Links Between a Biomarker Profile and Clinical Outcome Following Simultaneous Pancreas and Kidney Transplantation

Abstract

Introduction In sepsis, trauma and major surgery, where an explicit physiological insult leads to a significant systemic inflammatory response, the acute temporal evolution of biomarkers have been delineated. In these settings, Interleukin (IL) -6 and TNF-α are often the first pro-inflammatory markers to rise, stimulating production of acute phase proteins followed by peaks in anti-inflammatory markers. Patients undergoing SPKT as a result of diabetic complications already have an inflammatory phenotype as a result of uraemia and glycaemia. How this inflammatory response is affected further by the trauma of major transplant surgery and how this may impact on graft survival is unknown, despite the recognised pro-inflammatory cytokines' detrimental effects on islet cell function. This study aimed to determine the expression of biomarkers in the peri-operative period following SPKT and establish a correlation to clinical outcome.

Methods

The temporal patterns of pro- and anti-inflammatory cytokines (interleukin (IL) -6, -10 and TNF-α), inflammatory markers (WCC and CRP) and diabetes markers (insulin, C-peptide, glucagon and resistin) were serially measured at 8 time-points in the first 72 hours post-SPKT.

Results 46 patients were recruited to the study (November 2011- March 2014). Patterns of expression of inflammatory and diabetes markers were delineated. Levels of C-peptide, insulin and glucagon were raised significantly 30 minutes post pancreas perfusion and were significantly negatively related to prolonged CIT ($p < 0.05$, linear regression model).

Levels of IL-6 and IL-10 peaked significantly at 30 minutes and six hours respectively ($p < 0.05$, ANOVA). CRP levels rose rapidly in the post-operative period and correlated significantly with the Post-Operative Morbidity Survey ($p < 0.05$, Spearman Correlation).

Conclusions The temporal inflammatory marker signature after SPKT is comparable to the pattern observed following other physiological insults. We conclude that CIT is significantly related to early pancreatic endocrine function and that CRP provides an easily measurable predictor of recipient morbidity, demonstrating the potential use of targeted anti-inflammatory therapies in the peri-operative period.

Word Count 312

Introduction

In sepsis, trauma and major surgery, where an explicit physiological insult leads to a significant systemic inflammatory response, the acute temporal evolution of biomarkers have been delineated (Ferguson, Taheri et al. 1997, Smith and Giannoudis 1998, Gabay and Kushner 1999, Leung, Lai et al. 2000, Rivers, Kruse et al. 2007, Faix 2013). In these settings, Interleukin (IL) -6 and TNF-α are often the first pro-inflammatory markers to rise, stimulating production of acute phase proteins followed by peaks in anti-inflammatory markers (Faix 2013). Biomarkers are now used to improve specificity for risk stratification and predict outcomes at an early stage of the disease process (Mentula, Kylanpaa et al. 2005, Cuschieri, Bulger et al. 2010, Andaluz-Ojeda, Bobillo et al. 2012), estimate extent of tissue damage (Glaser, Sannwald et al. 1995, Desborough 2000, Leung, Lai et al. 2000) and provide targets for novel pharmacological therapies (Bernard, Vincent et al. 2001, Stenvinkel and Alvestrand 2002, Alejandro, Barton et al. 2008). In chronic diseases such as insulin dependent diabetes mellitus (IDDM) and end-stage renal failure (ESRF), specific pro-inflammatory markers (IL -4, -6, -8, TNF-α) are persistently raised (Stenvinkel and Alvestrand 2002, Chatzigeorgiou, Harokopos et al. 2010), leading to higher cardiovascular risk in this cohort (Kimmel, Phillips et al. 1998).

Simultaneous pancreas and kidney transplantation (SPKT) recipients suffer with an exacerbated inflammatory phenotype as a result of uraemia and glceamia which has contributed to multi-system morbidity, end-organ failure and at transplantation undergo high-risk surgery, which further aggravates the inflammatory response.This reaction is clinically akin to sepsis and major trauma, and provides the impetus for current guidelines to recommend a more detailed surveillance. There is an urgent and unmet need to identify and validate accurate biomarkers for the detection of complications to improve quality of life for

the patients and reduce costs for health care providers, while maintaining or improving current outcomes. Explicit clinical factors, which include a prolonged cold ischaemic time (CIT), recipient BMI greater than 30kg/m^2 (Sampaio, Reddy et al. 2010), recipient age greater than 45 years (Gruessner, Sutherland et al. 2004) and donor age greater than 40- 45 (Gruessner, Gruessner et al. 1993, Douzdjian, Gugliuzza et al. 1995) have all been identified as clinical markers correlating to poor outcomes. However, relevant biological profiling in this clinical context has not been explored and the temporal evolution and interactions of peri-operative inflammatory markers (IMs) have not been characterised.

In solid-organ transplantation, biomarkers have been investigated in relation to donation after brainstem death (DBD), the impact of ischaemia-reperfusion-injury (IRI) and in rejection (van der Hoeven, Ploeg et al. 1999, Bharat, Narayanan et al. 2007, Dziodzio, Biebl et al. 2014). High levels of IL-1, -2, -4, -10 and TNF-α in the peri-operative period correlate with reduced long-term graft survival and increased rates of rejection. During islet cell transplantation, β-cells produce pro-inflammatory cytokines leading to β-cell dysfunction, graft toxicity and islet-cell death (Kutlu, Cardozo et al. 2003, Barbe-Tuana, Klein et al. 2006, Lawrence, Naziruddin et al. 2011, Kanak, Takita et al. 2014). This has led to the introduction of anti-TNF-α agents being used as part of induction immunosuppression in some islet transplant centres, with evidence suggesting improved rates of graft survival (Bellin, Kandaswamy et al. 2008, Faradji, Tharavanij et al. 2008, Koh, Senior et al. 2010). In the setting of pancreatitis, the most widely available stressor model affecting the pancreas, raised levels of inflammatory cytokines (IL-6, IL-8, TNF-α and IL-10) have been observed and aid in prognosis prediction (Mentula, Kylanpaa et al. 2005). We propose that a measure of a panel of independent complementary biomarkers, including the markers with classic prognostic properties, together with novel inflammatory and diabetic markers may provide a more accurate prediction of outcome, compared with a few individual markers, thus improving risk stratification and clinical management of patients following SPKT.

Additionally, measuring both tissue and circulating biomarkers add valuable novel information to the study for diagnosing further surgical complications. Human adipose tissue is a metabolically active organ. The function and metabolic activity of the fat varies, depending on the location within the body (Baker, Silva et al. 2006), but higher levels of obesity correlate with higher levels of circulating IMs (Pou, Massaro et al. 2007) leading to increased cardio-vascular risk in the obese patient.

Within the abdomen, the omentum is an apron of fat, which like other deposits of fat is a physiologically and metabolically active organ (Liebermann-Meffert 2000). In surgery it is known as the "abdominal policeman" as it is often found to encase areas of intra-abdominal inflammation and infection. The omentum is also involved in the formation of peritoneal adhesions and the production of growth factors and cytokines (Liebermann-Meffert 2000, Wilkosz, Ireland et al. 2005). It reacts to a localised inflammatory insult via macrophage production of IMs (TNF-α, Resistin, plasminogen activator inhibitor-1 (PAI-1) and multiple interleukins (Chandra and Naik 2008)) resulting in a systemic effect (Vieira-Potter 2014). This role has been outlined in cases of intra-abdominal sepsis, but minimal evidence exists as to the role of the omentum in instigating a systemic inflammatory reaction in response to an elective intra-abdominal procedure (Collins, Hogan et al. 2009).

In SPKT, assumptions are also made regarding the production of endocrine markers by the allograft pancreas. Given that serum blood sugar levels tend to fall within the first hour post-pancreatic perfusion, and demonstrable primary pancreatic graft dysfunction is exceptionally rare, it is assumed that endocrine function is instantaneous and uniform, with the distinction between "impaired" or "delayed" graft function difficult to characterize.

Therefore, this study aimed to determine the temporal evolution of biomarkers in the peri-operative period following SPKT, assess the inflammatory response of omentum in relation to elective major surgery and establish a correlation of serum biomarkers to clinical outcome.

Methods

Study Centre

The study was undertaken at The Central Manchester University Hospitals NHS Trust and The University of Manchester. Appropriate ethical and Research and Development approvals were obtained. Recipient serum and omental biopsies were taken prospectively from SPKT recipients between November 2012 and March 2014.

