BioPAsSPoRT – Biomarkers for Patient Assessment and Stratification Post Renal Transplant

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

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- Dr Helen Sewell: Some sample analysis and data generation
- Michelle Wilson: Statistical analysis and modelling
- LTHT colleagues: biomarker and clinical expertise
- Randox collaborators: Provision of the equipment, manufacture of consumables and Midkine analysis

Abstract

Chronic kidney disease affects approximately 10% of the global population and the incidence is increasing annually with the rise in comorbidities. 2-4% of patients with chronic kidney disease are expected to progress to end stage kidney disease requiring renal replacement therapy in the form of dialysis or transplantation. Transplantation is the optimum treatment for end stage kidney disease both in terms of cost savings to the NHS and patient quality of life but organs are scarce. In order to expand the pool of available grafts, extended criteria donations are increasingly being used but these come with increased rates of complications such as delayed graft function. Currently delayed graft function is defined as the need for dialysis in the first week after transplant in the absence of hyperkalaemia. As there are no definitive guidelines on when it is necessary to dialyse a patient post-transplant, diagnosis of delayed graft function is therefore subjective to some extend and governed by the overseeing clinician.

Using the innovative multiplexing biochip technology from Randox, BioPAsSPoRT aims to combine the novel Aminoacylase-1 biomarker with other established and potential renal biomarkers to develop an assay that will provide a method to predict the development of delayed graft function early post-renal transplant and provide prognostic information to allow risk-based patient follow-up stratification.

An initial cohort of 241 patients was investigated and statistical analysis determined an optimal biomarker panel including ACY-1, sTNFR1, YKL-40 and cystatin C which was then manufactured into a prototype Renal Transplant Array to be validated in a multi-centre independent cohort of a further 320 patients.

Statistical modelling determines that the renal transplant array shows promise with regards to early detection of delayed graft function and prognosis with regards to dialysis-free survival with a serum sample taken day 1-3 post-transplant. A further prospective clinical trial will be designed to assess the final array before commercial availability.

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List of Abbreviations

ACY-1	Aminoacylase-1	**HD**	Haemodialysis
AUC	Area under curve	**HRP**	Horseradish peroxidase
C3DA	C3a-des-arginine	**IL-8**	Interleukin-8
CIT	Cold ischaemic time	**IRI**	Ischaemic reperfusion injury
CKD	Chronic kidney disease	**<LLOQ**	Below lower limit of quantification
CRP	C reactive protein	**MIP1A**	Macrophage inflammatory protein 1-α
CV	Coefficient of variation	**NGAL**	Neutrophil gelatinase-associated lipocalin
CYSC	Cystatin C	**NIHR**	National Institutes for Health Research
DBD	Donation after brain death	**PD**	Peritoneal dialysis
DCD	Donation after cardiac death	**ROC**	Receiver operating characteristic
DGF	Delayed graft function	**RRT**	Renal replacement therapy
ECD	Expanded criteria donor	**RTA**	Renal transplant array
EGF	Epidermal growth factor	**SCD**	Standard criteria donor
ESKD	End stage kidney disease	**sCr**	Serum creatinine
FABP1	Fatty acid binding protein-1	**sTNFR1**	Soluble TNF receptor 1
GFR	Glomerular filtration rate	**sTNFR2**	Soluble TNF receptor 2
eGFR	Estimated-GFR	**>ULOQ**	Above upper limit of quantification
mGFR	Measured-GFR	**WIT**	Warm ischaemic time

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1 Introduction

1.1 Chronic Kidney Disease and End-Stage Kidney Disease

Chronic kidney disease (CKD) is a decrease in kidney function and is defined by the National Kidney Foundation as abnormalities of kidney structure or function with implications for health or an albumin:creatinine ratio of >30mg/g for three months or more[1].

CKD can be divided into five classifications based on the estimated glomerular filtration rate (eGFR) measured in mL/min per 1.73m^2[1]. The classifications are described in Table 1.1.

Table 1.1 Stages of Chronic Kidney Disease

Stage	Description	eGFR
Normal kidney function	Healthy kidneys	≥90 mL/min per 1.73m^2
Stage 1	Kidney damage with no loss of function	≥90 mL/min per 1.73m^2
Stage 2	Kidney damage with mild loss of kidney function	60-89 mL/min per 1.73m^2
Stage 3	Mild to moderate loss of kidney function	30-59 mL/min per 1.73m^2
Stage 4	Severe loss of kidney function,	15-29 mL/min per 1.73m^2
Stage 5 (ESKD)	Kidney failure requiring dialysis or transplant	<15 mL/min per 1.73m^2

An eGFR of >60mL/min without any other indication of kidney disease does not warrant clinical intervention[2].

The current gold standard for measuring GFR is isotopic clearance but as it is a costly and more invasive method of monitoring GFR measuring serum creatinine (sCr) levels is used routinely in clinical practice. There are several

calculations to estimate GFR based on serum creatinine (and to some extent the newer biomarker cystatin C) but despite a generally good correlation with isotopically measured GFR, eGFR is not as accurate as measured GFR[3,4].

CKD is estimated to affect around 10% of the global population [5,6] although this varies by country. The most common causes of CKD are diabetes and hypertension[7], both of which are increasing in prevalence worldwide in part due to the rising rates of obesity. Earlier stages of CKD are estimated to be present in 5-7% of the global population[8] but it is likely higher given the lack of symptoms in early CKD[9].

Age is also a significant risk factor for CKD, with CKD rates of nearly 40% reported in the over 60 population in the US[10] compared to 12% in 40-59s. With life expectancy increasing, rates of CKD can be expected to increase in line with an aging population. Additionally there is some link to heritability[11], gender and ethnicity[12].

In the UK it is estimated that, based on eGFR, 15% of adults over the age of 35 had some degree of chronic kidney disease (stage 1-5) and 7% had stage 3-5 CKD[13]. With nearly 700million cases and 1.2million deaths[11] reported worldwide in 2017 CKD is a major burden on health services globally.

It is expected that approximately 2-4% of CKD patients go on to develop end stage kidney disease (ESKD) which requires renal replacement therapy (RRT) in the form of either dialysis or renal transplantation.

1.2 Renal Replacement Therapy

ESKD is the fifth and final stage in the progression of CKD, where the kidneys no longer function sufficiently to keep a patient alive, requiring renal replacement therapy in the form of dialysis or renal transplant. ESKD is diagnosed when the estimated glomerular filtration rate reaches <15mL/min or less and it is estimated that by 2030 the number of people utilising renal replacement therapy will grow to 5.5 million[14].

1.2.1 Dialysis

Dialysis is the process of removing excess water, solutes and toxins from the blood once the native kidneys are no longer able to do so. Diffusion is the underpinning principle of all forms of dialysis with a concentration gradient removing the waste products from the blood. The proportion of

people on dialysis worldwide is growing with the increase in kidney disease causing the demand for transplant to outstrip the supply of grafts.

In haemodialysis (HD) the patient's blood is run past a semi-permeable membrane which removes excess water and waste products. It can be performed in a dedicated dialysis centre or at home. Home haemodialysis appears to have the best patient outcomes in terms of quality of life, longer survival[15] and fewer hospitalisations[16]. It is also more cost effective than in-centre dialysis at around £35000 and £32000 per year respectively[17] suggesting that rates of home haemodialysis may increase in the future[18].

In peritoneal dialysis (PD) a permanent catheter allows the infusion of a dialysate into the abdomen of the patient where dialysis is by diffusion across the peritoneum. There is some evidence to suggest that patients utilising PD have at least equal clinical outcomes and quality of life improvements to those on HD[19]. PD is more cost effective than HD at around £15000 per annum[20] and can be performed at home by the patient as opposed to having to travel to a dedicated dialysis facility[21]. Despite this, patients utilising HD vastly outnumber PD patients in practice[22] with the rate of PD decreasing 0.5% between 2014 and 2018 and HD rates increasing 0.9%.

1.2.2 Renal transplantation

Renal transplant is the optimal form of renal replacement therapy as it provides the best long term outcome for patients both in terms of quality of life and better survival rates. It also has major cost savings when compared to dialysis[23], however since 1990 there has been around a 34% increase in renal transplantation in response to a substantial increase in ESKD[24]. In the UK the number of patients commencing RRT in response to ESKD has risen from 7453 in 2014 to 8075 in 2017, though they did decrease slightly in 2018 down to 7959[22]. In 2018 the number of transplants performed was 3664, compared to 3463 in 2017 and 3331 in 2016.

