428.4	2974.9
	CV (%)	**57.2**	**47.4**	**60.2**	**39.4**	**43.7**	**60.2**
ISOLATED TRAUMA	BP3	3526.9	1805.3	3078.5		5316.2	8129.7
	BP7	2130.5	2236.5	3876.8	3291.0	3916.8	
	BP9	3732.7	2920.6	2692.9	3219.7	3693.3	2466.1
	BP8	3008.6	1642.8	2357.0			
	BP10	1709.6	2206.7	2911.4	3262.4	4447.4	
	JM8	3353.1	1988.7	2920.6	3836.9	5104.3	
	JM12	2999.3	2198.2	2833.5	3439.7	3334.0	
	JM16	2792.5	1424.2	2797.0	2331.1	4768.4	
	BP11	4491.5	3609.2	5507.6	5414.7	6164.9	6313.4
	BP13	3652.7	3018.8	4289.5	3795.0	4462.5	
	BP14	4892.3	4056.7	6152.0	4791.1	6805.5	
	BP17	3673.4	2938.9			3688.7	
	BP23	4186.3	4316.4	3876.6			
	BP22	4657.4	2971.1	4122.2	5126.0	4102.2	
	BP24	3571.8	2480.5	3393.4	4657.4	5144.0	
	MEAN	3491.9	2654.3	3629.2	3924.1	4688.3	5636.4
	SD	884.0	859.4	1105.6	953.3	1013.1	2891.9
	CV (%)	**25.3**	**32.4**	**30.5**	**24.3**	**21.6**	**51.3**
POLYTRAUMA	NT1	822.4	2038.5	154.0	160.5		
	DL	329.2	1977.5	3883.5	832.6		
	BP2	6096.1	2756.9	2838.2		4357.5	5183.8
	BP4	2535.6	1452.4	1848.4	4196.0	4303.5	
	BP6	2905.6	3051.6	3289.3	3852.1	5400.8	
	JM2	849.8	997.3	1831.4	2896.0	5510.2	
	BP15	3194.4	2726.5	2706.7	4124.4	6365.3	
	BP16	1963.0	2391.6	3485.2	4720.1	6197.1	10046.2
	BP12		1486.1	1670.9	2681.9	3545.6	8382.7
	BP18	4167.4	2656.6	4540.8	4732.1		
	BP19	2829.7	1598.8	2205.5	3873.4	3612.4	1686.0
	BP20	4170.7	3053.5	4424.2	3878.5	5303.6	7390.6
	BP27	2608.3	1840.7	2475.4	4145.0	5598.5	1515.3
	BP28	2269.1	2113.5	3003.0	2339.1	3755.1	
	BP31	1593.0	1004.2	1183.5	2020.8	2135.5	

	MEAN	2595.3	2076.4	2636.0	3175.2	4673.8	5700.8
	SD	1533.2	685.3	1206.8	1415.9	1264.4	3545.0
	CV (%)	**59.1**	**33.0**	**45.8**	**44.6**	**27.1**	**62.2**

Table 10.1-2 Inter-individual variations in Angiogenin (pg/ml)