Study Design

This study was designed to:

1. Delineate the temporal evolution of IMs (IL-6 and -10, TNF-α, C-reactive protein (CRP), White Cell Count (WCC) and Amylase) and endocrine markers (C-peptide, insulin, glucagon and resistin) in the perioperative period following SPKT;

2. Evaluate specific factors which may affect the levels of inflammatory and endocrine markers;

3. Correlate biomarker levels with clinical outcomes.

All adult SPKT recipients were eligible for inclusion in the study.

Serum samples were taken and processed for analysis on eight occasions in the peri-operative period (pre-operatively, immediately prior pancreas perfusion, 30 minutes post

pancreas perfusion and at 6, 12, 24, 48 and 72 hours post-transplantation) (Chowdhury, Ghosh et al. 2010).

Two omental biopsies were taken intra-operatively, firstly on entering the abdomen and secondly prior to closing the abdomen. Specimens were processed in an automated processor and wax embedded for subsequent immunohistochemical analysis.

Sample Analysis

Serum Samples

Serum CRP, Amylase and WCC were measured prospectively by the biochemistry and haematology departments at the investigating unit.IL-6, IL-10 and TNF-α, were all measured in serum using ELISA development kits from R&D Systems (Abingdon, UK). Minimum detection limits were 1pg/ml, 5pg/ml and 2pg/ml respectively. Readings below these minimal levels were considered as 0 for analysis.

Insulin, C-peptide, glucagon and resistin were measured in bulk using a bioplex micro-array multi-bead based system (BioRad Life Science Group, USA). Minimum detection limits were 1.0pg/ml, 14.5pg/ml, 4.9pg/ml and 1.3pg/ml for insulin, C-peptide, glucagon, and resistin respectively. Beyond this level the software imputes likely doses, by extrapolating the calibration curves.

Omental Biopsy Analysis

Immunohistochemical analysis was performed for CD68 and CD206 positivity (M1 and M2 Macrophages respectively) to assess for changes in omental inflammatory response during

surgery. Slides were visualised on a Zeiss Axio Scope light microscope and quantified using ImageJ analysis software.

Patient Characteristics and Clinical Data

Patient, donor, organ, operative and outcome data were recorded to assess for potential confounders and factors affecting either the inflammatory or endocrine marker levels in the post-operative period, as well as to correlate biomarker levels with clinical outcomes. In addition, Multiple Organ Dysfunction Score (MODS) (Marshall, Cook et al. 1995) and Post-Operative Morbidity Survey Score (POMS) were calculated and recorded for correlation to peak biomarker levels.

Transplant Protocol

Induction immunosuppression was with alemtuzumab (Campath®, Sanofi, Paris, France), 30mg subcutaneous injection at induction of anaesthesia (repeated at 24 hours post-operatively) and methylprednisolone (Solu-Medrone®, Pfizer, New York). Transplant protocol, maintenance immunosuppression and standard post-operative care were as previously described (Ablorsu, Ghazanfar et al. 2008).

Statistical Analysis

Statistical analyses were carried out using SPSS (IBM SPSS Statistics 20, Armonk, New York). Continuous data are presented as mean (Standard Deviation) where normally distributed, or median (Interquartile range, IQR, 25^{th}- 75^{th} percentile) if skewed.

The temporal evolution of biomarkers in the peri-operative period have been graphically presented (95% C.I.) with variation in individual biomarker levels with time analysed using

Analysis of Variance (ANOVA) testing with Bonferroni Correction ($p < 0.05$ considered significant). Correlations of individual IMs were also made using Pearson correlation (p and r values presented). Univariate analyses and multiple regression analysis were carried out to assess the impact of potential confounders (DBD/Donor after cardiac death (DCD) status, further surgery within 72 hours of SPKT and pancreas CIT) on biomarker levels at specific time-points. Scatter plots were drawn to evaluate the relationship between biomarker levels and CIT. $P< 0.05$ for each independent variable was considered significant.

, Peak biomarker levels of IL-6, IL-10, Amylase and WCC were correlated to clinical outcomes (number of post-operative complications, length of hospital stay, length of critical care unit stay, days to mobilisation out of bed, time to tolerating normal diet, 72 hour MODS and days 5, 7 and 10 POMS) using Spearman Correlation. In the case of CRP, which did not reach peak within 72 hours, levels at 24, 48 and 72 hours were correlated with the clinical outcomes. $P< 0.05$ was considered significant.

Results

There were 69 recipients of SPKTs during the recruitment period. Of these, complete serum and omentum samples of 46 recipients were analysed.

Patient Demographics

Baseline recipient, donor and operative variables are detailed in Table 1.

Temporal Evolution of Biomarkers

Endocrine Markers

Figures 1a- d outline the temporal change in levels of resistin, C-Peptide, insulin and glucagon respectively beginning immediately prior to SPKT, and at intervals for the duration of 72 hours following surgery. Levels of C-peptide, insulin and glucagon raised significantly between pre-perfusion and 30 minutes post perfusion (C-peptide, 0pg/ml- 4899.82pg/ml (3510.44) $p< 0.001$; Insulin, 787.20pg/ml (982.68)- 2764.71pg/ml (1469.11) $p< 0.001$ and Glucagon, 244.76pg/ml (132.55)- 2879.23pg/ml (2091.00) $p < 0.001$). Resistin levels rose significantly between 30 minutes and six hours post-SPKT (13326.04pg/ml (4550.43)- 19696.76pg/ml (7227.48) $p< 0.001$), following which there were no significant changes in consecutive levels.

In uni-variate analysis, no potential confounder had a significant effect upon endocrine marker levels within the first 72 hours post-operatively ($p> 0.05$) Therefore, multivariate analysis with further variables was not conducted.

Inflammatory Markers

Levels of IL-6 (n= 45, one outlier excluded, Figure 2a) rose rapidly at the start of surgery, peaking at 30 minutes post-pancreas perfusion (92.27pg/ml (149.44), p= 0.001, when compared to pre-perfusion levels, 53.94pg/ml (134.43) and tended to decrease in concentration over the following 72 hours post-transplantation.

Levels of IL-10 (Figure 2b) began to rise prior to pancreas perfusion, but peaked significantly at six hours post-operatively ($p < 0.001$) when compared to pre-operative levels (2.89pg/ml (6.70) and 89.24pg/ml (89.66) respectively). Following this peak, levels fell significantly over the following six hours to 37.04pg/ml (37.39, p= 0.008) and continued to fall reaching baseline levels at 72 hours (2.89pg/ml (6.70) at baseline and 9.70pg/ml (20.76) at 72 hours, p= 0.819). Levels of TNF-α (Figure 2c) increased after start of surgery, before returning to baseline values within 12 hours of surgery, but none of these temporal changes were significant at any time point (p= 0.788). Levels of WCC (Figure 2d) rose significantly between 30 minutes and 6 hours post-transplantation (6.73×10^9/L (2.88×10^9/L) - 11.03×10^9/L (2.80×10^9/L, $p < 0.001$) and continued to rise to a peak at 24 hours (17.10×10^9/L (28.07)), though not significantly ($p > 0.05$) due to the large spread of results at that time-point. CRP levels (Figure 2e) rose rapidly in the post-operative period (significantly within the first 24 hours, $p < 0.001$), up to 72 hours post-transplantation (30 minutes and 72 hours; 3.73mg/L (4.29)-164.23mg/L (115.18), respectively, $p < 0.001$)., Levels of serum Amylase (Figure 2f) rose significantly between pre-pancreas perfusion levels, up to 6 hours post-surgery (38.52U/L (23.93)- 243.81U/L (221.98), p= 0.003) and peaked at 12 hours (299.62U/L (230.99)) before falling to baseline levels by 72 hours post-transplant (pre-operative levels35.76U/L (26.76), 72 hour levels 72.09 (48.47), p= 0.292).

When comparing levels of IMs at corresponding time-points there were no significant correlations between IM levels ($p > 0.05$). However, when correlations were made between peak IM levels, the data became highly significant between TNF-α and IL-6 ($p < 0.001$, $r = 0.556$) and TNF-α and IL-10 ($p = 0.001$, $r = 0.420$).

In uni-variate analysis, no potential confounder had a significant effect upon IM levels at any point within the first 72 hours post-operatively ($p > 0.05$). Therefore, multivariate analysis with these variables was not conducted.

Inflammatory Markers in Omentum

Analysis of intra-operative omental biopsies indicate acutely raised levels of both inflammatory macrophages, M1 (CD68+) and protective M2 (CD206+) macrophages when comparing the biopsies at the start and the end of surgery ($p = 0.003$ and $p < 0.001$ respectively). Figure 3a illustrates representative patient samples highlighting CD68+ and CD206+ staining and Figure 3b demonstrates the quantitative analysis of these findings.

