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Changes in gene expression following trauma are related to the age of transfused packed red blood cells

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Running head: Age of transfused blood and inflammation in polytrauma.
Abstract

**Background:** Transfusion of packed red blood cells (PRBCs) is associated with an increased incidence of nosocomial infections and an increased risk of death. The duration of storage prior to transfusion may influence these outcomes. Here we explore the association between the age of transfused PRBCs and specific patterns of inflammatory gene-expression in severely injured trauma patients.

**Methods:** Severely injured trauma patients requiring ICU treatment and receiving transfusion of PRBCs within 24 hours of the injury were recruited. Blood samples were obtained within 2 hours of the trauma, at 24 hours and at 72 hours. Messenger RNA was extracted from whole blood and gene expression quantified using quantitative polymerase chain reaction. The median age of the units of PRBCs transfused to each patient was recorded. The primary outcome measure was the change in candidate gene expression over the initial 72hrs.

**Results:** Sixty-four patients were studied. 53 (83%) patients were male and the median age was 40.5 years (IQR 31-59). Median ISS was 31.5 (IQR 23-43) and 55 (86%) patients suffered a blunt injury. 41 (64%) patients developed a nosocomial infection and 15 (23%) patients died before hospital discharge. Each patient received a median of 5 units of PRBCs (IQR 4-9.8) over the first 24hrs of hospital admission. The median age of the units of PRBCs transfused in each patient was 20 days (IQR 17-22). Older blood was associated with greater decreases in IL-12, IL-23, and RORγt (all \( P<0.05 \)) gene expression over the initial 24hrs, greater decreases in IL-12 gene expression over 72hrs and a rise in TGF-β gene expression over the first 72hrs. A multivariate analysis confirmed the independence of these associations.

**Conclusions:** Increasing the duration of storage of PRBCs prior to transfusion is associated with a pattern of gene expression consistent with more severe immunosuppression.

**Level of Evidence:** Retrospective observational study, level III.
Keywords: Blood transfusion · Helper T-cells · Age of blood · Polytrauma · TRIM
Background

The transfusion of packed red blood cells (PRBCs) has been consistently associated with an increased incidence of nosocomial infections and mortality.\(^1\)\(^{-3}\) In the United Kingdom (UK) PRBCs can be stored for a maximum of 35 days prior to transfusion and in some countries for up to 42 days. During this time ‘storage lesions’ may develop which include morphological changes, acidosis, decreased 2,3 diphosphoglycerate and adenosine triphosphate, lipid peroxidation and apoptosis.\(^4\) Although the currently available evidence is composed primarily of retrospective, non-randomised studies, there is a suggestion that the administration of older PRBCs is more likely to be associated with nosocomial infections and an increased mortality.\(^5\)\(^,\)\(^6\) The association between the duration of storage of PRBCs and poor outcome is particularly well described in trauma patients\(^7\)\(^{-12}\) and intensive care unit (ICU) patients.\(^13\)\(^{-18}\) However, the precise mechanism by which PRBCs stored for prolonged periods increase susceptibility to infections remains elusive.

It has been repeatedly demonstrated that messenger RNA (mRNA), assayed using real-time polymerase chain reaction (qRT-PCR), can be used to accurately quantify gene expression.\(^19\)\(^{-21}\) By carefully selecting for analysis key cytokines and transcription factors as candidate genes, it is possible to make inferences about overall immune competence, and in particular the activity of specific immune pathways and T helper (T\(_h\)) subtypes (Table 1).\(^22\) Recently we described an association between PRBC administration, a pattern of inflammatory and immune gene expression consistent with immunosuppression and an increased incidence of nosocomial infection in a cohort of severely injured trauma patients admitted to an ICU.\(^23\)

In this study we hypothesise that the transfusion of older PRBCs following severe trauma may be associated with more profound immunosuppression, thereby providing a mechanistic link
between longer storage times of PRBCs and the development of nosocomial infections. Furthermore, through the analysis of candidate gene expression, we explore which immune pathways are preferentially affected by the transfusion of older PRBCs.
Methods

This study was conducted at The Royal London Hospital, UK, a major trauma centre receiving tertiary referrals from across Greater London via road and air. Care is provided by a designated trauma team working to well-developed trauma protocols (Supplementary Protocols 1-3). The study was approved by the East London and City Research Ethics Committee. Deferred informed written consent was obtained from each patient or their next of kin.