There are two sources of kidneys for transplantation: live donors and deceased donors. Live donations account for approximately 30% of grafts in the UK (28.5% in 2018[23]). Transplants from live donors are associated with better post-transplant outcomes due to, in part, the extensive screening that live donors undergo pre-transplant leading to a superior quality of graft[25] over one from a deceased donor plus the inherently shorter ischaemic times involved with live donation[26]. Living donors are also more likely to be

rejected based on increased age whereas a high proportion of deceased donors are over the age of 60[27].

While a live donor organ is the ideal and live donation has steadily increased [22,28] is the demand far outweighs the supply and as a result there has been a significant increase in the use of grafts from expanded criteria donors (ECD) such as deceased donors, donors over the age of 60, or donors over the age or 50 with comorbidities[29]. In the UK from 2017-2018 there was an 8% increase in transplants from donation after brain-stem death (DBD) and a 5% increase in donation after cardiac death (DCD)[22].

These grafts come with an increased rate of complications including delayed graft function (DGF) and rejection. In the US between 1998 and 2012 rates of DGF in extended criteria donors (ECD) and standard criteria donors (SCD) were approximately 30% and 15% respectively[30]. It has been suggested that the increase in rates of DGF in DCD kidneys may be due to the inherently longer warm ischaemic times (WIT) that occur[31]. Kidneys from deceased donors also tend to have a lower GFR than those from live donors, with DCD grafts performing slightly worse than DBD and LD kidneys having the highest GFR[22].

With the demand for renal transplantation outstripping the supply of grafts available it is essential that when transplant does occur, treatment options ensure the best possible outcomes for the graft. The latest available figures show that 56% of patients with ESKD received a transplant in the UK by the end of 2018[22]. Despite being a 5% increase compared to 2017, that still leaves 44% of patients without a transplant.

1.3 **Delayed Graft Function**

DGF is often defined as the need for dialysis in the first week after transplantation, in the absence of hyperkalaemia. However, a review in 2008 highlighted that there have been as many as 18 definitions across different countries and between renal centres[32] which can lead to inconsistencies in how it is diagnosed and reported. It also means that diagnosis of DGF is dependent on the clinician treating the patient as opposed to objective criteria being met. DGF may be suspected if, post-transplant, the serum creatinine level of the patient does not decline as expected or if they do not produce significant amounts of urine[33].

The exact mechanisms and pathology underlying DGF are still unclear but injury can occur anywhere from pre-harvest of the organ up until surgery to transplant the graft itself. Ischaemia reperfusion injury(IRI) causes a cascade of events that culminate in necrosis and the activation of apoptotic pathways[34]. As the cause of an acute kidney injury, IRI is strongly linked to the development of DGF and, despite sometimes occurring in grafts from live donors, is more common in grafts from deceased donors[35] where circulation has been absent for longer with rates of DGF around 20% in deceased donors compared to ~5% in live donors[30]. It follows then that higher incidences of DGF have been observed with the increased use of expanded criteria donors.

It is generally observed that within grafts from deceased donors, DCD grafts have a higher rate of DGF than DBD kidneys with around 40-60%[36-38] among various DCD cohorts and 20-30%[36-38] in their DBD counterparts. That said, kidney health in the donor is more likely to impact on the development of complications, including DGF, than the DCD vs DBD consideration[39]

Development of DGF is linked with ischaemic time during transplant[40] which is also inherently longer in deceased donations. Warm ischaemic time is the time from harvest of the organ when the donor's circulation stops until when the organ is perfused with preservation solution while cold ischaemic time is from perfusion with preservation solution until reperfusion with the recipients circulation. Most research in this area has linked CIT to the development of DGF[41-44] although few studies have shown that that WIT may be similarly implicated[45,46]. It has been suggested that CIT has more of an impact on grafts from DCD donors than DBD donors so this should be taken into consideration when allocating grafts to recipients[37].

There has previously been some investigation into the link between donor age and the development of DGF that found correlation between the two[47] but other studies have found little difference in graft function based on biological age[48,49]. It has also been observed that a higher body mass index and longer duration of pre-transplant dialysis may be factors[50] and may be more likely in the case of repeat transplants[51].

In terms of patient outcomes, there have been associations with increased rejection rates[36,52] and a reduction in graft longevity[52] in addition to a reduction in patient life expectancy[53] when DGF is a factor. Conversely though, a large systematic review has determined that statistically the

difference in long-term survival rates between DCD and DBD grafts is negligible irrespective of DGF[36].

Possibly the duration of DGF may be a significant factor as it has been observed that DGF persisting for more than six days decreases the long-term survival of the donor kidney[54] and that the development of DGF may have more of an impact on graft survival than a significant HLA mismatch between donor and recipient[33]. There is no treatment for DGF beyond dialysing appropriately, being careful not to overdialyse as post-transplant dialysis poses a potential risk to the graft itself through hypotension and risk of clotting but also increases the duration of hospital stay[50]. Graft function may also be monitored through biopsies[55] although this is only as a last resort and where absolutely clinically necessary as it is invasive and potentially detrimental to the patient.

Attempts have been made to model risk of DGF development[56] as with organs being so scarce, a method enabling earlier diagnosis/prediction of DGF and consequently earlier and more timely intervention to prevent it or minimise the impact would be invaluable to improve the longevity of grafts and ensure the best possible outcome for recipients.

1.4 Biomarkers in renal transplantation

Biomarker is short for biological marker and is defined by the National Institutes of Health as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention” [57].

Discovery and evaluation of biomarkers is a valuable area of research in all fields of medicine to allow developments such as earlier intervention and improved patient outcomes. Often molecular biomarkers are detectable long before clinical manifestations of a problem are observable and so could indicate a complication before it becomes severe or irreparable[58].

There are a number of categories of biomarker, but in this study the markers of interest are being reviewed in an earlier diagnostic/predictive and a prognostic capacity for patients with DGF. A biomarker may be considered predictive if its presence or a change in its presentation can be used to predict if a patient is more likely to experience a particular clinical event than similar patients that lack the biomarker[59] or in the case of therapies, predicting who will respond.

Prognostic biomarkers can be used to identify the probability of a particular clinical event or outcome in a patient[60]. In this research the intention is to determine whether the biomarkers give any prognostic information with regards to the dialysis-free survival of patients and their overall survival which would allow clinicians to stratify patient care and tailor levels of monitoring appropriately to specific patient needs.

Currently the accepted circulating marker of renal function is serum creatinine. However, it has limitations as observable levels can be affected by factors non-specific to kidney function such as age, gender, and muscle mass, among others[61]. Serum creatinine levels may also indicate renal injury only after significant organ damage has occurred and later than other biomarkers can[62] meaning earlier intervention is possible with other biomarkers. When combined with other markers such as Cystatin C which is not affected by the same non-renal variables as sCr the estimated GFR is more accurate than when sCr is measured alone[62]. Many current renal biomarkers are monitored in urine as opposed to serum, however in DGF patients there may be little to no urine produced[63] and therefore samples to test may be scarce. Consequently it is more practical to monitor biomarkers in serum in this context.

New biomarkers of graft function in a renal-transplant context are still needed to improve post-operative management of patients and increase graft longevity. Research into alternative biomarkers with greater specificity and sensitivity than sCr and earlier potential to diagnose renal function decline is ongoing. Recent reviews suggest there are emerging biomarkers in several areas of research (i.e. proteomics, genomics etc) that have some utility in a renal-transplant context[64-66]. A number of promising combinations of biomarkers have been proposed to detect AKI earlier, including a NGAL and CYSC plasma panel and a urinary NGAL, interleukin-18 and kidney injury molecule-1 panel[64]. Despite not being directly applicable in this renal transplant context, that AKI can affect outcomes post-transplant makes these biomarkers potentially valuable. Detection of early AKI gives clinicians advanced notice of the potential development of DGF. Some emerging biomarkers may have the ability to predict DGF pre-transplant in either donor blood/urine or in the preservation fluid used when harvesting the kidney. Mitochondrial DNA and complement C5a have both been measured in the donor fluids while NGAL in preservation fluid has been shown to correlate with severity of IRI[66] and urinary lactate and pyroglutamate levels may have the ability to predict whether DGF would be prolonged[67].

In Leeds, our group[68] identified a novel biomarker, aminoacylase-1, in serum that has the potential to identify the development of delayed graft function earlier and may provide some long-term prognostic information for renal-transplant patients.

With multiple biomarkers offering complementary information that in combination have the potential to better inform a clinician about a patient it would be prohibitively inefficient to measure each individually, both in time and physical resources such as consumables and patient samples. Multiplexing allows multiple analytes to be measured simultaneously on a single assay using a single small sample thereby reducing the time, labour and reagents used to generate significantly more informative data. Biomarkers have previously been investigated via multiplexing in a renal context[69] which led to the discovery of biomarkers for other biological processes such as apoptosis, cell repair and inflammation being associated with declining GFR.