Biomarker Relationship with Cold Ischaemic Time

Endocrine Markers

In a simple linear regression model, C-peptide, insulin and glucagon levels were significantly inversely correlated with increasing CIT at every time point, up to 72 hours post-SPKT. Figures 4a-c highlight the correlations between C-peptide, insulin and glucagon respectively, with CIT at 72 hours. Furthermore, when assessed as a multiple regression model, CIT continues to exert a significant influence on insulin, C-peptide and glucagon levels within the first 72

hours (Table 2), but not at every time-point, as suggested by the simple linear regression model.

Inflammatory Markers

In a multiple regression model, increasing CIT did not significantly influence IL-6, IL-10, TNF-α, Amylase and CRP, at any time-point within the first 72 hours post-transplantation ($p > 0.05$). CIT did significantly influence WCC at 12 hours post-SPKT (mean level 11.77 x 10^9/L (3.42), $p = 0.021$, $r = -0.353$).

Correlation of Peak Inflammatory Marker Levels with Clinical Outcome

Clinical outcomes for this patient cohort are outlined in Table 3. CRP levels at 24, 48 and 72 hours post-operatively (91.19mg/L (38.03), 132.14mg/L (84.73), 164.23mg/L (115.18) respectively) correlated significantly with POMS on post-operative days 5, 7 and 10, the time taken for the patient to mobilise from the bed and the total number of post-operative complications suffered by the recipient during their inpatient stay (Table 4). However, peak levels of IL-6, IL-10, WCC and Amylase did not correlate with any clinically assessed outcome ($p > 0.05$).

Discussion

This study has demonstrated the inflammatory and endocrine marker profiles related to biomarker patterns after SPKT. In our patient group we have defined the temporal evolution of serum IM profiles (IL-6, IL-10, TNF- α, WCC, CRP and Amylase) and serum endocrine marker profiles (insulin, C-peptide, glucagon and resistin) up to 72 hours post-SPKT. Concurrently, this study has shown a significant rise in localised omental expression of CD68+ and CD206+ macrophages intra-operatively. In addition, we have demonstrated the significant negative impact of prolonged CIT on pancreatic endocrine function and finally we have presented data suggesting that consistently raised levels of post-operative CRP may be a valuable biomarker of morbidity following SPKT.

Biomarker profiles are associated with specific outcomes in a range of clinical settings (Glaser, Sannwald et al. 1995, Desborough 2000, Leung, Lai et al. 2000, Mentula, Kylanpaa et al. 2005, Cuschieri, Bulger et al. 2010, Andaluz-Ojeda, Bobillo et al. 2012). For the first time, this paper has shown similar profiles of IM levels following SPKT. Our paper demonstrates an early rise in TNF-α, despite the administration of pre-operative anti-inflammatory therapies, peaking prior to pancreas perfusion, suggesting the biggest systemic inflammatory insult during SPKT is not at the time of graft reperfusion and unlikely to be significantly related to IRI (as is commonly perceived). Instead, the most notable pro-inflammatory insult may be surgical trauma. The successive peaks in IL-6 and IL-10 that follow are consistent with previously published data regarding the chronological evolution of IMs following major physiological stressor events (Kellum, Kong et al. 2007, Malmstrom, Hansen et al. 2012, Kanak, Takita et al. 2014). Our data also suggest a strong correlation between the peaks in TNF-α and IL-6 and IL-10, confirming a relationship between these three biomarkers. Of note, all three cytokines return to baseline within 72 hours of surgery, despite the clinically

observed persistent pro-inflammatory state, suggesting other factors should be monitored for an improved prognosis of outcome.

Since macrophages are known to secrete CRP, it seems likely that CRP and indeed other biomarkers could be secreted locally into the circulation by the presence of omentum macrophages. Since these cells are classified into two broad classes based on their secretion profile and cell surface markers; analysis of the omental biopsies enabled us to identify the increased presence of both M1 (classical) and M2 (alternative) macrophages. The data confirm a significant increase in infiltration of intra-abdominal phenotypic pro-inflammatory CD68+, M1 sub-type together with an increase in the alternative CD206+, M2 macrophage sub-set, which is associated with anti-inflammatory cytokine production. Whether this is a response to the immuno-suppression or simply a reparative mechanism due to surgical trauma and whether the ratio of these two phenotypes could play a role in outcome remains to be elucidated. The data confirm the simultaneous initiation of a localised and speedy inflammatory response to surgery and a correlation of increased CRP with macrophage infiltration into the omentum. Our data also show rapid rises in insulin, C-peptide and glucagon levels immediately after pancreas allograft reperfusion which plateau within 24 hours post-surgery. Naziruddin et al (Naziruddin, Iwahashi et al. 2014) postulate that high levels of circulating C-peptide immediately after islet transplantation, rather than being a sign of immediate function, may be a sign of islet cell damage, perhaps due to the instant blood-mediated inflammatory reaction described in islet cell transplantation (Bennet, Sundberg et al. 1999). Given our results, we postulate that in solid-organ PT, the opposite is true. The strong correlations of insulin, C-peptide and glucagon with CIT suggest, that CIT negatively impacts on pancreatic endocrine function, and rather than higher levels of C-

peptide being a result of islet cell damage, we propose that higher levels detected with shorter CIT are likely to be due to improved islet cell function in solid-organ PT.

Pancreatic CIT has been strongly linked to recipient morbidity previously (Humar, Kandaswamy et al. 2000), but here we present biochemical evidence of the detrimental effect of prolonged CIT on pancreatic endocrine function. This variation in endocrine function observed in relation to CIT, highlights the presence of reduced graft function in PT attributable to a defined and modifiable variable, a previously unreported phenomenon. This finding should encourage the implementation of specific, islet-protective, peri-operative strategies in grafts with prolonged CIT. In addition, our data support previous suggestions by Barton et al (Barton, Rickels et al. 2012) for the use of specific anti-inflammatory induction of immunosuppressive factors to help reduce early graft dysfunction and improve long-term graft survival, given; firstly the inflammatory load on the recipient at the time of pancreas perfusion, and secondly, the known deleterious effects of pro-inflammatory cytokines on islet function and survival in isolated islet cell transplantation (Kutlu, Cardozo et al. 2003, Barbe-Tuana, Klein et al. 2006, Lawrence, Naziruddin et al. 2011, Kanak, Takita et al. 2014). This course of therapy has been used successfully in animal models (Yang, Chen et al. 2005) and in clinical islet transplantation (Kanak, Takita et al. 2014) and should perhaps be utilised in PT.

Monitoring biomarkers in a disease process provides the opportunity to modify current treatment strategies, and/or to target the negative clinical processes modulated by specific biomarkers, thus delivering a positive influence on patient outcome.

A second advantage of exploring biomarkers and delineating their profiles in a disease processes is the ability to monitor the disease or intervention progression and predict

outcome. Serum is readily available and the ability to correlate serological markers to patient outcome is of tremendous benefit, clinically and economically. In transplantation, the most important outcome measures are graft loss and patient mortality. Incidences of these outcome measures in SPKT are now reasonably low and have plateaued over the last 10 years (Gruessner and Gruessner 2013), making these poor comparators in clinical trials and routine clinical practice. Therefore, greater importance in assessing relevance of biomarkers in clinical practice should now be focused on the objective assessment of peri-operative morbidity, especially considering the prevalent nature of morbidity in our cohort (Bassetti, Salvalaggio et al. 2004) and the influence of peri-operative morbidity on long-term outcomes following major surgery (Khuri, Henderson et al. 2005). POMS is an objective, validated assessment tool to evaluate patient morbidity following major surgery (Bennett-Guerrero, Welsby et al. 1999) and our data demonstrate that CRP at 48 hours best correlates with a number of objective clinical outcome measures, including POMS on days 5, 7 and 10 (r= 0.461, 0.350 and 0.359 respectively) and the number of complications suffered by an individual as an inpatient (r= 0.394). It is interesting that a single and commonly available biomarker, CRP is statistically related to outcomes following SPKT in this study, but this finding is entirely consistent with studies in patients with acute pancreatitis (Wilson, Heads et al. 1989) and pancreatic resections (Welsch, Frommhold et al. 2008).