Patient selection

All adult trauma patients (>15 years) who met the local criteria for trauma team activation were eligible for enrolment into the Activation of Coagulation and Inflammation in Trauma (ACIT) 2 study when research personnel were present (8am to 8pm daily). ACIT2 is a study prospectively evaluating aspects of coagulation and inflammation in trauma patients (UKCRN ID: 5637). Those patients who were transferred to the Intensive Care Unit (ICU) following their initial resuscitation and treatment and also received PRBCs in the 24 hours following the trauma were eligible for inclusion in this study.

Exclusion criteria from ACIT2 were: arrival at hospital more than 2 hours after injury, transfer from another hospital, known severe liver disease, known bleeding diathesis, administration of >2000 ml of fluid prior to enrolment, a burn injury covering more than 5% of the total body surface area, infection with the human immunodeficiency virus, patients with neutropenia as a result of chemotherapy or patients receiving long-term treatment with corticosteroids.

Transfusion in the pre-hospital (Supplementary Protocol 1) and emergency department environment (Supplementary Protocols 2 and 3) in this institution is guided by well-developed
protocols. A ‘code red’ was declared either in the pre-hospital environment or in the emergency department if: systolic blood pressure was <90 mmHg, there was a poor response to initial fluid therapy or active haemorrhage was suspected.

All packed red cells administered are leukocyte deplete, include a standard SAGM additive solution and have a maximum age of 35 days.

**Data collection**

Data collected on each patient included measures of shock and tissue ischaemia (on scene haemodynamic variables and base excess levels) and injury severity as assessed by the injury severity score (ISS) (Table 2).

The timing and constituents of any blood products administered were recorded for each patient during the first 24 hours of their stay. The median age of the total number of PRBCs transfused over the first 24 hours following injury was calculated for each patient.

Patients were examined daily by the clinical team for the presence or absence of infection. Definitions of infection were agreed prospectively and based on the Center for Disease Control and Prevention definitions. Adjudication of infectious complications was performed in a blinded manner independently of the clinical team (by M.J.O’D. and H.D.T.T.). All positive blood cultures were reviewed in conjunction with the microbiology department and likely contaminants were excluded from the subsequent analysis. Survival was defined as survival to hospital discharge or transfer to a rehabilitation facility. In hospital death was recorded.
Selection of genes

Key constituent genes of specific immune pathways of interest (focusing on T_h and T_reg function) were selected (Table 1).

Blood Sampling & Laboratory Techniques

Blood samples were taken within 2 hours of the trauma, at 24 hours and at 72 hours after admission. At each time point blood was collected in a PAXGene™ blood RNA tube (PreAnalytix, Hilden, Germany). Samples were analysed for their integrity and reverse transcribed to cDNA as previously described. Gene expression was quantified using quantitative RT-PCR (qRT-PCR). qRT-PCRs were performed using Taqman assays (Applied Biosystems, Foster City, CA) and were carried out on a 7900HT, Life Tech (Applied Biosystems, Foster City, CA) as previously described. Each sample was assayed in triplicate. Reference genes (ATP5B and GAPDH) were selected empirically from a panel of six. Relative quantification was calculated using the standard delta delta methodology. Results were expressed as a normalised ratio of candidate gene / reference gene.

Statistical analysis

Discrete variables are expressed as counts with percentages in parenthesis and continuous variables as median and interquartile range. mRNA levels were treated as continuous variables. All statistical tests are two-sided and a P-value of $P<0.05$ was considered significant. $P$-values in univariate analysis are reported without correction for multiple comparisons. Differences in categorical variables were calculated using a chi-squared or Fisher’s exact test as appropriate, and the Kruskal-Wallis test or Wilcoxon rank sum test for continuous variables. Spearman’s rank correlation coefficient was used to describe correlation.
A number of multivariate linear regression models were constructed to test whether the age of the blood transfused was independently associated with changes in gene expression. Variables descriptive of the number of units of PRBCs transfused, median age of PRBCs transfused over the first 24 hours following injury, shock (0 hour and 24 hour base deficit (BD)), severity of injury and patient age were assessed as a univariate analysis. When a $P$-value for a univariate comparison was <0.25 this variable was then added to a multivariate linear regression model (Table 3). Those variables failing to meet this threshold were not added to the model for the gene expression variable. This ensured a common adjustment model with the same confounders considered in each case, with inclusion based on statistical probability. Backwards elimination was performed when appropriate.