A number of multiplexing platforms exist and in this study, through our collaboration with Randox Laboratories, we used the Randox Evidence Investigator with their biochip array technology.

1.4.1 Randox Evidence Investigator

The Randox Evidence Investigator is a semi-automated benchtop immunoanalyser utilising an innovative biochip technology allowing multiple tests to be performed simultaneously from a single patient sample which maximises efficiency of both time and resources. Figure 1.1 is an example image of the analyser used in this research but there are other models with a higher throughput and full automation suitable for a clinical chemistry environment.

Figure 1.1 Image of the Randox Evidence Investigator as used in this research (www.randox.com/evidence-investigator/)

Each biochip consists of a 9x9mm ceramic square with an array of discrete test regions (DTR) in which up to 49[70] different analytes can be detected in a single sample, along with some internal quality control sites. Antibodies to the biomarkers of interest are bound to the biochip within the discrete test regions, capturing the biomarker on the biochip. Captured biomarkers are detected following addition of conjugated analyte-specific antibodies in a sandwich immunoassay with subsequent chemiluminescent reaction when incubated with a detection solution and inserted into the analyser. Within the analyser is a Charge-Coupled Device (CCD) camera[70] that detects and records the light signal output from all the DTRs simultaneously. The CCD camera consists of a collection of light sensitive diodes that convert the chemiluminescent signal into electrical charge with brighter light signals generating a greater electrical charge. The electrical charge allows the quantification of the light emission which when compared to validated biomarker-specific calibration curves allows the biomarker concentrations in a sample to be determined.

There are 9 biochips per carrier (Figure 1.2) and 6 carriers can be analysed in a single assay which, when accounting for calibration and control samples, equates to 19 samples in duplicate per assay or 39 samples in singlicate.

Figure 1.2 Randox 9-well biochip carrier. Image taken and edited from the operator manual[71].

1.4.2 Biomarker multiplexing

Having already been linked to DGF diagnostic and prognostic potential, ACY-1 was the primary biomarker of interest in this research. Additional renal-related biomarkers already exist on Randox biochip arrays although initially developed in the context of CKD. Some of these have potential biological relevance in acute kidney injury (AKI) and renal transplantation and were therefore explored. In addition a biochip assay for YKL-40 was included and a commercially available research grade ELISA for Midkine.

A brief overview of the biomarkers investigated is as follows with statistically relevant biomarkers later discussed in further detail in Chapter 4.3:

Aminoacylase-1 is mammalian enzyme that catalyses the hydrolysis of N-acylated aminoacids and is involved in amino acid recycling and the urea cycle. It has been shown to have early predictive potential for the onset of DGF post renal transplant and provide some long-term prognostic information for patients that do develop DGF[68]. ACY-1 is the primary biomarker of interest in the BioPAsSPoRT project.

Chitinase-3-like protein 1, also known as YKL-40, is produced by a variety of cells, including macrophages and neutrophils[72], and plays a significant role in the activation of the innate immune system, tissue injury, repair and remodelling and inflammation[73]. Various studies have shown that increased urinary YKL-40 implies a higher level of damage to the kidney and therefore an increased likelihood of developing DGF[74] but also may be indicative of improved recipient outcomes, particularly among “high risk”

recipients[75] as an elevated presence of YKL-40 implies a greater degree of repair activity occurring. Conversely though this study associates the greater degree of repair activity with a reduced incidence of DGF. Although elevated in AKI, it is not clear whether YKL-40 is increased in response to the damage caused by the injury or through mediating the repair.

Epidermal growth factor is a small protein involved in such mechanisms as cell growth and differentiation[76]. Urinary EGF (uEGF) has already been shown to be a promising prognostic biomarker in CKD progression[77] and uEGF creatinine ratio has been suggested to identify patients at high risk of graft failure after transplant[78].

Fatty acid binding protein 1 is one of the primary forms of FABP1 expressed by the kidneys[79], being found in the proximal tubules, and is responsible for fatty acid transport and regulation[80]. Increased urinary levels have been found in response to kidney injury, including IRI[81] and it has been suggested as a biomarker of early kidney injury warranting further investigation[80]. FABP1 is also known as FABP-liver and is therefore not a renal-specific biomarker. Urinary FABP1 has previously been used as a biomarker of renal injury after transplant but as utility is limited in DGF as patients often produce little to no urine[81] it is worth assessing in serum.

D-Dimer is a by-product of clot breakdown and is elevated in patients with renal impairment and correlates with serum creatinine levels in CKD patients[82].

Macrophage inflammatory protein 1-alpha is a cytokine involved in the recruitment of macrophages in response to AKI and the development of inflammation in CKD progression[83]. High levels of MIP1-α have been noted in pre-transplant end-stage kidney disease patients with a reduction post-transplant[84] and therefore merit exploration for this renal-transplant panel.

Soluble TNF receptors 1 and 2 have roles in inflammatory responses and have been associated with the progression of CKD and increased mortality irrespective of other clinical variables such as age and gender[85,86]. sTNFR1 has also been linked to the development of DGF and increased death-censored graft loss[87] while elevated sTNFR2 is associated with quicker rates of kidney function decline[88].

Interleukin-8 is a chemotactic cytokine released by macrophages to recruit other immune cells to the site of infection. It is raised in patients requiring dialysis and has been shown to be a strong predictor of mortality in end-stage kidney disease patients[89].

C3A Desarginine is a form of C3a which mediates inflammatory responses and has a role in complement activation. It has been observed to be elevated by ischaemic reperfusion injury and in chronic kidney disease[90].

C-Reactive protein is an acute phase protein elevated in many diseases with inflammation as a pathology such as CKD. CRP is also associated with a faster decline of kidney function[88] and correlated with an increase in serum creatinine and reduced eGFR[91].

Neutrophil gelatinase-associated lipocalin is a protein involved in the innate immune response to bacterial infection. Within research it is also a well-established biomarker for acute kidney injury and chronic kidney disease[92] although not used in routine practice. Urinary NGAL has been associated with the development of DGF but serum/plasma NGAL appears to be a better predictor with a higher sensitivity and specificity[93]. As with other urinary biomarkers, it is subject to the limitations of potential anuria in DGF.

Cystatin C is an emerging biomarker of renal function that has some advantages over sCr. It is a small protein filtered by the kidneys and not released back into circulation[94] meaning as renal function is impaired CYSC levels begin to rise. As CYSC has been shown to correlate well with creatinine clearance[95] and is not affected by external factors such as age, gender and acute illness, it has potential to replace sCr as the routine renal function biomarker. It has been suggested that CYSC could be substituted into formulae for eGFR that are currently based on sCr with relative ease[96]. Furthermore there is some evidence to suggest that using kinetic eGFR calculations with plasma CYSC may be able to diagnose DGF early after renal transplant[97].

Midkine plays a role in inflammation and is rarely elevated in adults unless an inflammatory pathology is ongoing[98] such as CKD. It been associated with ischaemia/reperfusion damage[99] in the kidneys and therefore may have some value in the context of this research.

1.5 Aims and Objectives

The BioPAsSPoRT project, with funding from Innovate UK and with Randox Laboratories as the industrial lead, aims to provide a multiplex biochip array that will provide renal transplant clinicians with detailed information about a

patient from a single assay that allows personalised monitoring and stratified care post-renal transplant.

There is currently no definitive guidance as to when to dialyse a patient post-renal transplant and is therefore left at the discretion of the clinician, leading to subjectivity in the decision to dialyse. The primary objective of BioPAsSPoRT is to evaluate a panel of biomarkers including ACY-1 in terms of clinical utility in DGF. Ultimately this would allow the development, manufacture and independent validation of a multiplex biochip assay for the Randox Evidence series of analysers that will allow clinicians to predict and diagnose early complications that arise following renal transplant, allowing them to intervene earlier and adjust the fluid balance and potentially avoid unnecessary dialysis. It will also provide prognostic information allowing the stratification of patients for follow-up and to improve patient outcomes and quality of life in the longer term. Importantly the overall study also includes a health economic analysis performed concurrently by the University of Leeds.

The specific aims of this MSc project which forms part of the BioPAsSPoRT study are to:

- Measure an initial selection of biomarkers including ACY-1 and some panels relating to renal function already available on Randox biochip assays in an initial cohort of ~200 renal transplant patient samples from the Leeds Multidisciplinary Research Tissue Bank.
- Assess combinations of biomarkers to optimise diagnostic and prognostic utility in the context of DGF and select an optimised combination for inclusion on a novel Renal Transplant biochip assay (to be manufactured by Randox).
- Independently validate the prototype assay in a multicentre prospectively collected patient cohort of ~300 patients.

The ultimate aim is that following longer-term clinical testing it will be commercially available for use in hospitals, creating a standard criteria to combat post renal-transplant complications.