We acknowledge limitations of this study in that

the sample size is small, and the numbers of concomitant variables associated with outcomes in SPKT are large, limiting the analysis which could be performed. Therefore, to conduct a detailed multiple regression analysis, a larger cohort would need to be investigated. Nevertheless, in our analysis we have addressed the most clinically relevant variables affecting this cohort, with the aim of proposing factors affecting biomarker levels and the predictive capabilities of specific biomarkers to outcome post-SPKT. This study only

begins to elucidate the inflammatory response to SPKT, modulated by the background of immunosuppression and clearly warrants further study, as our findings suggest this approach could have wide clinical implications for improved patient stratification and better outcome.

Our cohort was treated with two doses (30mg each), of subcutaneous alemtuzumab, administered 24 hours apart and a single dose of methylprednisolone at the time of anaesthetic induction. Glucocorticoids have been linked with reducing circulating pro-inflammatory cytokines (Petrovsky, McNair et al. 1998), whilst alemtuzumab is a monoclonal antibody which induces antibody mediated lysis of the cell. Therefore, with the conventional view of macrophages being an inflammatory cell, one would have expected to see a reduction in macrophage numbers, and consequently inflammatory cytokines, following immunosuppression induction. Conversely, the opposite was noted. This may be explained in two ways; first possibly due to the sub-cutaneous administration of alemtuzumab, therefore likely reducing overall absorption, compared to the intra-venous route (Hale, Rebello et al. 2004), which would explain the delayed anti-inflammatory response and the persistently low IM levels measured 24 hours onwards. However, alemtuzumab has also been reported to stimulate a type 1 hypersensitivity reaction that would increase circulating pro-inflammatory cytokines. Although clinical anaphylaxis is rare, the acute pro-inflammatory tendency may be contributing to the acute pro-inflammatory state observed. Therefore, perhaps a specific anti-inflammatory induction regimen would also negate the acute pro-inflammatory effects of alemtuzumab.

Second, in recognition of the growing importance of alternative macrophage polarization in disease, and the characteristics and phenotypic diversity macrophages that underlie the cells' actions, as well as the contribution of resident versus infiltrating macrophages/monocytes, and indeed, the role of macrophage mediators, a larger

prospective study is needed to confirm our preliminary results of a significantly increased presence of both M1 and M2 macrophages within the context of SPKD. Whether the surgical insult, or patient stratification affects macrophage phenotypic switch in the omental and/or other tissues, or whether outcome is determined by macrophage function, remains to be elucidated. These further studies could have a novel direction towards the potential of macrophages as a therapeutic target in SPKT and provide promising strategies to manage patients following transplantation.

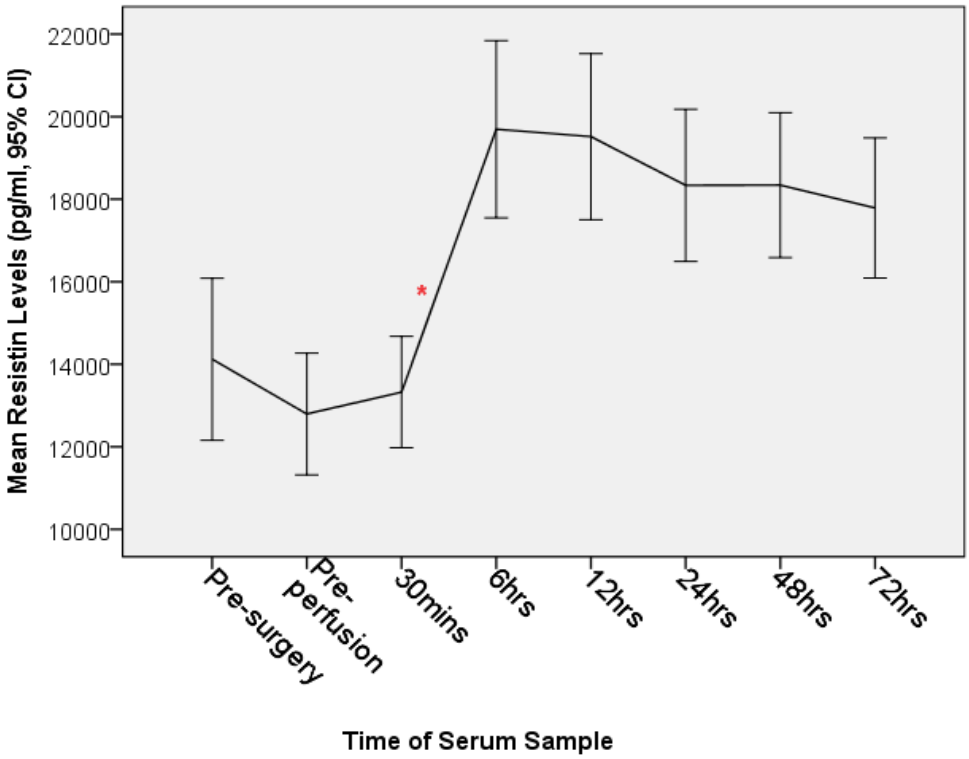
Conclusion

. The temporal signature of specific biomarkers, namely IL-6, IL-10 and TNF-α after SPKT are comparable to patterns observed following major surgery, trauma and sepsis.Our study has two key findings; firstly, that CIT is significantly related to early pancreatic endocrine function, and secondly, that CRP provides an easily measurable predictor of recipient morbidity at an early post-operative period. Therefore, we suggest guidelines should recommend an aim to reduce CIT, not only to reduce post-operative morbidity, but also to optimise peri-operative pancreatic graft function. These findings provide evidence for the use of targeted anti-inflammatory therapies and supportive measures such as low-dose insulin in the peri-operative period to minimise islet cell stress and damage and perhaps improve long-term graft survival and opens an avenue for future studies in this surgical setting, to further elucidate the role of inflammatory cytokines and the phenotypic diversity of macrophages within the context of SPKD.

Table 1. Baseline Recipient, Donor and Operative variables

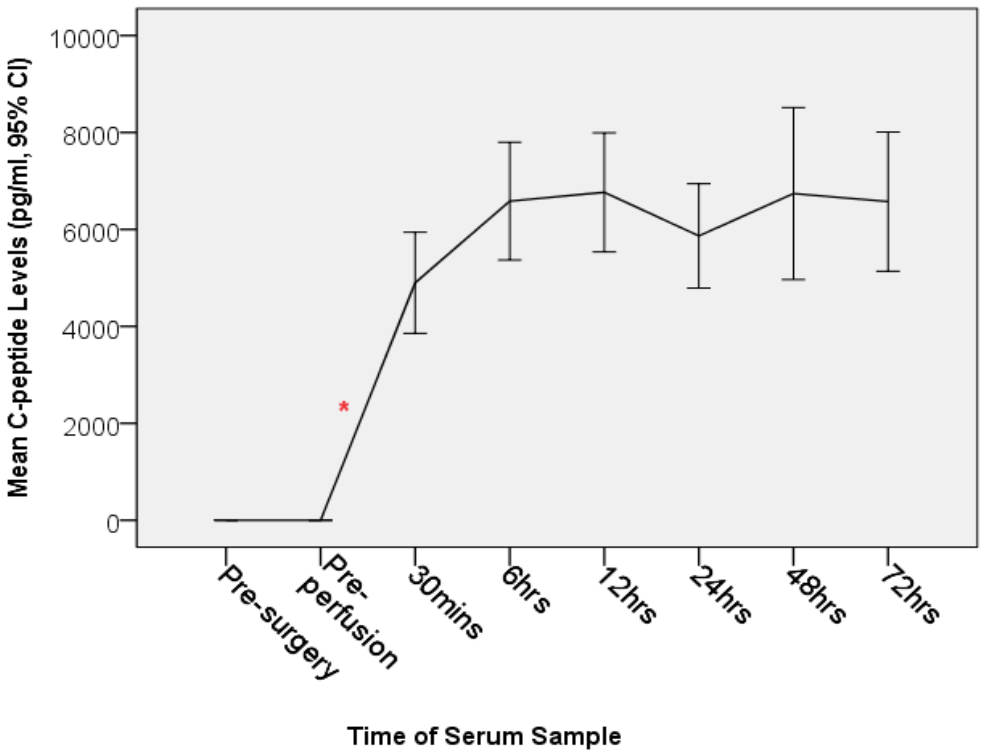
RECIPIENT VARIABLES	**N= 46**
Age (years)	42.69 ± 7.02
Male	26 (56.5%)
Ethnicity	1 Afro-Caribbean (2.2%) 45 White Caucasian (97.8%)
BMI kg/m^2	25.31 ± 3.01
Years diabetic	27.09 ± 3.01
Pre-dialysis	18 (39.1%)
Time on dialysis (months)	16.58 ± 26.07
Time on waiting list (days)	646.11 ± 418.93
DONOR VARIABLES	
Age (years)	33.57 ± 12.37
Male	32 (69.6%)
Ethnicity	1 Mixed, White/ Asian (2.2%) 1 Indian (2.2%) 1 European Other (2.2%) 43 White Caucasian (93.5%)
BMI kg/m^2	23.78 ± 2.55
DBD **DCD**	37 (80.4%) 9 (18.4%)
Donor WCC (n= 18 and 18) $^{\Phi}$	12.00 IQR 8.93- 12.03
Donor Amylase (n= 15 and 16) $^{\Phi}$	52.00 IQR 34.00- 133.00
Time from admission to retrieval of organs$^{\Phi}$ **(hrs)**	52.00 IQR 29.75- 78.75
P-DRI	1.72 ± 0.77
P-PASS	10.78 ± 2.35
OPERATIVE VARIABLES	
P CIT	694.74 ± 184.73
K CIT	836.30 ± 203.10
Pancreas fattiness grade	2.00 ± 1.12
Mismatch >3	28 (60.9%)
Induction Agent	46 campath (100%)
Pancreas Preservation Solution	42 UW (91.3%) 4 HTK (8.7%)
Length of Operation (mins)	334.15 ± 72.09