Data analysis was performed using the JMP (version 11) statistical software (SAS, Cary, NC, USA).
**Results**

**Patients**

A total of 112 ICU patients with severe traumatic injury as an admission diagnosis were enrolled to ACIT2 between September 2010 and October 2012. This group has been described in detail previously. From this group data were available for 64 patients that received a PRBC transfusion within 24 hours of admission. 53 (83%) patients were male and median patient age was 40.5 years (IQR 31-59). Median ISS was 31.5 (IQR 23-43) and 55 (86%) patients suffered blunt injury. Median base deficit on admission was 4.9 mEq/L (IQR 2.4-8.8) and at 24 hours was 0.7 mEq/L (IQR -0.7-2.6). 40 (63%) patients developed infectious complications and 15 (23%) patients died before hospital discharge.

**Blood transfusion**

Each patient received a median of 5 units (IQR 4-9.8) of PRBCs over the first 24 hours of hospital admission. Median age of the transfused PRBCs for each patient was 20 days (IQR 17-22). Patients also received a median of 4 units (IQR 0-8) fresh frozen plasma (FFP) and 1 unit (IQR 0-1) of platelets.

**Univariate analysis of age of PRBCs transfused and gene expression**

At baseline (blood sample within 2 hours of the trauma) there was an association between Foxp3 mRNA levels and the median age of the transfused PRBCs ($r^2=0.18$, $P=0.001$) and also between GATA-3 mRNA levels and the median age of the transfused PRBCs ($r^2=0.09$, $P=0.03$). No other associations were detected between gene expression at baseline and the median age of the PRBCs.
Change in gene expression was expressed by taking a ratio of both the 24 hour or the 72 hour mRNA level to the baseline mRNA level. There was an association between decreasing IL-12 ($r^2=0.10$, $P=0.03$), IL-23 ($r^2=0.10$, $P=0.03$), RORγt ($r^2=0.19$, $P=0.003$), Foxp3 ($r^2=0.09$, $P=0.04$) and GATA-3 ($r^2=0.16$, $P=0.007$) mRNA over 24 hours and increasing age of the transfused PRBCs.

There was an association between decreasing IL-12 mRNA over 72 hours ($r^2=0.20$, $P=0.01$) and increasing age of the transfused PRBCs. There was also an association between increasing TGF-β mRNA over 72 hours ($r^2=0.16$, $P=0.03$) and increasing age of the transfused PRBCs.

No associations were detected between changes in IL-10, TNFα, IFN-γ, T-bet, and IL-27 gene expression and the age of the transfused PRBCs.

**Multivariate analysis of age of PRBCs transfused and gene expression**

Multivariate linear regression models were created to test for independent associations between change in gene expression and the median age of transfused PRBCs in cases where a univariate association was detected. The selection of predictor variables is outlined in the methods and presented in Table 3. For decreasing IL-12 over 24 and 72 hours, decreasing RORγt over 24 hours and increasing TGFβ over 72 hours an independent association between change in gene expression and the median age of the PRBCs transfused was also present (Table 3). In the case of decreasing Foxp3, GATA3 and IL-23 over 24 hours the variation in gene expression levels was better explained by variations in patient age as opposed to variations in the median age of the PRBCs (Table 3). Supplementary Table 1 demonstrates the model, and model performance with all predictor covariates included.
Age of PRBCs and infections

No associations were detected between the age of PRBCs transfused and nosocomial infections or in-hospital mortality.
Discussion

In this study, we have assayed gene expression for a selected panel of interlinked cytokines and transcription factors, in order to investigate the relationship between the duration of storage of PRBCs prior to transfusion and the resultant immunomodulation. We found that a pattern of altered gene expression consistent with greater immunosuppression is associated with the transfusion of older PRBCs. Reduced activity in the pro-inflammatory T\textsubscript{h}1 and T\textsubscript{h}17 pathways is evidenced by reduced gene expression of the prototypical T\textsubscript{h}1 polarising cytokine, IL-12, of the T\textsubscript{h}17 promoting cytokine, IL-23, and the T\textsubscript{h}17 specific transcription factor ROR\textgreek{g}t. The increased expression of the apoptotic and anti-inflammatory gene, TGF-\textbeta, with increasing age of transfused PRBCs further points to the immunosuppressive nature of the response observed following the transfusion of older PRBCs.