	DONOR ID	**Day 0**	**Day 1**	**Day 3**	**Day 5**	**Day 7**	**Day 14**
HEAD INJURY	BH1		293396.4	527543.0	465445.6	491441.3	548302.3
	BH3		371712.5	432621.8	424407.5	358168.0	478831.7
	BH4	238786.6	304346.1				
	BH6	372118.7	429036.3	553018	451943.7	443424.0	261546.8
	BH8		328711.9	410598.6	441088.4	500405.3	136835.6
	BH9		465445.6	398353.8	341966.7	512274.3	608554.7
	BH10	309180.8	295693.1	406852.8	387846.1	238242.6	160944.1
	BH11	136521.0	208118.2	289960.0	338194.3	408724.6	387230.2
	BH13		229098.1	115324.4	144349.3	82789.72	130022.0
	BH16		122670.0	123478.8	118975.6		62201.8
	BH17	611544.5	517253.4	380322.7	527549.7	558278.9	504201.3
	BH18	364986.5	351665.0	551418.1	605056.2	476843.1	533596.5
	BH20	488198.3	574348.1	663867.4	569395.8	584287.3	
	BH21	356672.2	473544.7	529468.9	545786.1	600033.9	
	MEAN	359751.1	354645.7	414063.7	412461.9	437909.4	346569.7
	SD	144967.9	126622.7	162699.2	149214.4	150524.7	200433.9
	CV (%)	**40.3**	**35.7**	**39.3**	**36.2**	**34.4**	**57.8**
ISOLATED TRAUMA	BP3	440661.2	420373.4	464456.2		612692.0	561676.4
	BP7	378766.9	363934.7	389190.6	370365.2	381906.5	
	BP9	525806.4	488742.3	479710.1	400957.9	472848.1	386756.6
	BP8	379249.3	365122.2	383843.6			
	BP10	334517.5	289207.6	395302.1	457421.7	387486.1	
	JM8	261991.0	222207.2	362985.9	239055.6	266173.5	
	JM12	377321.4	312319.1	363460.2	336126.1	325838.6	
	JM16	429202.9	299685.9	424400.8	343982.3	397536.3	
	BP11	539652.1	537339.5	634060.0	537628.3	393963.3	508161.3
	BP13	551850.8	556230.7	464109.0	448022.6	510429.0	
	BP14	555353.8	414080.1	492667.3	464657.8	503636.5	
	BP17	304752.9	351665.0			385104.2	
	BP23	350796.1	495569.9	540657.4			
	BP22	594189.4	401158.7	563419.6	706850.6	504264.1	
	BP24	501276.2	259352.3	367424.1	365852.8	315473.8	
	MEAN	435025.9	385132.6	451834.8	424629.2	419796.3	485531.4
	SD	103791.4	101365.4	83953.3	123035.1	95985.5	89628.8
	CV (%)	**23.9**	**26.3**	**18.6**	**29.0**	**22.9**	**18.5**
POLYTRAUMA	NT1	472345.4	391735.4	424429.4	374775.7		
	DL	329158.3	419255.2	297973.4	378740.4		
	BP2	408724.1	336248.0	311456.8		332459.6	404387.5
	BP4	410514.8	243408.4	315152.4	406426	356889.2	
	BP6	355433.9	396272.5	373540.0	350358.4	219742.5	
	JM2	310765.7	378988.8	354222.9	331987.2	348432.1	
	BP15	378789.2	430532.9	429464.6	491827.0	159695.5	
	BP16	142332.3	137655.0	147430.6	106331.9	159695.5	128641.5
	BP12		266945.8	378661.7	277697.4	390905.1	366304.0
	BP18	297874.0	277289.6	408220.1	429398.8		
	BP19	320754.3	371586.7	292690.8	351013.3	275456.6	288356
	BP20	388966.5	409976.1	428270.7	372377.0	369447.6	332935.9
	BP27	251576.9	253364.3	538276.6	423790.2	344307.8	357138.8
	BP28	357584.0	506508.8	364955.9	458854.9	324274	
	BP31	381437.7	341891.7	394242.3	584483.5	509006.8	
	MEAN	343304.1	344110.6	363932.5	381290.1	315859.4	312960.6
	SD	80015.9	93454.8	87507.8	109207.3	99706.3	98116.3
	CV (%)	**23.3**	**27.2**	**24.0**	**28.6**	**31.6**	**31.4**

Table 10.1-3 Inter-individual variations in Follistatin (pg/ml)

	DONOR ID	Day 0	Day 1	Day 3	Day 5	Day 7	Day 14
HEAD INJURY	BH1		3219.1	1372.0	907.8	955.7	1025.1
	BH3		2426.9	4654.0	5782.3	2598.0	1570.4
	BH4	4226.7	4281.7				
	BH6	925.3	1465.0	2086.4	855.4	1295.4	771.9
	BH8		3159.0	4872.4	2933.8	820.3	661.1
	BH9		3558.4	1954.2	2271.5	1498.8	1150.1
	BH10	2279.7	2994.3	2703.5	2369.7	2748.0	1507.2
	BH11	2974.1	2123.5	2008.0	4438.7	683.3	960.1
	BH13		945.9	2516.4	1288.9	1729.0	1417.9
	BH16		4809.3	12605.7	9027.7		2025.6
	BH17	2588.5	2356.1	1107.4	1447.6	862.3	768.6
	BH18	5073.7	4722.6	11186.9	5257.8	4344.7	846.7
	BH20	1544.4	1479.0	2988.5	1281.8	1758.8	
	BH21	2240.5	2903.0	2278.1	2032.6	1781.7	
	MEAN	2731.6	2888.8	4025.6	3068.9	1756.3	1155.0
	SD	1358.5	1193.1	3672.5	2420.7	1050.5	425.9
	CV (%)	**49.7**	**41.3**	**91.2**	**78.9**	**59.8**	**36.9**
ISOLATED TRAUMA	BP3	1892.7	2745.1	4679.8		1216.7	3801.1
	BP7	1533.1	2209.4	5417.5	3516.6	2281.8	
	BP9	1837.3	1159.1	1351.3	3572.9	5681.9	5062.5
	BP8	2112.1	2415.6	1594.8			
	BP10	1632.5	2521.6	2340.3	1690.6	1621.5	
	JM8	4224.1	5707.5	8172.2	6910.3	2570.6	
	JM12	1848.4	2062.5	1431.5	1364.2	1138.4	
	JM16	2132.9	3436.1	1875.9	1343.0	1132.0	
	BP11	1829.0	5569.1	1742.6	2002.5	1957.4	2320.2
	BP13	2951.5	1228.4	2477.0	4018.1	2872.0	
	BP14	1656.6	1353.4	1457.1	1160.0	1247.1	
	BP17	1892.2	3199.8			1785.6	
	BP23	1926.4	992.8	1363.2			
	BP22	2388.7	1937.6	1922.4	1886.9	2479.8	
	BP24	1393.2	4071.5	3332.5	1897.0	1530.8	
	MEAN	2083.4	2707.3	2797.0	2669.3	2116.6	3727.9
	SD	700.8	1477.4	1997.7	1723.1	1220.1	1372.6
	CV (%)	**33.6**	**54.6**	**71.4**	**64.6**	**57.6**	**36.8**
POLYTRAUMA	NT1	2222.7	2954.5	1785.5	3894.8		
	DL	1012.3	810.2	688.5	793.5		
	BP2	1453.1	1966.9	1168.6		884.4	1394.5
	BP4	1335.8	1467.8	2357.7	3619.5	2143.2	
	BP6	6238.9	2557.6	925.4	1634.7	1063.1	
	JM2	2824.7	3057.5	3523.5	1766.3	1203.8	
	BP15	1634.3	2212.9	4368.3	1272.0	1707.5	
	BP16	5701.0	2499.9	2477.0	2463.9	1736.2	1899.5
	BP12		5203.7	3262.6	2161.2	1126.1	870.5
	BP18	2627.1	2161.2	1598.1	1336.0		
	BP19	2619.6	3598.2	2630.8	2319.6	2244.2	2511.1
	BP20	2651.6	2671.9	3820.4	1091.6	1286.0	3543.9
	BP27	5508.7	4312.3	2565.7	3342.6	2312.8	4583.1
	BP28	2277.3	3070.5	2105.1	5093.7	2989.8	
	BP31	2682.0	2307.7	1973.2	9602.3	2277.3	
	MEAN	2913.5	2723.5	2350.0	2885.1	1747.9	2467.1
	SD	1678.3	1081.2	1064.3	2290.4	650.7	1390.0
	CV (%)	**57.6**	**39.7**	**45.3**	**79.4**	**37.2**	**56.3**