Values are absolute (%) or mean (± Standard Deviation, SD) unless otherwise stated. $^{\Phi}$Median (Interquartile range, IQR). BMI, Body Mass Index; DCD, Donor after cardiac death; DBD, Donor after brainstem death; WCC, White cell count; P-DRI, Pancreas donor risk index; P-PASS, Pre-procurement pancreas allocation score; P CIT, Pancreas cold ischaemic time; K CIT, Kidney cold ischaemic time; UW, University of Wisconsin solution; HTK, Histidine-tryptophan-ketoglutarate solution

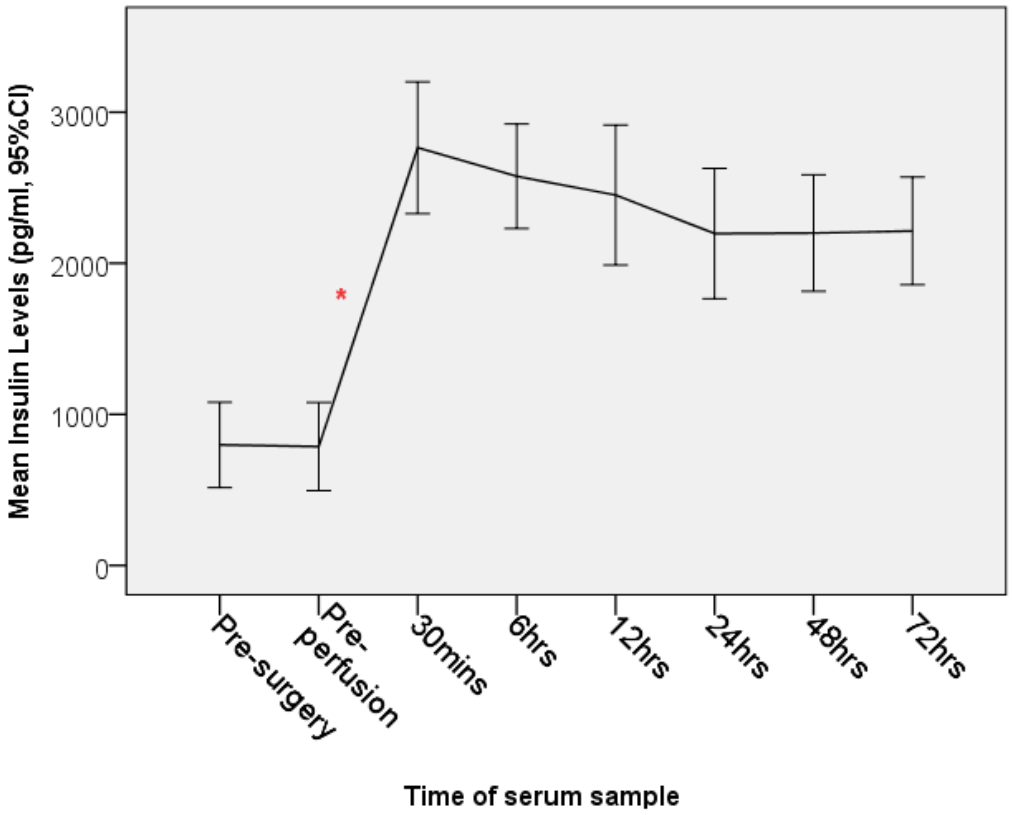


(a) Resistin

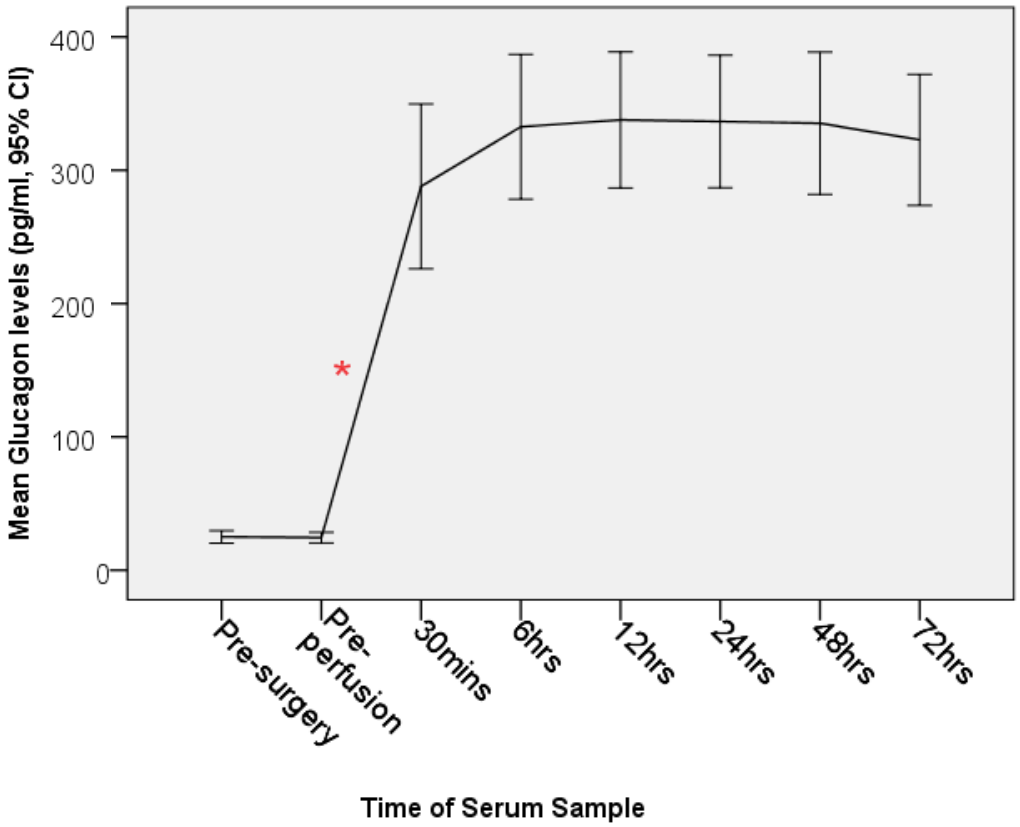
Figure 1a- d. Mean Resistin (a), C-peptide (b), Insulin (c) and Glucagon (d) levels (pg/ml 95% CI) respectively in the peri-operative period post-SPKT (Pre-surgery, pre-pancreas perfusion and then at 30 minutes and 6, 12, 24, 48 and 72 hours post-transplant, *denotes significant change in levels, p< 0.001, ANOVA).