There is now convincing evidence that the transfusion of PRBCs is independently associated with an excess of nosocomial infections\textsuperscript{1} and also with increased mortality.\textsuperscript{2,6} The transfusion of PRBCs stored for longer periods was originally thought to be associated with equivocal effects as these transfusions failed to consistently improve tissue oxygenation.\textsuperscript{13} However, there is now an increasing body of evidence associating the use of older PRBCs with harm. Transfusion of older blood has been associated with an excess of infectious complications and multiple organ failure in trauma patients\textsuperscript{7,8} and following cardiac surgery, transfusion of PRBCs stored for more than 28 days has been associated with a 2.5 fold increase in pneumonic complications.\textsuperscript{27,28} Following colorectal cancer surgery transfusion of PRBCs stored beyond 20 days more than doubled the risk of postoperative infections.\textsuperscript{29} In addition, the duration of storage of PRBCs has been independently associated with an increased risk of death in trauma patients\textsuperscript{12} and following cardiac surgery.\textsuperscript{30} Interestingly, the mortality data suggests that older PRBCs exert
a detrimental effect only when transfused in larger quantities with smaller volume transfusion of older blood having little effect on mortality. However, these data are largely retrospective and consequently prone to potential bias. The results of a large randomised controlled trial prospectively assessing the impact of blood stored less than seven days versus a standard transfusion policy on mortality and nosocomial infections are awaited.

Prolonged storage of PRBCs can induce ‘storage lesions’ the clinical consequences of which are unclear. In particular, whether these lesions could be responsible for increasing susceptibility to nosocomial infections and increasing mortality remains uncertain. Our group has recently described an association between the transfusion of PRBCs and an excess of infectious complications following major trauma. Importantly, we also offered a plausible mechanism for the detrimental effect of PRBCs by describing a pattern of gene expression that was consistent with immunosuppression and was independently associated with transfusion. Here we demonstrate that the administration of older PRBCs exacerbates this transfusion induced immunosuppressed phenotype, thereby perhaps providing a mechanism for further increasing susceptibility to nosocomial infections.

A reduction in IL-12, IL-23 and RORγt gene expression in association with an increase in TGF-β gene expression is consistent with an immunosuppressed phenotype. IL-12 is a pro-inflammatory cytokine, produced primarily by dendritic cells and macrophages, that vigorously promotes the differentiation of naïve T cells into an IFN-γ producing T\(_{h1}\) phenotype. T\(_{h1}\) cells are an essential link between innate and adaptive immunity and are particularly important for effective bactericidal activity on intracellular pathogens. IL-23 is a cytokine, again produced primarily by dendritic cells and macrophages, that promotes differentiation to a T\(_{h17}\) phenotype. RORγt is a transcription factor specific to terminally differentiated T\(_{h17}\) cells.
Th17 cells are a branch of the adaptive immune system and appear to deal primarily with organisms inadequately subdued by Th1 or Th2 immunity and that seem to require a very robust inflammatory response. TGF-β is produced by multiple lineages of leukocytes and stromal cell. Although a paracrine source of TGF-β may be necessary for Th17 development this cytokine is better known for inducing widespread apoptosis and limiting the pro-inflammatory Th1 response.

The data presented are consistent with an exaggeration of the changes in gene expression that we have previously observed in response to blood transfusion following severe traumatic injury. Although the individual biomarkers descriptive of the specific immune pathways differ between the two studies, the transfusion of PRBCs alone was associated with features suggestive of reduced activity in the Th1 and Th17 pathways whilst the additional data reported here suggest that transfusing older PRBCs may potentiate this response. Whilst we previously reported that expression of the prototypical anti-inflammatory gene, IL-10, increases in response to blood transfusion in trauma patients, in this cohort IL-10 gene expression was not associated with the age of the transfused PRBCs. It is plausible that this study was underpowered to detect such an association. We did however detect greater increases in another potent anti-inflammatory gene, TGF-β, in those patients receiving older PRBCs.