Table 10.1-4 Inter-individual variations in TGF-β2 (pg/ml)

	DONOR ID	Day 0	Day 1	Day 3	Day 5	Day 7	Day 14
HEAD INJURY	BH1		488.7	341.6	361.4	359.9	313.4
	BH3		430.8	346.1	543.6	681.1	587.8
	BH4	529.7	536.7				
	BH6	467.0	548.9	473.6	636.6	589.6	470.3
	BH8		526.3	600.3	370.6	463.6	642.2
	BH9		500.6	526.3	671.8	766.9	668.0
	BH10	631.2	623.9	485.3	492.1	460.3	411.5
	BH11	434.1	562.9	857.9	633.0	651.4	490.4
	BH13		220.8	395.4	453.9	285.4	355.2
	BH16		315.8	281.4	279.4		299.5
	BH17	271.5	318.3	362.1	295.5	662.5	262.1
	BH18	232.9	197.5	197.5	192.2	216.9	271.1
	BH20	197.5	194.0	202.7	187.0	201.0	
	BH21	238.3	211.5	211.5	190.5	188.7	
	MEAN	375.3	405.5	406.3	408.3	460.6	433.8
	SD	161.5	156.1	186.0	176.9	208.4	148.9
	CV (%)	**43.0**	**38.5**	**45.8**	**43.3**	**45.2**	**34.3**
ISOLATED TRAUMA	BP3	198.1	513.4	581.4		541.0	297.1
	BP7	441.6	268.7	385.7	249.4	2386.7	
	BP9	1390.0	689.2	556.7	585.0	295.6	798.7
	BP8	174.5	1057.9	975.2			
	BP10	868.4	630.1	599.3	381.0	622.8	
	JM8	267.6	3119.2	759.7	915.2	998.3	
	JM12	602.9	886.7	345.2	1563.9	1439.4	
	JM16	1771.9	606.5	2623.1	830.3	279.4	
	BP11	315.0	207.6	356.9	215.0	457.7	293.9
	BP13	281.0	209.1	222.4	154.3	276.3	
	BP14	184.6	217.9	213.5	183.2	212.0	
	BP17	222.2	202.7			166.3	
	BP23	256.5	231.2	213.3			
	BP22	1441.2	2912.9	1249.0	1714.0	2183.6	
	BP24	2829.5	2220.3	1981.0	1736.4	5072.9	
	MEAN	749.7	931.6	790.2	775.2	1148.6	463.2
	SD	779.5	994.2	717.6	629.9	1394.0	290.6
	CV (%)	**104.0**	**106.7**	**90.8**	**81.3**	**121.4**	**62.7**
POLYTRAUMA	NT1	344.9	215.1	198.1	238.1		
	DL	348.9	301.6	234.2	322.0		
	BP2	285.4	271.4	285.4		218.9	281.4
	BP4	279.4	249.8	338.5	259.6	299.5	
	BP6	263.5	222.7	263.5	419.2	218.9	
	JM2	380.5	303.6	561.2	271.4	261.5	
	BP15	179.0	191.7	184.6	170.6	270.0	
	BP16	203.3	203.3	210.6	166.5	196.0	254.3
	BP12		240.1	195.7	190.5	202.7	265.6
	BP18	194.0	180.0	171.4	185.2		
	BP19	278.4	213.3	194.0	234.7	216.9	199.2
	BP20	4946.1	4603.8	10908.4	5125.6	4235.2	825.0
	BP27	3180.2	2106.3	1097.2	890.5	848.1	3118.9
	BP28	1740.1	1862.6	2106.3	1557.4	1301.9	
	BP31	3507.3	2564.1	2187.2	1877.4	967.4	
	MEAN	1152.2	915.3	1275.8	850.6	769.8	824.1
	SD	1572.0	1302.1	2749.8	1345.8	1153.3	1147.8
	CV (%)	**136.4**	**142.3**	**215.5**	**158.2**	**149.8**	**139.3**