2 Materials and Methods

2.1 Study Design

Randox Laboratories successfully transferred the research grade ELISA from the original ACY-1[68] study onto their biochip platform to be used in this research consisting of two phases.

Phase 1 explored the new ACY-1 biochip in addition to a selection of biomarkers already commercially available on Randox biochips. Following statistical analysis, the optimal combination of biomarkers was selected to comprise be included as a novel Renal Transplant array (RTA).

Phase 2 was primarily to validate the performance of the prototype RTA biochip in an independent cohort of samples procured. Additional substudies into stability of samples under various conditions were also conducted.

A graphical representation of the study progression from initial discovery of ACY-1 to the end of the current project can be found in Figure 2.1.

All studies were approved by Leeds East Research Ethics Committee (ref: 20-YH-0103 279008).

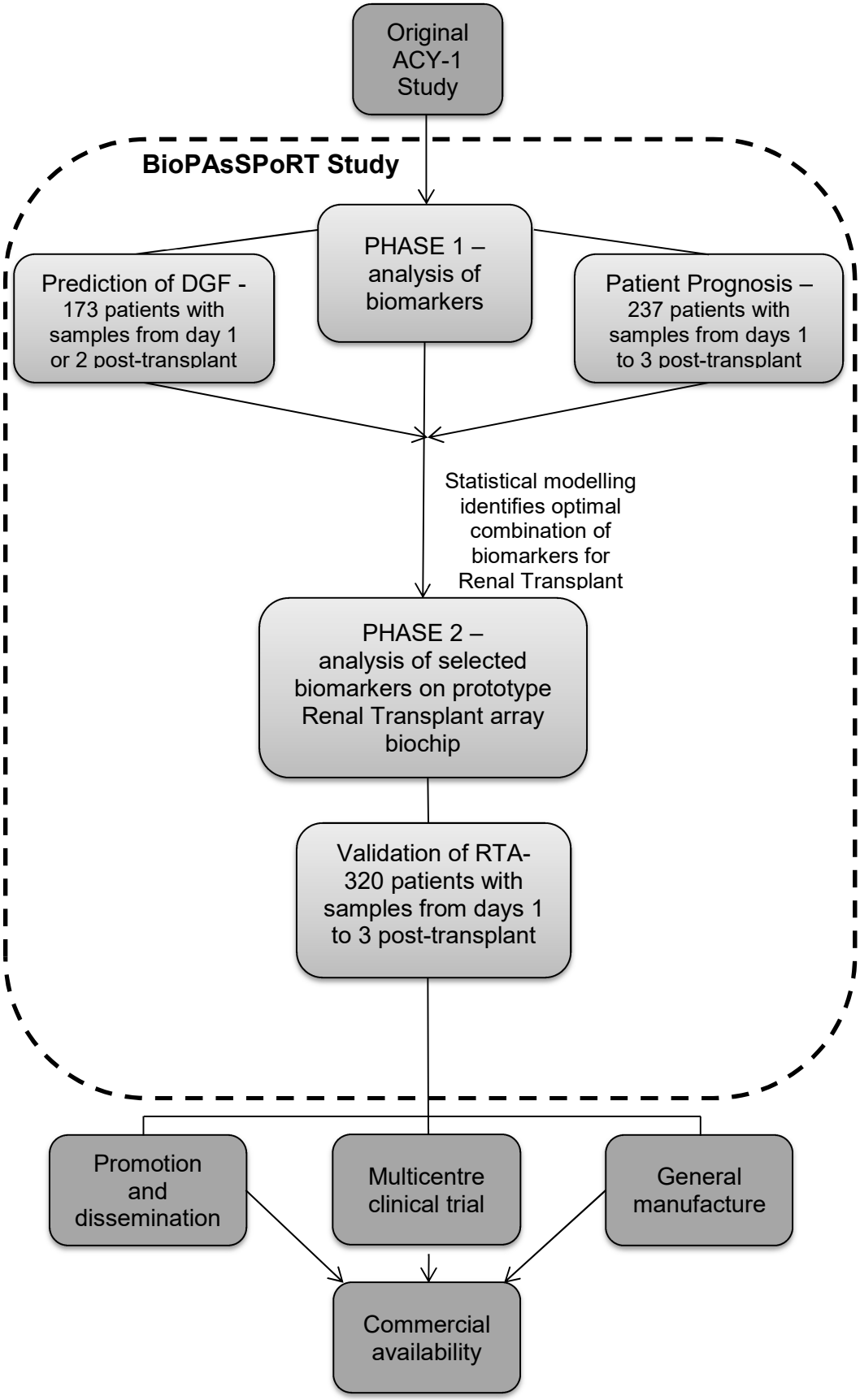


Figure 2.1 Study design overview including the precursor research to this study by Welberry-Smith et al[68] and potential next steps once the study concludes

2.1.1 Patient samples and clinical data acquisition

Venous blood samples were collected from renal-transplant patients during standard blood draws in red-top, non-gel Greiner serum clot-activator tubes and allowed to clot for 45 minutes (max 2 hours) before being centrifuged at 2000g for 10 minutes at 20°C. Serum was aliquoted into 500µl Eppendorf tubes and stored at -80°C until analysis. Processing was the same for all samples and relevant clinical data was also collected for all patients on standardised forms with informed consent.

The 379 serum samples used in Phase 1 were from 241 patients previously recruited and with samples already stored in the Leeds Multidisciplinary RTB. Samples were collected pre-operatively and at least three times per week post-transplant with samples collected on days 1 to 3 post-transplant being used in this study.

Phase 2 samples consisted of 724 serum samples (days 1 to 3 post-transplant) sourced from the Leeds NIHR Research Tissue Bank from a total of 320 patients treated at 10 clinical sites across the UK [100].

Prior to analysis samples were allowed to thaw completely at room temperature before being mixed gently with a vortex. All samples were randomised and analysed blind in duplicate.

2.1.2 Data generation

All sample analysis was performed in duplicate irrespective of platform used and samples were predominantly analysed in Leeds using the Randox Evidence Investigator. Midkine was also selected by Randox as a potential biomarker of interest, but as there is currently a lack in the appropriate biochip technology for Midkine, analysis was performed at Randox using a commercially available Midkine ELISA (cat: MKELISA – Cellmid, Australia).

In Phase 1, the commercially available biochip arrays CKD1 and CKD2 were used together with prototype ACY-1 biochips. A smaller pilot study was also carried out to explore the potential use of YKL-40 biochips as described in Chapter 4.2.1. An overview of the biomarkers investigated and the array used, along with practical assay parameters such as dilution and range is presented in Table 2.1.

Table 2.1 Phase 1 biomarker and assay details outlining the analytes, the abbreviations by which they are referred and the array that they are present on. Relevant assay parameters are also included. Range is the range of the standard curve without any dilution after reconstitution of lyophilised standards. Dilution is the baseline dilution of samples as per the analytical protocol, not accounting for any further dilution that may be necessary.

Biomarker	Abbreviation	Array	Range	Dilution	LLOQ
Aminoacylase-1	ACY-1	ACY-1	0-1000 ng/mL	200x	4.74 ng/mL
Chitinase-3-like protein 1	YKL-40	YKL-40	0-714 ng/mL	4x	54.5 ng/mL
Epidermal growth factor	EGF	CKD1	0-400 pg/mL	neat	6.94 pg/mL
Fatty acid binding protein 1	FABP1	CKD1	0-400 ng/mL	neat	0.29 ng/mL
D-Dimer	DDIMER	CKD1	0-1000 ng/mL	neat	3.47 ng/mL
Macrophage inflammatory protein 1-alpha	MIP1A	CKD1	0-1500 pg/mL	neat	3.73 pg/mL
Soluble TNF receptor 1	sTNFR1	CKD1	0-10 ng/mL	neat	0.04 ng/mL
Soluble TNF receptor 2	sTNFR2	CKD1	0-20 ng/mL	neat	0.07 ng/mL
Interleukin-8	IL-8	CKD1	0-600 pg/mL	neat	3.00 pg/mL
C3DesArg	C3DA	CKD2	0-100 µg/mL	200x	0.35 µg/mL
C-Reactive protein	CRP	CKD2	0-60µg/mL	200x	0.47 µg/mL
Neutrophil gelatinase-associated lipocalin	NGAL	CKD2	0-2 µg/mL	200x	0.01 µg/mL
Cystatin C	CYSC	CKD2	0-5 µg/mL	200x	0.03 µg/mL

In Phase 2, samples were analysed on a prototype Renal Transplant Array biochip (Table 2.2) with biomarkers showing the most promising utility.