An association was also detected between decreasing Foxp3 and GATA-3 and the age of the PRBCs. These mediators are transcription factors specific to the immunosuppressive T<sub>reg</sub> cell response and the Th2 response respectively and as such these findings would not support the hypothesis that transfusion of older PRBCs induces a greater immunosuppressive response. However, an association was also detected between these variables at baseline and the age of PRBCs prior to transfusion. Therefore the validity of the association at 24 hours is questionable.
Furthermore, the multivariate analysis suggested that patient age was a more important variable in explaining gene expression variation in GATA3 and Foxp3 than the median age of the PRBCs. Whilst this may also be true for the change in IL-23 gene expression over 24 hours an additional marker of reduced T\textsubscript{h}17 activity, ROR\textgreek{t}, remained independently associated with the transfusion of older PRBCs.

Although we hypothesise that the described gene expression profiles may potentially lead to an increased risk of subsequent infectious complications this study was not powered to detect such an association. These data do, however, provide a plausible mechanistic link between older PRBCs reducing the effectiveness of essential bactericidal functions and thereby rendering patients more susceptible to later infectious insults. This is an important distinction from suggesting that older PRBCs may directly introduce infection to the host. We previously reported a median time lag of 7 days between the appearance of immunosuppressive gene expression patterns and the detection of a bacteraemia\textsuperscript{23} which supports the concept that these changes are associated with an enhanced susceptibility to later infectious insults as opposed to representing an early immune response to infection.

This study has a number of limitations. Retrospective observational cohort studies are prone to systematic biases, unknown confounders and the possibility of Type 1 errors, and must be viewed as hypothesis generating. While the expression of a number of inflammatory mediators are significantly associated with the age of blood transfused, the accompanying the \textit{r}\textsuperscript{2} values that we present are low implying that age of blood might account for only a small proportion of the variation in the gene expression. However, there will be multiple influences present in trauma patients influencing gene expression and it would be surprising if any single factor, such as age of blood, had a dominant effect on the inflammatory response. Our data
suggests that the age of blood has a small, yet significant effect on gene expression and could contribute to clinical outcomes. Importantly, unlike many other factors influencing the inflammatory response to major trauma, it is potentially modifiable. It is possible that unknown confounding variables were omitted from our multivariate regression analysis despite the inclusion of variables descriptive of shock on admission and at 24 hours, the ISS, the volume of transfused PRBCs and a common adjustment model with the same confounders considered in each case. The inclusion of patients receiving both large and small volume transfusions may obscure important associations as it has been suggested that small volume transfusion of old blood does not adversely affect mortality.\textsuperscript{12} In addition to this the variability between patients receiving large and small volumes of PRBCs may mean that differences between the calculated (median) age of the blood transfused in different patients may be underestimated. Patient numbers preclude meaningful sub-analyses. There were some baseline associations between Foxp3 and GATA-3 gene expression and the age of transfused PRBCs, which introduces bias in the interpretation of alterations in T\textsubscript{reg} and T\textsubscript{h}2 cell types at 24 hours. It is possible that the observed changes in mRNA levels are explained solely, or in part, by the relative abundance of specific cell types collected in the whole blood samples.\textsuperscript{38} However, all expression data from the candidate genes were normalised to the reference genes ATP5B and GAPDH which were demonstrated to be expressed at stable levels in these patients thus minimising the effect of relative cellular subtype abundance in individual samples. It is also possible that residual allogenic leukocytes, nucleated erythrocytes, residual plasma and platelets persist even in leukdepleted PRBCs and these can confound the analysis through contamination. However, it has been demonstrated previously that the post mortem analysis of patients receiving a massive transfusion failed to detect any foreign DNA.\textsuperscript{39} It is plausible that small variations in the qRT-
PCR assay could have led to changes in the amplified signal despite having made every effort to standardise the process; (templates (primers and probes), mRNA (concentrations and integrity), the reverse transcription step and reference genes) as well as a single operator (H.D.T.T.).