Table 10.1-5 Influence of trauma groups on PDGF-AA levels (Mann-Whitney U, p-value)

		Head Injury	Isolated Trauma	Polytrauma	Control
Admission	Head Injury	N/A	p<0.05	p=0.20	p<0.05
	Isolated Trauma	p<0.05	N/A	p=0.03	p=0.10
	Polytrauma	p=0.20	p=0.03	N/A	p=0.02
	Control	p<0.05	p=0.10	p=0.02	N/A
		Head Injury	Isolated Trauma	Polytrauma	Control
Day 1	Head Injury	N/A	p<0.05	p<0.05	p<0.05
	Isolated Trauma	p<0.05	N/A	p=0.09	p<0.05
	Polytrauma	p<0.05	p=0.09	N/A	p<0.05
	Control	p<0.05	p<0.05	p<0.05	N/A
		Head Injury	Isolated Trauma	Polytrauma	Control
Day 3	Head Injury	N/A	p<0.05	p=0.09	p<0.05
	Isolated Trauma	p<0.05	N/A	p=0.06	p=0.21
	Polytrauma	p=0.09	p=0.06	N/A	p<0.05
	Control	p<0.05	p=0.21	p<0.05	N/A
		Head Injury	Isolated Trauma	Polytrauma	Control
Day 5	Head Injury	N/A	p<0.05	p=0.23	p<0.05
	Isolated Trauma	p<0.05	N/A	p=0.38	p=0.57
	Polytrauma	p=0.23	p=0.38	N/A	p=0.15
	Control	p<0.05	p=0.57	p=0.15	N/A
		Head Injury	Isolated Trauma	Polytrauma	Control
Day 7	Head Injury	N/A	p<0.05	p<0.05	p=0.06
	Isolated Trauma	p<0.05	N/A	p=0.83	p=0.33
	Polytrauma	p<0.05	p=0.83	N/A	p=0.18
	Control	p=0.06	p=0.33	p=0.18	N/A
		Head Injury	Isolated Trauma	Polytrauma	Control
Day 14	Head Injury	N/A	p=0.94	p=0.92	p=0.73
	Isolated Trauma	p=0.94	N/A	p=1.0	p=0.41
	Polytrauma	p=0.92	p=1.0	N/A	p=0.41
	Control	p=0.73	p=0.41	p=0.41	N/A

Table 10.1-6 Influence of trauma groups on ANG levels (Mann-Whitney U, p-value)

Admission		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.16	p=0.95	p=0.18
	Isolated Trauma	p=0.16	N/A	p<0.05	p=0.98
	Polytrauma	p=0.95	p<0.05	N/A	p<0.05
	Control	p=0.18	p=0.98	p<0.05	N/A
Day 1		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.53	p=0.90	p=0.19
	Isolated Trauma	p=0.53	N/A	p=0.37	p=0.36
	Polytrauma	p=0.90	p=0.37	N/A	p<0.05
	Control	p=0.19	p=0.36	p<0.05	N/A
Day 3		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.88	p=0.16	p=0.87
	Isolated Trauma	p=0.88	N/A	p<0.05	p=0.66
	Polytrauma	p=0.16	p<0.05	N/A	p=0.05
	Control	p=0.87	p=0.66	p=0.05	N/A
Day 5		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.79	p=0.29	p=0.92
	Isolated Trauma	p=0.79	N/A	p=0.62	p=0.62
	Polytrauma	p=0.29	p=0.62	N/A	p=0.17
	Control	p=0.92	p=0.62	p=0.17	N/A
Day 7		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.38	p<0.05	p=0.26
	Isolated Trauma	p=0.38	N/A	p<0.05	p=0.76
	Polytrauma	p<0.05	p<0.05	N/A	p<0.05
	Control	p=0.26	p=0.76	p<0.05	N/A
Day 14		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.31	p=0.55	p=0.79
	Isolated Trauma	p=0.31	N/A	p<0.05	p=0.31
	Polytrauma	p=0.55	p<0.05	N/A	p<0.05
	Control	p=0.79	p=0.31	p<0.05	N/A