Table 2.2 Phase 2 biomarker and assay details outlining analytes and abbreviations by which they are referred. Relevant assay parameters are also included. Range is the range of the standard curve without any dilution. Dilution is the baseline dilution of samples as per the analytical protocol, not accounting for any further dilution that may be necessary.

Biomarker	Abbreviation	Array	Range	Dilution	LLOQ
Aminoacylase-1	ACY-1	RTA	0-500 ng/mL	50x	1.79 ng/mL
Soluble TNF receptor 1	sTNFR1	RTA	0-80 ng/mL	50x	0.51 ng/mL
Chitinase-3-like protein 1	YKL-40	RTA	0-3000 ng/mL	50x	54.5 ng/mL
Cystatin C	CYSC	RTA	0-10 µg/mL	50x	0.025 µg/mL

2.1.3 Phase 1

The primary aim of Phase 1 was to determine an optimal combination of biomarkers for the renal-transplant panel to maximise early diagnostic/predictive and prognostic utility with regards to patients returning to dialysis, either in the first week after transplant as with DGF or permanently following loss of graft function. In order to do this selected biomarkers were evaluated both individually and in combination to assess the potential to diagnose DGF early and the long term prediction of patients returning to dialysis.

A sample from day 1 or 2 post-transplant from each patient was analysed to generate results for early prediction analysis and a sample from days 1-3 post-transplant was used for the long-term prognostic analysis.

Samples were analysed using a prototype ACY-1 biochip alongside an additional ten biomarkers on the already commercially available Randox biochip arrays CKD1 and CKD2, with Midkine being analysed at Randox Laboratories. Based on a review of the literature, YKL-40 was also investigated in a small subset of 67 samples as a pilot study to determine its worth as a potential biomarker for the RTA.

Initially a direct comparison between the results generated on the original ELISA[68] and the new biochip assay was performed (Chapter 3.1) to ensure the validity of the previous results and ensure the integrity of the samples given the difference in storage time.

Due to the limited range of the CYSC biochip and a lack of evidence of dilutional linearity, the Leeds CYSC data generated originally on the Seimens platform[68] was used for the statistical analysis instead of the newly generated Evidence Investigator results. This is further discussed as a technical development in Chapter 3.5.

2.1.4 Phase 2

Phase 2 samples were analysed using the prototype RTA manufactured by Randox Laboratories following Phase 1 statistical analysis and accounting for the technical issues encountered such as widening the assay range. The RTA comprised of ACY-1, sTNFR1, YKL-40 and CYSC. Again, due to there being no available midkine biochip, midkine was analysed at Randox by ELISA. All samples were randomised and analysed blind in duplicate.

Of the 320 patients sampled in Phase 2, 152 patients had day 1 samples and 46 had day 2 samples to be used in the diagnostic model (n=198) with a further 60 patients with day 3 samples to add for the prognostic model (n=258). Measurements were generated for all biomarkers in all samples with the exception of two midkine measurements in one day 1 sample and one day 3 sample.

A number of technical sub-studies were also performed to assess sample integrity over time, compare plasma vs serum and sample stability under various processing conditions. These are discussed in depth in Chapter 3.2.

2.1.5 Study clinical endpoints

The endpoints of the study used for the assessment of the biomarkers were the development of DGF, defined as the need to return to dialysis within the first week after transplant in the absence of hyperkalaemia, and failure of the graft at a later timepoint necessitating a permanent return to dialysis.

2.2 Analysis of samples by Randox Evidence Investigator

2.2.1 Equipment and reagents used

- Randox Evidence Investigator (IE-17472) and PC with EvInvest software (Version 2.1.0). Randox, Crumlin
- Evidence Investigator assay kit (Array kit catalogue and lot information described in Table 2.3). Randox, Crumlin
- Control sample kits appropriate for the relevant assay (Control sample kit catalogue and lot information described in
- Table 2.4). Randox, Crumlin
- Randox Thermoshaker. Randox, Crumlin
- Roller shaker
- Assortment of micropipettes and tips with volumes to cover a range of 5µl-1000µl
- Brand Handystep repeater pipette and 5ml/2.5ml syringe tips
- Deionised water
- 500ml Wash bottle
- Plastic microtubes
- 10ml brown glass bottle

Table 2.3 Consumable kit information

Array	Catalogue	Lot
Aminoacylase-1	EV4321	0396
Chronic Kidney Disease 1	EV4189	0392/0362
Chronic Kidney Disease 2	EV4171	0147/0148
CTK CST 42 (YKL-40)	EV4253	0976
Renal Transplant Array (prototype)	EV4321	2220

Table 2.4 Control kit information.

Array	Catalogue	Lot
Aminoacylase-1	EV4322	0341
Chronic Kidney Disease 1	EV4188	0516
Chronic Kidney Disease 2	EV4170	0119
CTK CST 42 (YKL-40)	EV4254	0977
Renal Transplant Array (prototype)	EV4322	2219

Reagents provided in the assay kit are as follows:

- 9 concentrations of lyophilised calibration to generate a standard curve
- 6 foil packaged chip trays
- Wash buffer concentrate
- Assay diluent
- Sample diluent (if samples are to be diluted)
- Conjugate
- Luminol-EV840
- Peroxide

Reagents provided in the controls kit are as follows:

- Three sets of 3 levels of control sample for a total of 9 QC samples per kit (low, mid and high for each biomarker)

Prior to the commencement of an assay a plate plan was populated with the locations that the calibration samples, controls and serum samples were to be loaded in.

2.2.2 Preparation of standard curve and serum samples

The lyophilised standard curve and quality control samples were reconstituted in 1mL deionised water and rolled on a roller shaker for between 30 and 120 minutes.

Samples were diluted as per array-specific instructions in an appropriate volume microtube using kit-provided sample diluent. Table 2.5 outlines the necessary sample preparation for each array.

If further dilution was required to bring the sample into the working range of the assay this was done in a separate microtube following the initial dilution.

Table 2.5 Sample preparation Array kits for the Randox Evidence Investigator, the standard dilution factor applied to all samples run on the array and the sample and diluent volume required to meet that dilution requirement.

Array	Dilution Factor	Sample volume (µl)	Diluent volume (µl)
Aminoacylase-1	200	5	995
Chronic Kidney Disease 1	Neat	100	0
Chronic Kidney Disease 2	200	5	995
CTK CST 42	4	60	180

2.2.3 Sample loading

Once samples had been prepared up to six Biochip carriers were inserted into the thermoshaker carrier tray before 200µl assay diluent was added to each of the wells using a Brand Handystep repeater pipette. 100µl calibrator, standard or sample as per the pre-prepared plate plan was loaded before the biochips were incubated in a preheated thermoshaker at 37°C for 60 minutes at 370 rpm.

2.2.4 Wash procedure

16mL wash buffer concentrate provided was decanted into a wash bottle and topped up to 500ml with deionized water to form a working wash buffer.

After the sample incubation period, the contents of the carrier were tipped into the sink before filling each well with wash buffer. This action constitutes one “wash”. The full wash procedure comprises of two “quick washes” where the wash buffer is not incubated in the wells for any time but immediately tipped away and four “soaks” where the buffer is incubated at room temperature on the biochips with agitation for two minutes before being tipped out. At the end of a full wash cycle, the biochips were removed one at a time and blotted firmly onto lint free absorbent towel before being replaced in the biochip carrier.

2.2.5 Detection

Following washing 300µl of kit provided assay-specific HRP-conjugate antibody mix was added to each well using the Handystep repeater pipette and the carrier was incubated in the thermoshaker at 37°C for 60 minutes at 370rpm

2.2.6 Reading and data generation

Fifteen minutes before the end of the conjugation incubation, signal reagent was prepared by adding kit provided Luminol-EV840 and peroxide in a 1:1 ratio in a container protected from light. A wash procedure was performed following conjugate incubation, omitting the blotting step. The biochip trays remained in wash buffer until ready to be imaged by the Evidence Investigator. One at a time the biochip tray was removed from the carrier before being blotted firmly on lint free towel and 250µl read buffer was added to each well using the repeater pipette. The biochip tray was then protected from light using tin foil and incubated at room temperature without agitation for two minutes before being inserted into the Investigator for imaging. When all biochips had been read, data generated was available for export and interpretation.

3 Technical Investigations

A number of investigations into technical aspects relating to the assay or samples were conducted to ensure that accurate and reliable data was being produced during the course of the research. Studies into biomarker stability under different sample conditions were also performed in order to provide information about the validity of data which is essential prior to the biochip being developed commercially.

3.1 ACY-1 platform comparison

In order to determine the comparability between the prototype ACY-1 biochip and the research-grade ACY-1 ELISA used in the original study the data generated from the original cohort of 379 samples on both platforms was assessed for correlation. Only 378 were able to be compared as in one case insufficient sample remained to be analysed on the biochip.