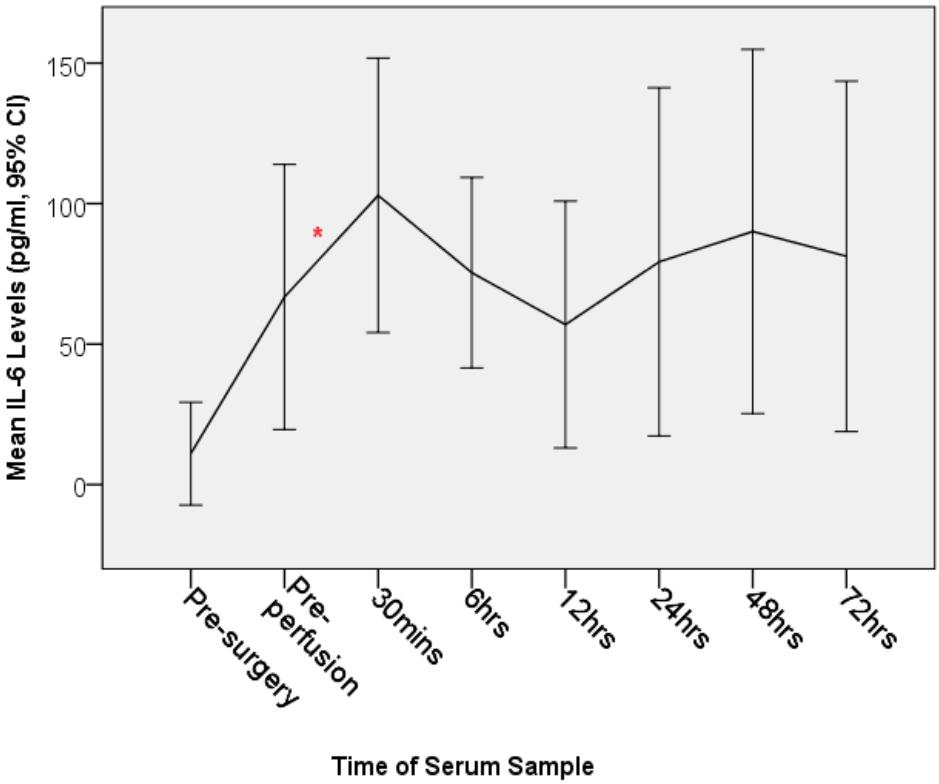
(b) C-Peptide



(c) Insulin

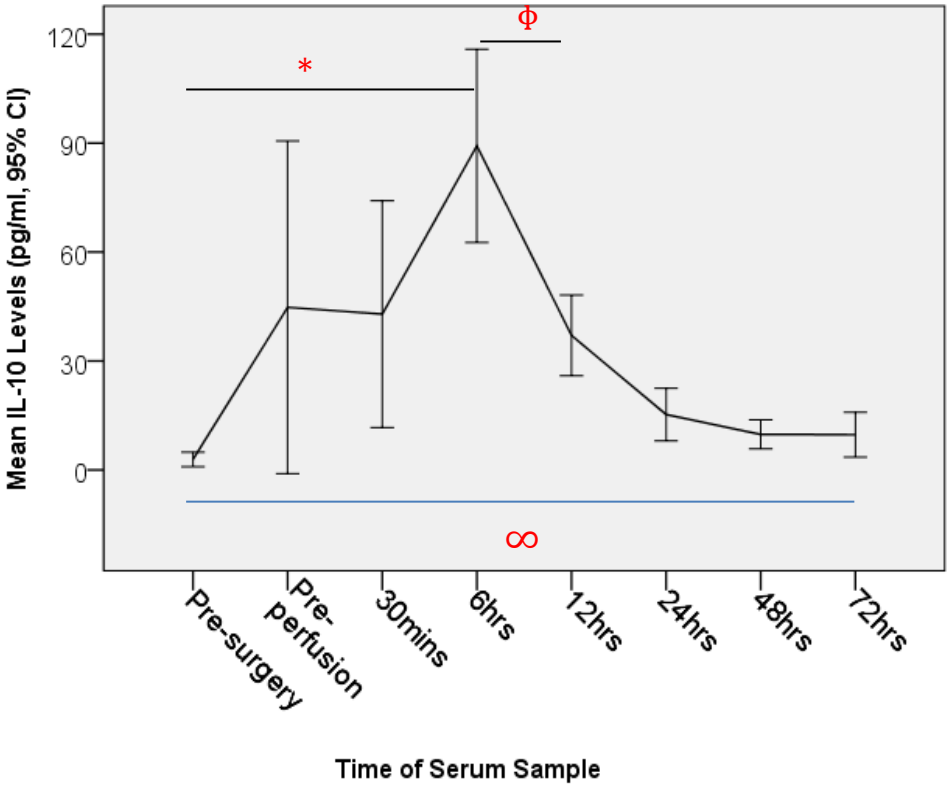


(d) Glucagon

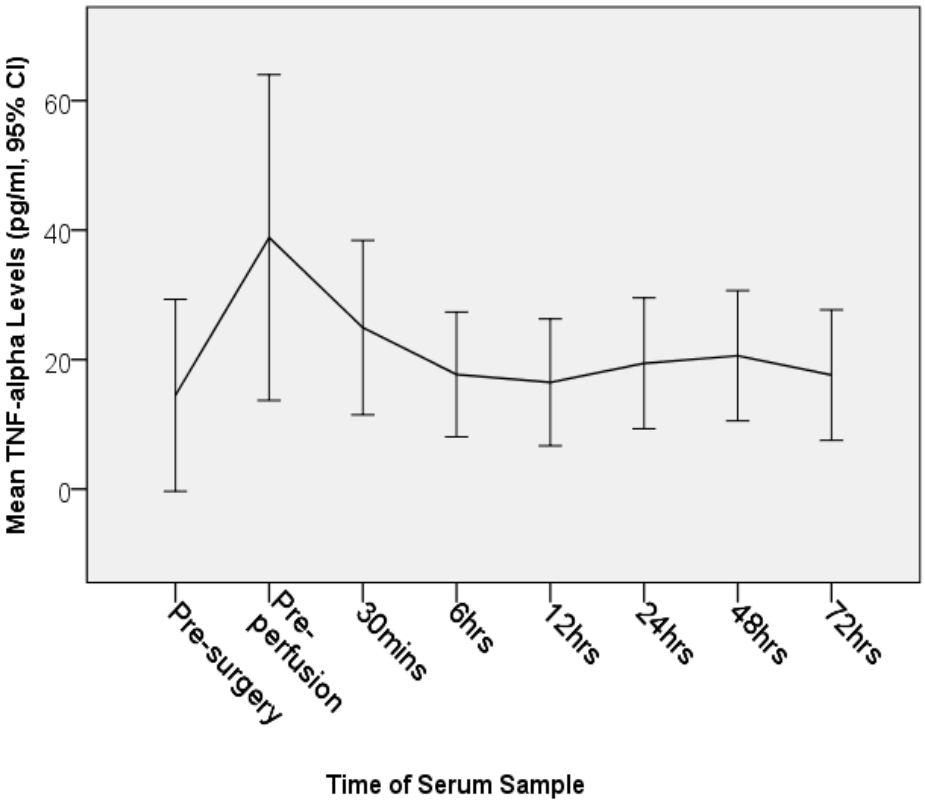


(a) IL-6, *p=0.001

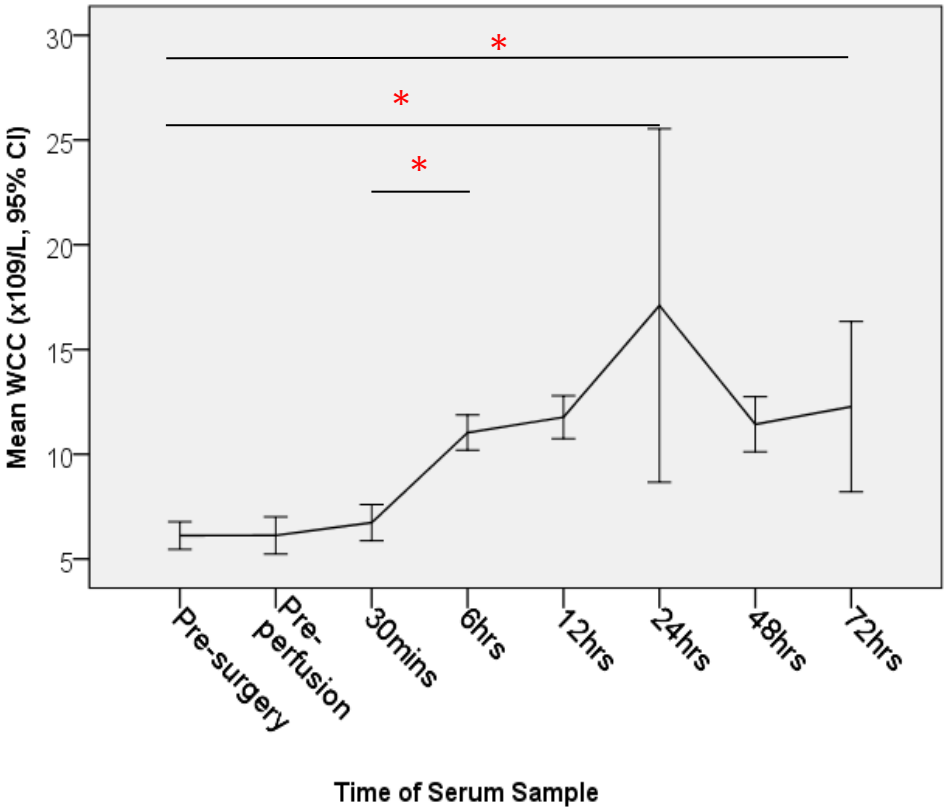
Figures a- f. Mean levels of IL-6, IL-10, TNF-alpha, WCC, CRP and Amylase respectively, in the peri-operative period post SPKT. Significant changes in temporal evolution within the first 72 hours post-operatively are indicated.