Table 10.1-7 Influence of trauma groups on Follistatin levels (Mann-Whitney U, p-value)

Admission		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.14	p=0.84	p=0.07
	Isolated Trauma	p=0.14	N/A	p=0.15	p=0.53
	Polytrauma	p=0.84	p=0.15	N/A	p<0.05
	Control	p=0.07	p=0.53	p<0.05	N/A
Day 1		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.49	p=0.66	p<0.05
	Isolated Trauma	p=0.49	N/A	p=0.66	p=0.06
	Polytrauma	p=0.66	p=0.66	N/A	p<0.05
	Control	p<0.05	p=0.06	p<0.05	N/A
Day 3		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.24	p=0.30	p<0.05
	Isolated Trauma	p=0.24	N/A	p=0.88	p=0.21
	Polytrauma	p=0.30	p=0.88	N/A	p=0.20
	Control	p<0.05	p=0.21	p=0.20	N/A
Day 5		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.89	p=0.92	p=0.15
	Isolated Trauma	p=0.89	N/A	p=0.96	p=0.31
	Polytrauma	p=0.92	p=0.96	N/A	p=0.15
	Control	p=0.15	p=0.31	p=0.15	N/A
Day 7		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.33	p=0.69	p=0.67
	Isolated Trauma	p=0.33	N/A	p=0.42	p=0.33
	Polytrauma	p=0.69	p=0.42	N/A	p=0.43
	Control	p=0.67	p=0.33	p=0.43	N/A
Day 14		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p<0.05	p<0.05	p=0.14
	Isolated Trauma	p<0.05	N/A	p=0.20	p<0.05
	Polytrauma	p<0.05	p=0.20	N/A	p=0.41
	Control	p=0.14	p<0.05	p=0.41	N/A

Table 10.1-8 Influence of trauma groups on TGF-β2 levels (Mann-Whitney U, p-value)

Admission		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.61	p=0.79	p<0.05
	Isolated Trauma	p=0.61	N/A	p=0.76	p=0.10
	Polytrauma	p=0.79	p=0.76	N/A	p=0.09
	Control	p<0.05	p=0.10	p=0.09	N/A
Day 1		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.16	p=0.46	p<0.05
	Isolated Trauma	p=0.16	N/A	p=0.37	p=0.25
	Polytrauma	p=0.46	p=0.37	N/A	p<0.05
	Control	p<0.05	p=0.25	p<0.05	N/A
Day 3		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.11	p=0.42	p<0.05
	Isolated Trauma	p=0.11	N/A	p=0.13	p=0.21
	Polytrauma	p=0.42	p=0.13	N/A	p<0.05
	Control	p<0.05	p=0.21	p<0.05	N/A
Day 5		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.34	p=0.51	p<0.05
	Isolated Trauma	p=0.34	N/A	p=0.55	p=0.24
	Polytrauma	p=0.51	p=0.55	N/A	p<0.05
	Control	p<0.05	p=0.24	p<0.05	N/A
Day 7		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.45	p=0.89	p=0.07
	Isolated Trauma	p=0.45	N/A	p=0.19	p=0.40
	Polytrauma	p=0.89	p=0.19	N/A	p<0.05
	Control	p=0.07	p=0.40	p<0.05	N/A
Day 14		Head Injury	Isolated Trauma	Polytrauma	Control
	Head Injury	N/A	p=0.82	p=0.42	p<0.05
	Isolated Trauma	p=0.82	N/A	p=0.44	p=0.17
	Polytrauma	p=0.42	p=0.44	N/A	p=0.10
	Control	p<0.05	p=0.17	p=0.10	N/A

10.2. Growth factor dynamics and inflammatory response

10.2.1. Summary of R and p-values between Growth Factors and Hospital Parameters

Table 10.2-1 Summary R and p-values of relationship between CRP and Growth Factors

Growth Factor	Trauma Group	R value	p-value
PDGF-AA	Head Injury	-0.04	0.80
	Polytrauma	-0.19	0.24
	Isolated Trauma	-0.45	0.14
	Combined	-0.11	0.27
Angiogenin	Head Injury	-0.03	0.85
	Polytrauma	+0.02	0.98
	Isolated Trauma	-0.58	0.05
	Combined	-0.13	0.18
Follistatin	Head Injury	+0.54	<0.01
	Polytrauma	+0.58	0.29
	Isolated Trauma	-0.13	0.68
	Combined	+0.36	<0.01
TGF-β2	Head Injury	+0.07	0.59
	Polytrauma	+0.14	0.41
	Isolated Trauma	+0.17	0.60
	Combined	+0.13	0.19

Table 10.2-2 Summary R and p-values of relationship between WCC and Growth Factors