We describe an association between the transfusion of older PRBCs to severely injured patients and gene expression patterns suggestive of more severe immunosuppression. These data are hypothesis generating and provide a plausible mechanistic link between the transfusion of older PRBCs and an increased incidence of infectious complications.

**Author Contribution**

H.D.T.T., K.B., J.R.P., R.M.P., H.C.O., C.J.H and M.J.O’D. contributed to the study design and data analysis. H.D.T.T., & M.E.V. performed the data collection. H.D.T.T. & H.C.O performed the laboratory analysis. All authors contributed to the preparation of the manuscript.

**Acknowledgements**

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References


Table 1. Selected cytokines and transcription factors and their related pathways

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Immune Pathway</th>
<th>Contributes to anti (-) or pro (+) Inflammatory phenotype*</th>
</tr>
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<tbody>
<tr>
<td>TNFα</td>
<td>Common end product of many innate and adaptive immune pathways</td>
<td>+</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>T_h1 effector cytokine</td>
<td>+</td>
</tr>
<tr>
<td>IL-12</td>
<td>Promotes differentiation to T_h1 effector cells</td>
<td>+</td>
</tr>
<tr>
<td>T-bet</td>
<td>Transcription factor utilised by T_h1 cells</td>
<td>+</td>
</tr>
<tr>
<td>IL-23</td>
<td>Promotes differentiation to T_h17 phenotype</td>
<td>+</td>
</tr>
<tr>
<td>IL-27</td>
<td>Inhibits differentiation to T_h17 phenotype</td>
<td>-</td>
</tr>
<tr>
<td>RORγT</td>
<td>Transcription factor utilised by T_h17 cells</td>
<td>+</td>
</tr>
<tr>
<td>IL-10</td>
<td>Anti-inflammatory cytokine produced by many T cell subtypes and some macrophages</td>
<td>-</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Transcription factor utilised by naturally occurring CD4+ CD25+ T_reg cell</td>
<td>-</td>
</tr>
<tr>
<td>GATA-3</td>
<td>Transcription factor utilised by T_h2 cells</td>
<td>-</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Induces widespread apoptosis, promotes T_reg and T_h17 cell development</td>
<td>-</td>
</tr>
</tbody>
</table>

*Cytokines and transcription factors will have diverse actions under different conditions and the descriptions above are primarily for illustrative purposes and are not exhaustive.
<table>
<thead>
<tr>
<th>Table 2. Patient Characteristics</th>
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<tr>
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<td></td>
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<tr>
<td>Male, n (%)</td>
</tr>
<tr>
<td>ISS, median (IQR)</td>
</tr>
<tr>
<td>Presence of TBI, n (%)</td>
</tr>
<tr>
<td>Blunt Injury, n (%)</td>
</tr>
<tr>
<td>Penetrating Injury, n (%)</td>
</tr>
<tr>
<td>On Scene sBP, median (IQR) mmHg</td>
</tr>
<tr>
<td>Emergency Department sBP, median (IQR) mmHg</td>
</tr>
<tr>
<td>Base deficit (0hr), median (IQR) mEq/L</td>
</tr>
<tr>
<td>Lactate (0hr), median (IQR) mmol/L</td>
</tr>
<tr>
<td>pH (0hr), median (IQR)</td>
</tr>
<tr>
<td>Base deficit (24hr), median (IQR) mEq/L</td>
</tr>
<tr>
<td>Number of PRBCs transfused, median (IQR) u</td>
</tr>
<tr>
<td>Massive Transfusion (≥10 units/24hrs), n (%)</td>
</tr>
<tr>
<td>All nosocomial Infections, n (%)</td>
</tr>
<tr>
<td>Pneumonia, n (%)</td>
</tr>
<tr>
<td>BSI, n (%)</td>
</tr>
<tr>
<td>Wound Infection, n (%)</td>
</tr>
<tr>
<td>UTI, n (%)</td>
</tr>
<tr>
<td>Survival To Hospital Discharge, n (%)</td>
</tr>
</tbody>
</table>