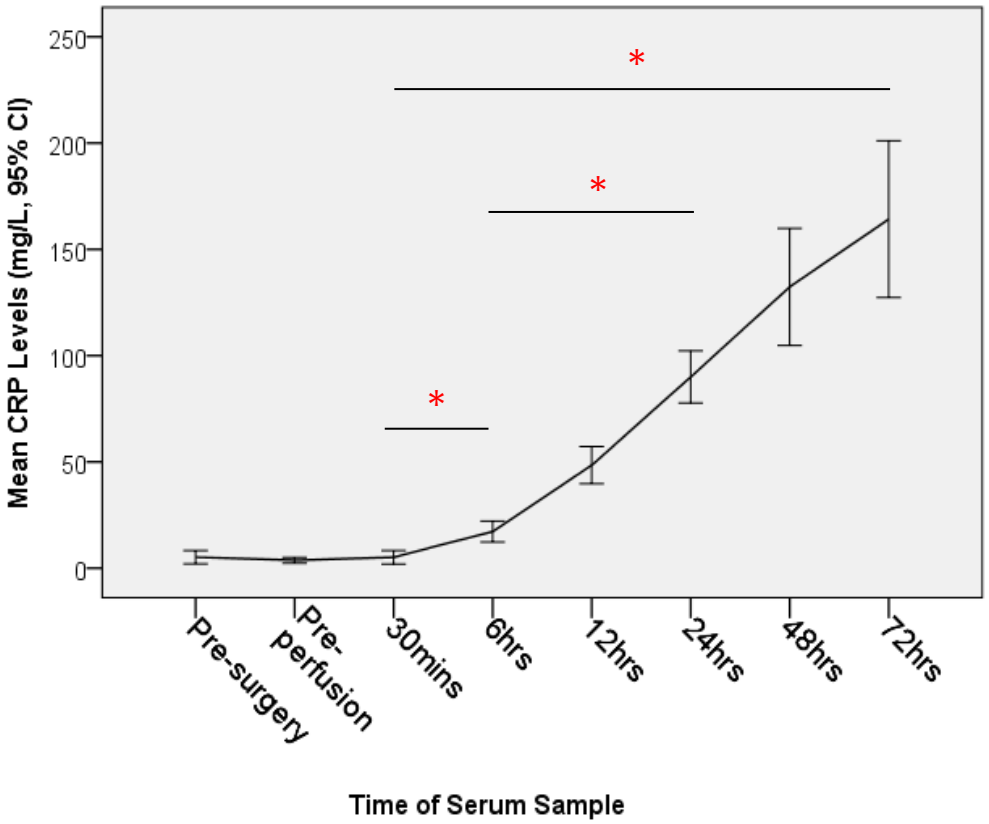
(b) IL-10 *p<0.001, ϕp= 0.008, ∞p= 0.819



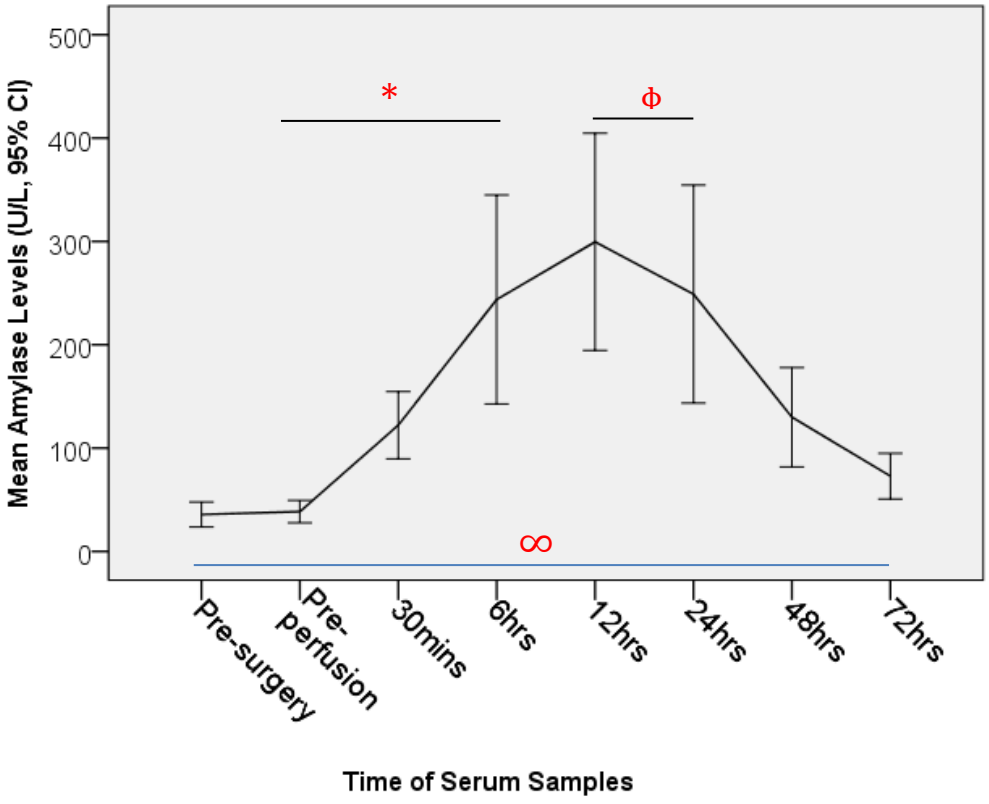
(c) TNF-α



d. WCC * p <0.001



(e) CRP * p <0.001



(f) Amylase * p= 0.003, Φ p= 0.014, ∞ p= 0.292

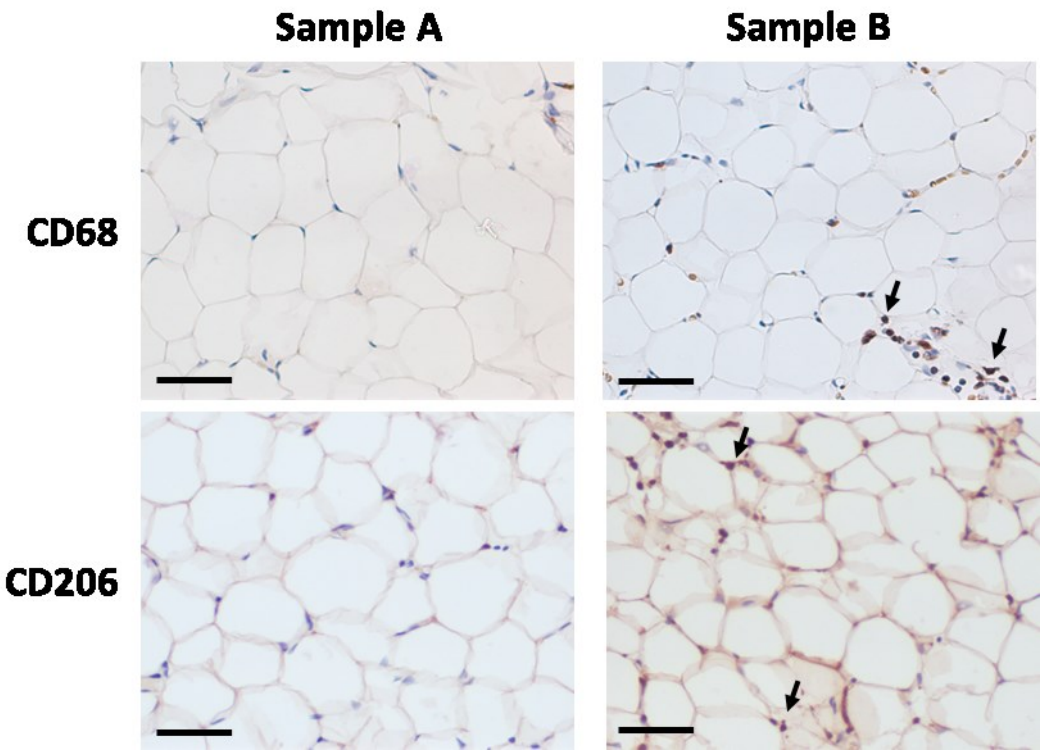


Figure 3a.