Growth Factor	Trauma Group	R value	p-value
PDGF-AA	Head Injury	+0.27	0.02
	Polytrauma	+0.10	0.02
	Isolated Trauma	+0.10	0.45
	Combined	+0.20	0.01
Angiogenin	Head Injury	+0.02	0.88
	Polytrauma	+0.02	0.90
	Isolated Trauma	+0.18	0.32
	Combined	+0.07	0.39
Follistatin	Head Injury	-0.14	0.26
	Polytrauma	+0.04	0.73
	Isolated Trauma	-0.28	0.13
	Combined	-0.09	0.24
TGF-β2	Head Injury	-0.06	0.61
	Polytrauma	+0.23	0.07
	Isolated Trauma	+0.19	0.30
	Combined	+0.21	0.01

Table 10.2-3 Summary R and p-values of relationship between Platelets and Growth Factors

Growth Factor	Trauma Group	R value	p-value
PDGF-AA	Head Injury	+0.69	<0.01
	Polytrauma	+0.55	<0.01
	Isolated Trauma	+0.85	<0.01
	Combined	+0.61	<0.01
Angiogenin	Head Injury	-0.06	0.63
	Polytrauma	-0.11	0.39
	Isolated Trauma	+0.38	0.03
	Combined	-0.02	0.78
Follistatin	Head Injury	-0.31	<0.01
	Polytrauma	-0.08	0.54
	Isolated Trauma	-0.01	0.94
	Combined	-0.20	0.01
TGF-β2	Head Injury	+0.52	<0.01
	Polytrauma	+0.14	0.27
	Isolated Trauma	+0.04	0.81
	Combined	+0.32	<0.01

10.2.2. Growth Factor and Clinical complications (SIRS Score)

Table 10.2-4 Summary SIRS score (Head Injury Group).

SIRS?	Patient ID	Admission	Day 1	Day 3	Day 5	Day 7	Day 14
Yes	BH1	2	1	1	2	1	1
Yes	BH3	1	0	2	0	1	0
No	BH6	1	0	1	0	0	0
Yes	BH8	2	2	0	2	2	1
Yes	BH9	1	0	0	0	0	2
Yes	BH11	2	3	1	1	2	2
No	BH13	1	1	1	1	1	1
No	BH16	0	1	0	1	1	1
No	BH17	1	0	1	1	1	1
Yes	BH18	2	0	0	0	3	1
Yes	BH20	1	0	1	2	1	
Yes	BH21	2	0	1	2	2	

SIRS is present when score is ≥2

Table 10.2-5 Summary SIRS score (Polytrauma Group).

SIRS?	Patient ID	Admission	Day 1	Day 3	Day 5	Day 7	Day 14
Yes	NT1	1	2	1	0	1	4
Yes	DL	0	2	1	0	0	0
Yes	BP2	2	0	0	2	3	2

Yes	BP4	0	1	3	2	1	2
Yes	BP6	1	0	1	3	2	2
Yes	BP15	2	1	0	0	0	
Yes	BP16	0	1	0	0	3	2
No	BP12	0	1	1	1	1	1
Yes	BP18	2	1	1	1		
No	BP19	1	0	0	0	1	1
Yes	BP20	2	1	0	3	2	1
No	BP27	1	1	0	0	0	
No	BP28	0	0	0	1	1	
Yes	BP31	2	1	0	0		

SIRS is present when score is ≥2

Table 10.2-6 Summary SIRS score (Isolated Trauma Group).

SIRS?	Patient ID	Admission	Day 1	Day 3	Day 5	Day 7	Day 14
No	BP3	1	1	1	1	1	0
No	BP7	1	0	0	0	0	
Yes	BP9	2	1	0	0	0	1
No	BP10	0	0	0	0	0	0
No	BP11	0	0	0	0	0	0
No	BP13	1	0	0	0	0	
No	BP14	1	0	0	0	0	
No	BP17	1	0	0	0	0	
Yes	BP23	2	2	0			
No	BP22	1	0	0	0	1	
Yes	BP24	2	1	1	1	3	

SIRS is present when score is ≥2

Table 10.2-7 Comparison of time and group matched PDGF-AA levels between patient who developed SIRS and patients who did not.

Trauma Group	Time point	p-value
Head Injury	Admission	0.36
	Day 1	0.50
	Day 3	0.73
	Day 5	0.23
	Day 7	0.31
	Day 14	0.03
Polytrauma	Admission	0.87
	Day 1	0.16
	Day 3	0.32
	Day 5	0.76
	Day 7	0.26

	Day14	0.28
Isolated Trauma	Admission	0.84
	Day 1	0.68
	Day 3	0.14
	Day 5	0.32
	Day 7	0.60
	Day 14	0.22

Mann-Whitney test, p-value<0.05 is statistically significant

Table 10.2-8 Comparison of time and group matched angiogenin levels between patient who developed SIRS and patients who did not.