Overall an excellent correlation was apparent with a Spearman's rank correlation coefficient of 0.93. The ELISA concentrations were approximately double those of the Investigator due to differences in standardisation and only a small number of samples appeared to be outliers.

These outlier samples were reanalysed on the Evidence Investigator biochip, by ELISA using a standard curve generated from Randox calibration material and by ELISA using a standard curve generated using recombinant ACY-1 (Biotechne) similar (but not identical) to that used in the original research study[68]. Additionally, five samples were included that had had good agreement between the platforms to use as positive control samples for reference. When reassayed it was shown that the biochip results were correct and the data from the original ELISA assay for those 5 samples was inaccurate, possibly due to pipetting errors or similar.

Once this data was removed from the scatterplots the correlation slightly improved further to 0.95. Figure 3.1 is a scatter plot used to visually compare the data once the outlying data was removed. Due to a difference in standardisation materials used on the different platforms data are not directly comparable so a line of equality was not expected however there is definite evidence of a linear relationship between the results.

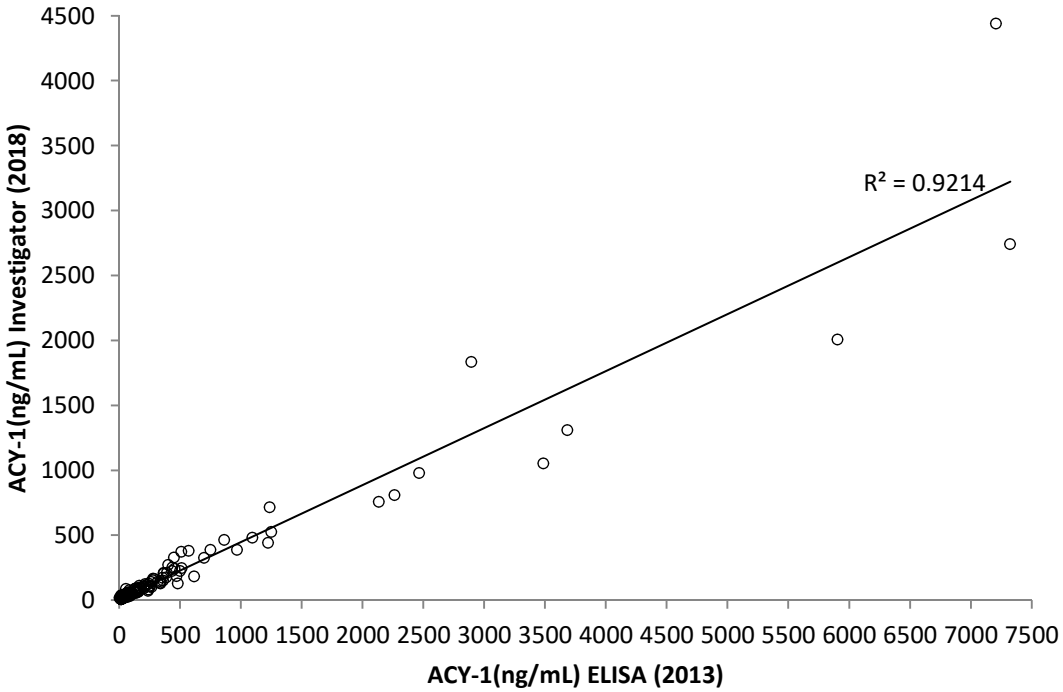


Figure 3.1 Scatterplot comparing final ACY-1 results generated on the original research-grade ELISA with those generated using the Randox Evidence Investigator.

3.2 Technical sub-studies

A number of investigations into technical aspects that need to be taken into consideration have been or are currently being conducted. Samples were processed in accordance with the general SOP for serum acquisition but it is important to investigate whether there is any impact to samples or data generated if external factors cause deviations from the usual processing method.

Sub-studies include investigating various pre-analytical conditions and their impact on the measured biomarkers (Table 3.1), stability of analytes after having undergone multiple cycles of freezing and thawing (Table 3.8) and the effect of long-term storage on sample stability (Table 3.10).

3.2.1 Pre-analytical sample processing conditions and impact on analytes

The conditions investigated in this stability study are used in the context of an inpatient renal transplant unit where the delay to samples reaching the lab are likely to be minimal as opposed to an outpatient or GP setting where it could be more than 24 hours before processing could occur.

Gel separator and non-gel tubes are being investigated as both are routinely used in hospitals and potentially going to be used going forward despite only non-gel tubes being used to collect the study samples already obtained. Although serum has been used it is important to determine if plasma would yield the same results.

Irrespective of storage or delay to processing the samples were processed by centrifuge at 2000g for 10 minutes at 20^{o}C. All sample processing was undertaken by the Sample Processing Team.

Figure 3.2 shows a flow chart of sub-study design while Table 3.1 outlines the technical aspects of the pre-analytical condition studies, blood collection tubes used and storage and analysis conditions for these studies. Blood collection tube types, volumes and catalogue numbers can be found in Table 3.2. The intention for each study was to recruit up to 20 patients, although these figures were not always met.

Values above the upper limit of quantification were assigned the upper limit of quantification for the purpose of statistical analysis, however no widely accepted consensus exists on how to handle such data. One sample had at least one YKL-40 replicate >ULOQ and 3 samples had at least one CYSC replicate >ULOQ.