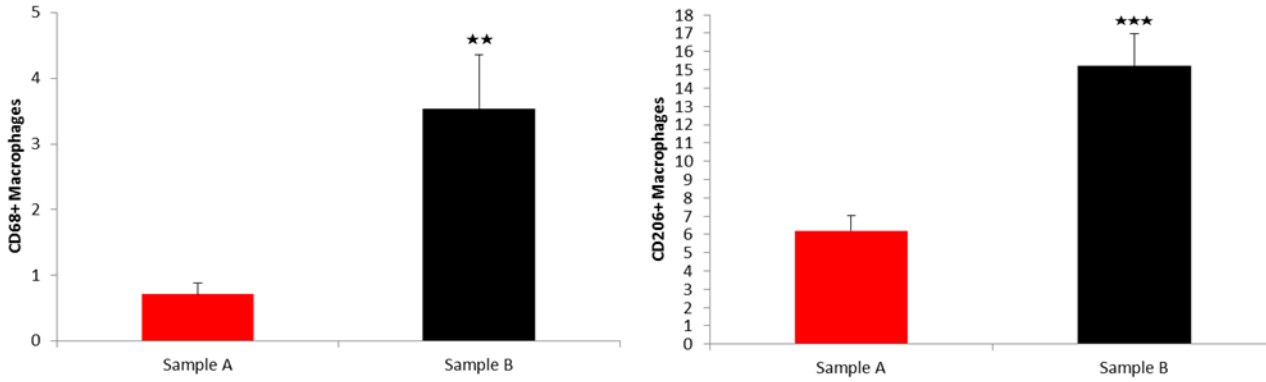
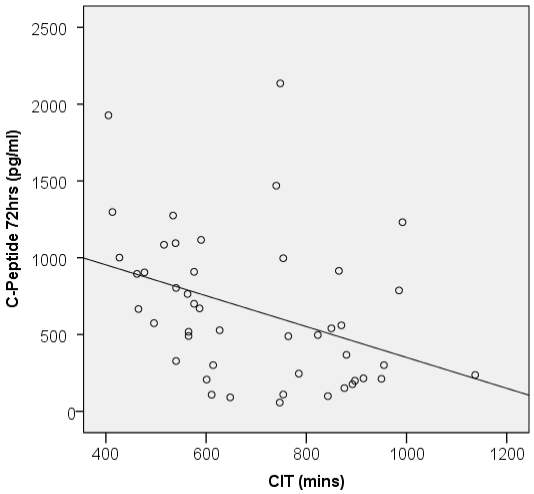
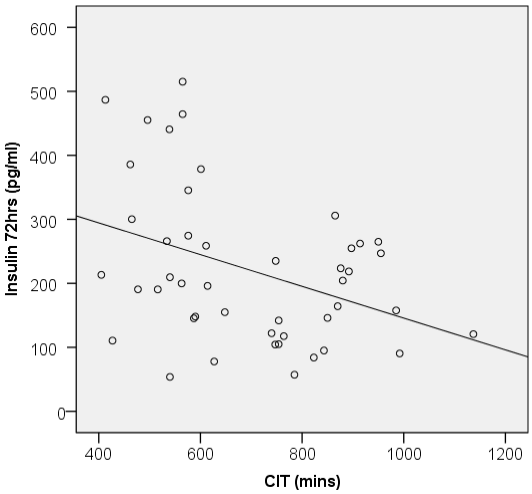


Figure 3b.

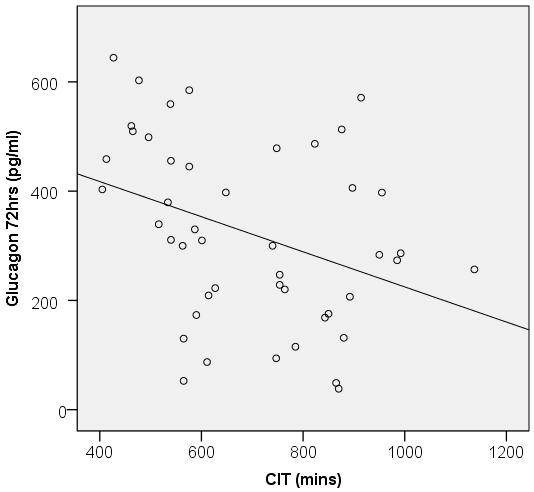
Figure 3. a) Sections from representative patient samples showing positive staining for inflammatory macrophage marker CD68 and anti-inflammatory macrophage marker CD206 Sample A, biopsied at the start of surgery, and Sample B, biopsied at the end of surgery, macrophages are indicated by arrows, bars =50µm. b) Quantification results of CD68+ and CD206+ macrophage staining in omentum (**p= 0.003 and ***p <0.001, T-test).



a. C-peptide



b. Insulin



c. Glucagon

Figures 4a- c. Linear correlation of Cold Ischaemic Time with a) C-peptide (p= 0.008, r= -0.384), b) Insulin (p= 0.009, r= -0.382) and c) Glucagon (p= 0.014, r= -0.359) at 72 hours post- transplantation

CIT, Cold Ischaemic Time

Table 2. Analysis of multiple regression model, investigating the effect of CIT on serum C-peptide, Insulin and Glucagon levels in the peri-operative period following SPKT.

Time Post-SPKT	C-Peptide	Insulin	Glucagon
30 minutes	P= 0.003 R= -0.528	P= 0.025 R= -0.348	P= 0.003 R= -0.479
6 hours	P= 0.093 R= -0.437	P= 0.029 R= -0.532	P= 0.082 R= -0.335
12 hours	P= 0.038 R= -0.466	P= 0.021 R= -0.356	P= 0.117 R= -0.432
24 hours	P= 0.006 R= -0.482	P= 0.002 R= -0.483	P= 0.086 R= -0.400
48 hours	P= 0.018 R= -0.507	P= 0.009 R= -0.416	P= 0.007 R= -0.528
72 hours	P= 0.035 R= -0.483	P= 0.002 R= -0.476	P= 0.033 R= -0.374

Table 3. Clinical outcomes at discharge

Outcome Measure	N= 46
1 month pancreas survival	39 (84.8%)
Critical Care Unit Length of Stay (days)[ϕ]	6.50 IQR 4.00- 8.00
Total Hospital Length of Stay (days)[ϕ]	18.00 IQR 14.00- 29.75
Number of Complications per patient[ϕ]	1.00 IQR 1.00- 2.00
Time to Mobilisation (days)[ϕ]	3.98 IQR 2.00- 6.00
Time to Tolerating Normal Diet (days) [ϕ]	7.00 IQR 5.00- 9.00
24 hour MODS	3.38 ±1.63
48 hour MODS	3.02 ± 1.79
72 hour MODS	2.53 ± 1.75
Post-Operative Morbidity Survey Score, Day 5[ϕ]	3.00 IQR 2.00- 4.00
Post-Operative Morbidity Survey Score, Day 7[ϕ]	2.00 IQR 0.00- 3.00
Post-Operative Morbidity Survey Score, Day 10[ϕ]	1.00 IQR 0.00- 2.75

Values are absolute (%) or mean ± Standard Deviation (SD) if normally distributed, or [ϕ] Median (Interquartile range, IQR). MODS, Multiple Organ Dysfunction Score.

Table 4. Correlations of clinical outcome measures and CRP at 24, 48 and 72 hours post operatively (Spearman's Correlation, p and r values stated).

Clinical Outcome Measure	CRP 24 hours	CRP 48 hours	CRP 72 hours
POMS, Day 5	P= 0.012 R=0.371	P= 0.001 R= 0.461	P= 0.041 R= 0.306
POMS, Day 7	P= 0.067 R= 0.273	P= 0.019 R= 0.350	P= 0.004 R= 0.308
POMS, Day 10	P= 0.024 R= 0.335	P= 0.015 R= 0.359	P= 0.041 R= 0.306
Time to Mobility	P= 0.017 R= 0.356	P= 0.005 R= 0.416	P= 0.201 R= 0.194
Number of Complications	P= 0.021 R= 0.344	P= 0.007 R= 0.394	P= 0.013 R=0.367

POMS, Post-Operative Morbidity Survey