Trauma Group	Time point	p-value
Head Injury	Admission	0.12
	Day 1	0.64
	Day 3	0.17
	Day 5	0.28
	Day 7	0.52
	Day14	0.09
Polytrauma	Admission	0.18
	Day 1	0.89
	Day 3	1.00
	Day 5	0.76
	Day 7	0.71
	Day 14	0.83
Isolated Trauma	Admission	0.68
	Day 1	1.00
	Day 3	0.73
	Day 5	0.10
	Day 7	0.30
	Day 14	0.22

Mann-Whitney test, p-value<0.05 is statistically significant

Table 10.2-9 Comparison of time and group matched follistatin levels between patient who developed SIRS and patients who did not.

Trauma Group	Time point	p-value
Head Injury	Admission	0.36
	Day 1	0.31
	Day 3	0.87
	Day 5	0.50
	Day 7	0.54
	Day 14	0.83

Polytrauma	Admission	0.61
	Day 1	**<0.01**
	Day 3	0.16
	Day 5	0.36
	Day 7	0.13
	Day 14	0.83
Isolated Trauma	Admission	0.68
	Day 1	0.31
	Day 3	0.21
	Day 5	0.51
	Day 7	0.60
	Day 14	0.22

Mann-Whitney test, p-value<0.05 is statistically significant

Table 10.2-10 Comparison of time and group matched TGF-β2 levels between patient who developed SIRS and patients who did not.

Trauma Group	Time point	p-value
Head Injury	Admission	0.17
	Day 1	1.00
	Day 3	0.73
	Day 5	0.73
	Day 7	0.68
	Day 14	0.14
Polytrauma	Admission	0.40
	Day 1	0.57
	Day 3	1.00
	Day 5	0.76
	Day 7	0.85
	Day 14	0.83
Isolated Trauma	Admission	0.22
	Day 1	0.22
	Day 3	0.91
	Day 5	0.10
	Day 7	0.43
	Day 14	0.22

Mann-Whitney test, p-value<0.05 is statistically significant

10.2.3. Growth Factor and Fracture Healing

Table 10.2-11 Rate of fracture healing

Trauma Group	Patient ID	Fracture location	Weeks to heal	Revision surgery
Head Injury	BH1	Distal radius	8	n/a
Head Injury	BH9	Femur	18	n/a
Head Injury	BH11	Distal radius	10	n/a
Head Injury	BH17	Femur	8	n/a
Polytrauma	BP2	Pelvic	18	n/a
Polytrauma	BP4	Tibia	49	n/a
Polytrauma	BP6	Femur	7	n/a
Polytrauma	BP15	Ulna	38	Revision surgery with iliac crest bone graft
Polytrauma	BP16	Pelvic	10	n/a
Polytrauma	BP12	Femur	19	n/a
Polytrauma	BP18	Ulna	46	Revision surgery exchange plate
Polytrauma	BP19	Pelvic	10	n/a
Polytrauma	BP20	Femur	13	n/a
Polytrauma	BP27	Tibia	20	n/a
Polytrauma	BP28	Tibia plateau	14	n/a
Isolated Trauma	BP3	Tibia	34	n/a
Isolated Trauma	BP7	Tibia	28	Exchange nail for delayed union
Isolated Trauma	BP9	Tibia	10	n/a
Isolated Trauma	BP8	Tibia	n/a (Excluded as recruited to drug trial post discharge)	n/a
Isolated Trauma	BP10	Ankle	21	n/a
Isolated Trauma	BP11	Tibia	11	n/a
Isolated Trauma	BP13	Tibia	84	Revision surgery with iliac crest bone graft
Isolated Trauma	BP14	Tibia	14	n/a
Isolated Trauma	BP17	Ankle	9	n/a
Isolated Trauma	BP23	Femur	8	n/a
Isolated Trauma	BP22	Femur	7	n/a
Isolated Trauma	BP24	Femur	16	n/a

Table 10.2-12 Comparative analysis of growth factor dynamics between patients with normal healing versus delayed healing. Statistical significance is p<0.05, Mann-Whitney.

Growth Factor	Time point	p-value
PDGF-AA	Admission	0.40
	Day 1	0.46
	Day 3	0.56
	Day 5	0.67
	Day 7	1.00
	Day 14	0.51
Angiogenin	Admission	0.57
	Day 1	0.81
	Day 3	0.62
	Day 5	0.75
	Day 7	0.92
	Day 14	0.28
Follistatin	Admission	0.40
	Day 1	0.19
	Day 3	0.03
	Day 5	0.34
	Day 7	0.29
	Day 14	0.51
TGF-β2	Admission	0.16
	Day 1	<0.05
	Day 3	0.08
	Day 5	<0.05
	Day 7	0.60
	Day 14	0.83

10.3. Mesenchymal Stem Cell (MSC) and Trauma

Table 10.3-1 Matched sample values for CFU-F count and areas for both MNC and Direct Plating Methods