Data are expressed as median (interquartile range) or n (%). ISS, Injury Severity Score; TBI, Traumatic Brain Injury; sBP, Systolic Blood Pressure; PRBCs, Packed Red Blood Cells; BSI, Blood stream Infection (bacteraemia or fungaemia); UTI, Urinary Track Infection. *Patients divided into two groups based on median age of PRBCs transfused in first 24 hours; ≤20 days vs. >21 days.
### Table 3. Multivariate Regression Analysis For Prediction of Gene Expression

<table>
<thead>
<tr>
<th>Response Variable</th>
<th>Predictor Variables on Univariate Analysis for the Model</th>
<th>Whole Model</th>
<th>Predictors Independently Associated With Response Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median age of blood</td>
<td>Quantity of PRBCs transfused</td>
<td>Baseline ED</td>
</tr>
<tr>
<td>ΔROX;</td>
<td>r² = 0.19</td>
<td>r² = 0.89</td>
<td>r² = 0.67</td>
</tr>
<tr>
<td>24 / 0 HR</td>
<td>P = 0.003</td>
<td>P = 0.06</td>
<td>P = 0.55</td>
</tr>
<tr>
<td>ΔIL-12;</td>
<td>r² = 0.18</td>
<td>r² = 0.86</td>
<td>r² = 0.65</td>
</tr>
<tr>
<td>24 / 0 HR</td>
<td>P = 0.003</td>
<td>P = 0.16</td>
<td>P = 0.53</td>
</tr>
<tr>
<td>ΔIL-25;</td>
<td>r² = 0.18</td>
<td>r² = 0.84</td>
<td>r² = 0.65</td>
</tr>
<tr>
<td>24 / 0 HR</td>
<td>P = 0.003</td>
<td>P = 0.16</td>
<td>P = 0.50</td>
</tr>
<tr>
<td>Δexp3;</td>
<td>r² = 0.06</td>
<td>r² = 0.006</td>
<td>r² = 0.80</td>
</tr>
<tr>
<td>24 / 0 HR</td>
<td>P = 0.003</td>
<td>P = 0.93</td>
<td>P = 0.36</td>
</tr>
<tr>
<td>ΔGATA3;</td>
<td>r² = 0.16</td>
<td>r² = 0.02</td>
<td>r² = 0.80</td>
</tr>
<tr>
<td>24 / 0 HR</td>
<td>P = 0.007</td>
<td>P = 0.39</td>
<td>P = 0.75</td>
</tr>
<tr>
<td>ΔIL-12;</td>
<td>r² = 0.21</td>
<td>r² = 0.04</td>
<td>r² = 0.80</td>
</tr>
<tr>
<td>72 / 0 HR</td>
<td>P = 0.003</td>
<td>P = 0.41</td>
<td>P = 0.75</td>
</tr>
<tr>
<td>ΔGF-β;</td>
<td>r² = 0.16</td>
<td>r² = 0.005</td>
<td>r² = 0.003</td>
</tr>
<tr>
<td>72 / 0 HR</td>
<td>P = 0.003</td>
<td>P = 0.09</td>
<td>P = 0.18</td>
</tr>
</tbody>
</table>

Multivariate regression analysis of gene expression using median age of blood transfused, transfusion requirement, selected markers of tissue injury, tissue ischemia and age. Each univariate P-value and r² value is displayed. * Indicates P-value obtained following backwards elimination. † Indicates P-value obtained prior to exclusion via backwards elimination. ‡ Indicates dynamic variables, or changes in cytokine expression over time period displayed. ED: Base deficit (at baseline (admission) and 24HR, 2HR after injury), ISS: Injury Severity Score. Quantity of PRBCs transfused, number of units of packed red blood cells (PRBCs) transfused in first 24HR post-injury. X indicates that univariate analysis did not meet threshold of P < 0.25 for inclusion in multivariate regression analysis model.
Legend

Figure 1: Graphs A-C Association between the median age of packed red blood cells (PRBCs) transfused in the first 24 hours following injury and the change in candidate gene expression over 72 hours (graphs A & B) or 24 hours (graph C). Results displayed as best-fit line (solid) and 95% confidence interval (dashed). All results are expressed as a relative quantification ratio between candidate and reference genes.