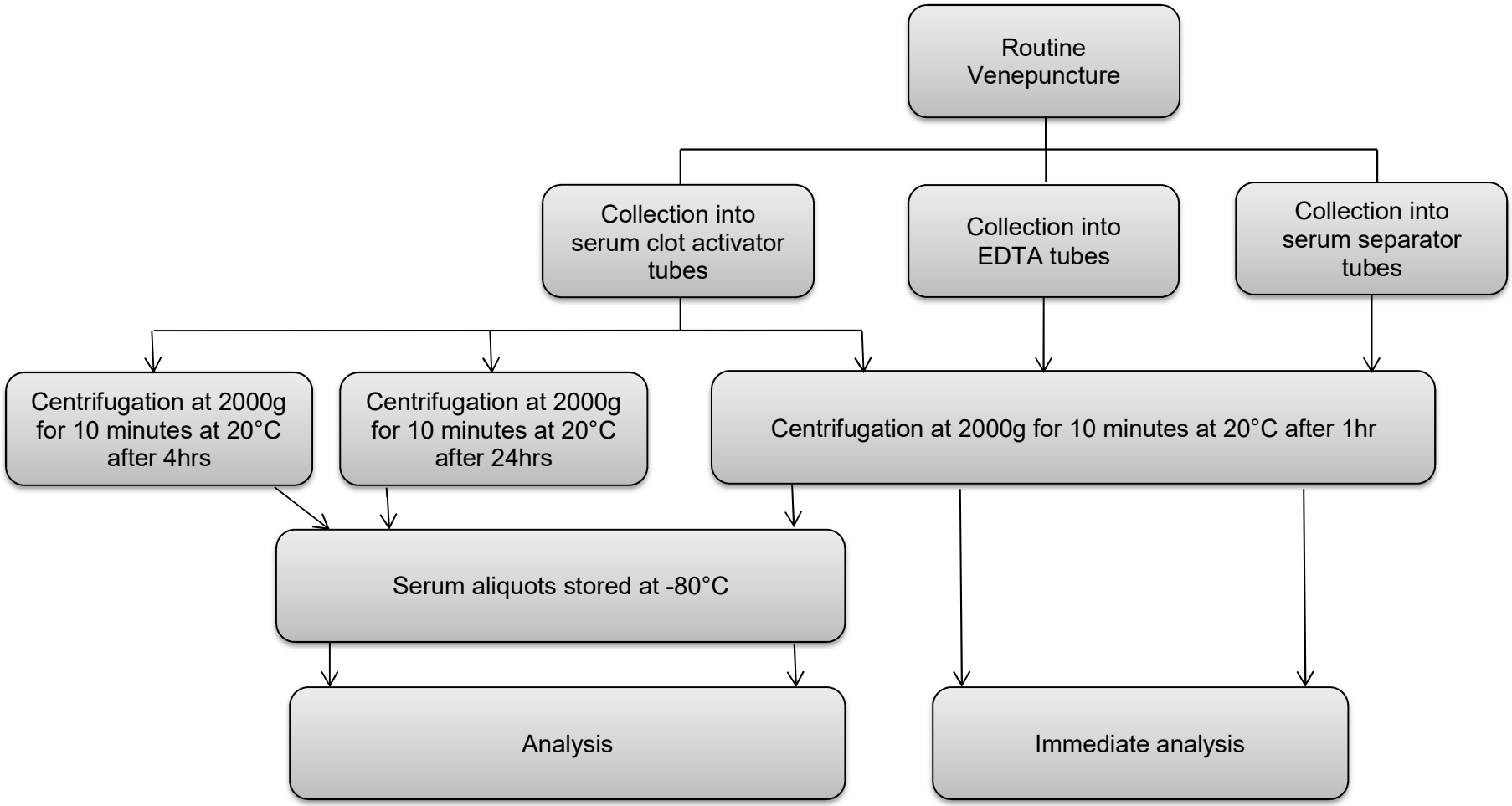


Figure 3.2 Sub-study design flowchart

Table 3.1 Sub-studies investigating pre-analytical condition impacts on analytes

Investigation (results)	Blood tubes	Count	Processing	Serum/Plasma Storage	Analysis
Gel separator vs non-gel tube (Table 3.3)	Greiner Z serum clot activator	1x 6mL	Process within 1-2 hours post venepuncture. Centrifuge at 2000g for 10 minutes at 20°C	4°C until analysis for a maximum of 2 hours	Immediate analysis
	Greiner Z serum separator clot activator	1x 3.5mL			
Serum vs Plasma (Table 3.4)	Greiner Z serum clot activator	1x 6mL			
	EDTA (plasma)	1x 4mL			
Fresh vs Frozen (Table 3.5)	Greiner Z serum clot activator	1x 8mL		4°C until analysis for a maximum of 2 hours	Immediate analysis
				-80°C	After storage for a minimum of 24 hours
Analysis Delay (Table 3.6)	Greiner Z serum clot activator	1x 6mL		4°C until analysis for a maximum of 2 hours	Immediate analysis
	Greiner Z serum separator clot activator	1x 3.5mL		4°C	After storage for 24 hours
	EDTA (plasma)	1x 4mL			
Processing delay (Table 3.7)	Greiner Z serum clot activator	1x 6mL	1 hour post venepuncture	-80°C	After storage for a minimum of 24 hours
		1x 4mL	4 hours post venepuncture		
		1x 4mL	24 hours post venepuncture		
	Greiner Z serum separator clot activator	1x 3.5mL	1 hour post venepuncture		
		1x 3.5mL	24 hours post venepuncture		

Table 3.2 Blood tube catalogue numbers

Blood tube	Volume (mL)	Catalogue number
Greiner Z serum clot activator	4	454204
	6	456092
	8	455701
Greiner K2EDTA	4	454209
Greiner Z serum separator clot activator	3.5	454071

3.2.1.1 Gel-separator vs non-gel separator tubes

Wilcoxon's signed rank test was performed on matched pairs of serum samples (Table 3.3) and there was no statistical significance in the differences between ACY-1 or sTNFR1 concentrations irrespective of whether a separator or non-separator blood collection tube was used and there is very little variability in the ranges of concentrations. YKL-40 had a p-value of significance and when plotted against each other the results for gel and non-gel readings correlate strongly (R^2=0.99) suggesting that the difference is proportional. CYSC is also significantly different but does not correlate as well (R^2=0.62). Correlation did improve when the outlying point was removed (R^2=0.73) however the significant difference between the data sets remains.

Table 3.3 Gel vs non-gel serum collection tubes study Wilcoxon's matched pairs results comparing two matched samples per patient in duplicate. All samples analysed fresh. Values are median (range). (n=26 samples from 13 patients).

Investigation	Biomarker	Non-gel (n=26)	Gel (n=26)	p value
Gel vs non-gel tubes	ACY-1 (ng/mL)	6.10 (2.97-114.81)	6.31 (3.21-109.26)	0.303
	sTNFR1 (ng/mL)	31.78 (14.80-71.85)	31.64 (16.62-65.37)	0.291
	YKL-40 (ng/mL)	361.87 (153.30-861.70)	362.57 (149.61-829.03)	0.018
	CYSC (µg/mL)	8.06 (5.56-11.68)	7.38 (2.98-11.13)	0.010

3.2.1.2 Serum vs plasma

Wilcoxon's signed rank test was performed on matched pairs of serum and plasma samples (Table 3.4). The concentrations of sTNFR1, YKL-40 and CYSC appear not to be affected significantly irrespective of whether serum or plasma is analysed. ACY-1 on the other hand shows a significant difference in serum or plasma although correlation is very strong between the data sets (R^2=1.00) suggesting they are proportionally related.

Table 3.4 Serum vs plasma study Wilcoxon's matched pairs results comparing two matched samples per patient in duplicate. All samples analysed fresh. Values are median (range). (n=26 samples from 13 patients).

Investigation	Biomarker	Serum (n=26)	Plasma (n=26)	p value
Serum vs plasma	ACY-1 (ng/mL)	6.10 (2.97-114.81)	8.33 (3.65-160.04)	<0.001
	sTNFR1 (ng/mL)	31.78 (14.80-71.85)	31.92 (14.44-71.85)	0.525
	YKL-40 (ng/mL)	361.87 (153.30-861.70)	345.02 (155.26-909.19)	0.150
	CYSC (µg/mL)	8.06 (5.56-11.68)	7.65 (4.98-10.86)	0.245

3.2.1.3 Fresh vs Frozen

Wilcoxon's signed rank test was performed on matched pairs of serum samples (Table 3.5). One sample was analysed immediately after processing and the other after at least 24 hours but not more than 14 days stored at -80°C. There was no statistically significant difference observed in any of the biomarkers between serum samples analysed fresh and samples stored at -80°C for between 24 hours and 14 days suggesting that biomarkers are not compromised by short term storage.

Table 3.5 Fresh vs frozen stability study Wilcoxon's matched pairs results comparing two matched serum samples per patient in duplicate, one of which was analysed immediately and the other after being stored for at least 24hrs at -80°C. Values are median (range). (n=25 samples from 13 patients) One replicate was lost due to analysis error.

Investigation	Biomarker	Fresh (n=25)	Frozen (n=25)	p value
Fresh vs Frozen	ACY-1 (ng/mL)	5.79 (2.97-114.81)	6.75 (2.71-36.36)	0.874
	sTNFR1 (ng/mL)	31.38 (14.80-71.85)	30.31 (14.53-64.02)	0.127
	YKL-40 (ng/mL)	328.17 (153.30-861.70)	319.77 (88.19-917.67)	>0.999
	CYSC (µg/mL)	8.02 (5.56-11.68)	8.15 (1.93-10.62)	0.367

3.2.1.4 Analysis delay: Serum and plasma

Wilcoxon's signed rank test was performed on matched pairs of serum and plasma samples (Table 3.6) that had been processed immediately and then one sample in the pair analysed immediately and the other stored at 4°C for 24 hours before analysis. For the serum produced from non-gel serum separator collection tubes, sTNFR1 had no significant difference in the measurements with delay in analysis, however ACY-1, YKL-40 and CYSC all had very significant p-values. The median values for the delayed analysis samples is lower than those analysed immediately, as is the range of concentrations observed. Conversely only ACY-1 showed a significant difference with delay in analysis using serum collected in gel separator blood tubes.

By contrast there was no statistically significant difference in any of the measurements from plasma samples. Nevertheless, in all cases (gel tube, non-gel tube and EDTA plasma) the median and ranges for all biomarkers shows lower concentrations even if the p-value was insignificant suggesting that it is important that samples not be left standing for long periods

Table 3.6 Analysis delay stability study Wilcoxon's matched pairs results comparing two matched samples per patient in duplicate, one of which was processed and analysed immediately and the other after being processed immediately but the serum/plasma stored for 24hrs at 4°C before analysis. Values are median (range). (n=26 samples from 13 patients).

Investigation	Biomarker	Immediate analysis (n=26)	Delayed analysis (n=26)	p value
Analysis delay: Serum clot activator tube	ACY-1 (ng/mL)	6.10 (2.97-114.81)	5.11 (1.93-101.62)	0.001
	sTNFR1 (ng/mL)	31.78 (14.80-71.85)	27.74 (15.41-62.80)	0.171
	YKL-40 (ng/mL)	361.87 (153.30-861.70)	341.04 (131.39-824.31)	0.002
	CYSC (µg/mL)	8.06 (5.56-11.68)	7.05 (4.44-9.20)	<0.001
Analysis delay: Serum separator clot activator tube	ACY-1 (ng/mL)	6.31 (3.21-109.26)	5.11 (2.09-103.71)	0.006
	sTNFR1 (ng/mL)	31.64 (16.62-65.37)	30.66 (14.60-62.91)	0.099
	YKL-40 (ng/mL)	362.57 (149.61-829.03)	316.75 (118.37-932.22)	0.960
	CYSC (µg/mL)	7.38 (2.98-11.13)	7.15 (4.54-10.13)	0.159
Analysis delay: EDTA plasma tube	ACY-1 (ng/mL)	8.33 (3.65-160.04)	8.22 (3.26-181.19)	0.134
	sTNFR1 (ng/mL)	31.92 (14.44-71.85)	28.99 (16.90-62.18)	0.096
	YKL-40 (ng/mL)	345.02 (155.26-909.19)	327.38 (129.51-944.07)	0.960
	CYSC (µg/mL)	7.65 (4.98-10.86)	7.62 (5.24-8.85)	0.601

3.2.1.5 Processing delay

Wilcoxon's signed rank test was performed on matched pairs of serum samples (Table 3.7), of which one sample in each pair was processed and analysed immediately and the other that was processed after a 24hr delay at room temperature and then analysed. With the exception of sTNFR1 in samples from non-gel tubes and CYSC in samples from gel tubes, none of the biomarkers had significant p-values, suggesting that a delay of up to 24hrs between venepuncture and processing has little to no effect on biomarker integrity. Even in the statistically significant CYSC there is very little difference in the observed concentrations (median and range).