GROUP	PT ID	CFU-F MNC/dish	Total Area MNC / dish (cm^2)	Average colony size MNC (cm^2)	CFU-F Direct/dish	Total Area Direct / dish (cm^2)	Average colony size Direct (cm^2)
POLYTRAUMA	BT010 Day 0	17.33	4.76	0.27	40.33	21.30	0.53
	BT010 Day 2	1.00	3.33	3.33	68.00	31.96	0.47
	BT010 Day 22	25.33	15.15	0.60	88.00	52.33	0.59
	BT015 Day 0	19.00	12.35	0.65	16.67	9.83	0.59
	BT015 Day 2	8.67	3.97	0.46	23.33	5.64	0.24
	BT016 Day 0	19.33	10.53	0.54	57.67	17.22	0.30
	BT016 Day 4	89.33	25.84	0.29	122.00	27.07	0.22
	BT016 Day 25	203.67	64.96	0.32	83.33	28.12	0.34
	BT017 Day 0	11.67	13.82	1.18	12.33	7.61	0.62
	BT017 Day 32	121.33	49.16	0.41	68.00	40.02	0.59
	BT018 Day 0	53.67	14.80	0.28	51.00	25.52	0.50
	BT018 Day 5	123.33	66.96	0.54	98.00	63.97	0.65
	BT019 Day 0	179.33	62.54	0.35	184.67	65.05	0.35
	BT019 Day 15	76.67	32.04	0.42	166.67	71.80	0.43
	BT020 Day 0	67.33	9.17	0.14	140.33	45.96	0.33
	BT020 Day 2	46.33	8.22	0.18	139.00	47.58	0.34
	BT021 Day 0	100.00	35.04	0.35	85.67	31.94	0.37
	BT021 Day 4	35.67	24.22	0.68	19.67	22.97	1.17
	BT021 Day 12	25.67	6.90	0.27	76.67	37.90	0.49
	BT025 Day 0	64.67	4.90	0.08	131.67	47.27	0.36
	BT025 Day 2	110.67	29.78	0.27	148.33	24.95	0.17
	BT025 Day 4	76.50	8.72	0.11	128.67	58.88	0.46
	BT027 Day 0	32.33	3.16	0.10	42.00	3.96	0.09
	BT027 Day 5	63.67	30.13	0.47	88.00	35.52	0.40
	BT028 Day 0	20.33	30.41	1.50	48.00	61.92	1.29
	BT028 Day 2	50.00	49.12	0.98	57.67	55.38	0.96
	BT028 Day 5	50.33	41.72	0.83	38.00	46.45	1.22
	BT029 Day 0	11.33	24.94	2.20	14.33	7.02	0.49
	BT029 Day 3	1.67	3.06	1.83	20.00	26.65	1.33

	BT031 Day 0	27.00	27.50	1.02	64.33	17.10	0.27
	BT031 Day 3	19.33	8.26	0.43	26.00	23.08	0.89
	BT031 Day 11	22.67	14.26	0.63	93.67	35.78	0.38
	BT033 Day 0	18.33	44.59	2.43	33.67	33.31	0.99
	BT033 Day 2	7.67	6.07	0.79	8.00	19.41	2.43
	BT033 Day 8	32.00	24.23	0.76	16.00	11.69	0.73
	BT034 Day 0	42.00	20.82	0.50	58.67	14.20	0.24
	BT034 Day 8	37.33	13.40	0.36	48.00	23.63	0.49
	BT034 Day 12	75.00	57.09	0.76	59.33	31.49	0.53
ISOLATED TRAUMA	BT006 Day 0	0.33	0.00	0.00	1.33	3.85	2.89
	BT006 Day 3	3.00	2.02	0.67	4.67	12.09	2.59
	BT007 Day 0	52.00	3.37	0.06	140.33	66.37	0.47
	BT007 Day 3	43.00	1.01	0.02	141.00	49.08	0.35
	BT008 Day 0	38.00	19.31	0.51	62.33	34.25	0.55
	BT008 Day 8	90.67	26.61	0.29	123.67	29.80	0.24
	BT009 Day 0	48.67	11.38	0.23	64.33	34.11	0.53
	BT009 Day 12	165.00	28.69	0.17	90.33	38.44	0.43
	BT009 Day 16	146.67	35.45	0.24	41.67	8.62	0.21
	BT011 Day 0	17.00	6.53	0.38	54.00	16.78	0.31
	BT011 Day 9	21.00	8.30	0.40	31.67	7.05	0.22
	BT013 Day 0	3.33	6.05	1.82	2.00	0.21	0.11
	BT013 Day 3	48.00	9.30	0.19	36.67	21.21	0.58
	BT014 Day 0	54.33	24.63	0.45	47.33	14.19	0.30
	BT014 Day 7	39.67	14.23	0.36	37.67	18.00	0.48
CONTROL	BT023	50.00	5.40	0.11	101.00	4.39	0.04
	BT024	43.67	1.10	0.03	12.67	15.65	1.24
	BT026	28.00	3.37	0.12	40.67	6.45	0.16