Table 3.7 Processing delay stability study Wilcoxon's matched pairs results comparing two matched serum samples per patient in duplicate, one of which was processed within one hour and analysed immediately and the other stored for 24hr at room temperature after venepuncture before processing and analysed immediately. Values are median (range). (n=20 samples from 10 patients).

Investigation	Biomarker	No processing delay (n=20)	Processing delay (n=20)	p value
Processing delay: Serum clot activator tube	ACY-1 (ng/mL)	11.95 (2.69-75.90)	12.09 (4.43-83.20)	0.143
	sTNFR1 (ng/mL)	28.92 (15.76-54.55)	30.89 (19.78-59.25)	0.036
	YKL-40 (ng/mL)	987.28 (116.45-3276.0)	915.65 (133.97-3276.0)	0.541
	CYSC (µg/mL)	6.95 (3.44-11.87)	7.15 (3.57-11.87)	0.140
Processing delay: Serum separator clot activator tube	ACY-1 (ng/mL)	12.07 (2.47-90.20)	10.42 (2.63-90.40)	0.860
	sTNFR1 (ng/mL)	34.72 (15.60-49.09)	34.35 (19.24-56.60)	0.169
	YKL-40 (ng/mL)	1041.38 (91.80-3276.0)	1091.24 (105.85-3276.0)	0.284
	CYSC (µg/mL)	6.31 (3.16-11.87)	6.24 (4.11-11.87)	0.030

3.2.2 Freeze-thaw stability

The effect of multiple cycles of freezing and thawing was investigated in case a previously used sample may be needed for repeat analysis. Samples were thawed at room temperature for one hour before being returned to the freezer for a minimum of one hour. This was repeated for the appropriate number of cycles before analysis took place. This was only carried out for serum as this was the main fluid proposed for the eventual routine measurement of patient samples.

Figure 3.3 shows a flow chart of sub-study design while Table 3.8 outlines the technical aspects of the pre-analytical condition studies, blood collection tubes used and storage and analysis conditions.

Table 3.8 Freeze-thaw stability study investigating the impact of multiple freeze-thaw cycles on samples

Investigation	Blood tubes	Processing	Storage	Analysis
Freeze-Thaw stability	Greiner Z serum clot activator	Process within 1-2 hours post venepuncture. Centrifuge at 2000g for 10 minutes at 20°C	-20°C -80°C	Aliquots analysed having undergone either 1 or 4 freeze thaw cycles.

Blood tubes used in this study were 1x 6mL Greiner serum clot activator (cat:456092).

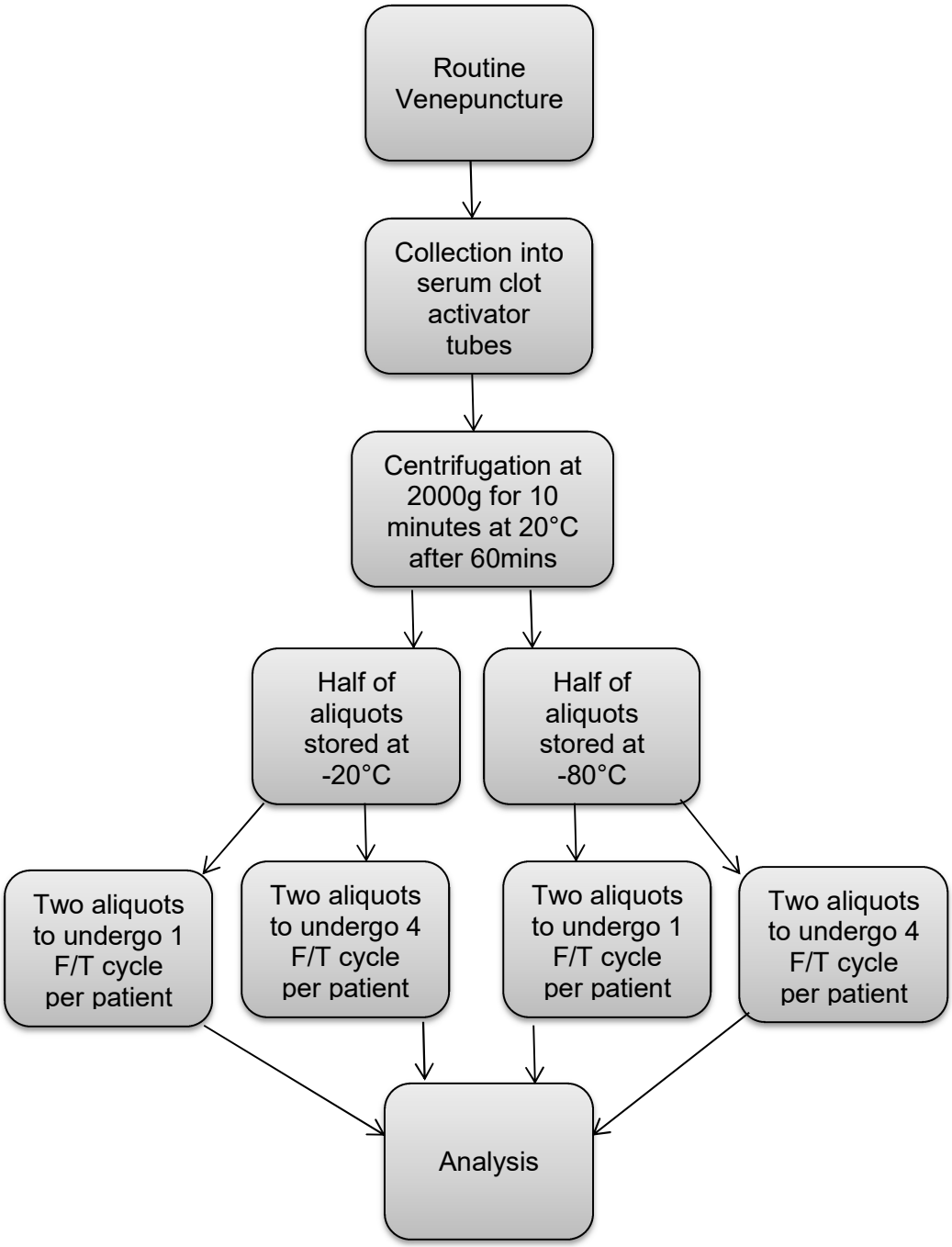


Figure 3.3 Freeze/thaw stability study flowchart investigating biomarker stability at -20 and -80 after undergoing 1 and 4 freeze/thaw cycles

Matched pair analysis was undertaken using a two-tailed Wilcoxon's signed rank test (Table 3.9). The p values for all biomarkers did not indicate significant difference between samples that have undergone one freeze-thaw cycle and samples that have undergone 4, although there is a slight reduction in measured concentrations (median and range) seen in the samples after undergoing 4 freeze-thaw cycles. This does imply that there is potential to rerun the same samples if necessary in practice although it would not be recommended.

Table 3.9 Freeze-thaw stability study Wilcoxon's matched pairs results comparing two matched serum samples per patient in duplicate, one of which underwent 1 freeze-thaw cycle and the other 4 freeze-thaw cycles. Values are median (range). All (n=20 samples from 10 patients).

Investigation	Biomarker	1 freeze-thaw cycle (n=20)	4 freeze-thaw cycles (n=20)	p value
1 vs 4 freeze-thaw cycles -20°C	ACY-1 (ng/mL)	9.99 (2.97-107.26)	9.35 (3.04-96.39)	0.330
	sTNFR1 (ng/mL)	31.26 (15.79-53.76)	31.20 (18.65-54.37)	0.4091
	YKL-40 (ng/mL)	1029.83 (118.05-3276.0)	919.93 (83.68-3276.0)	>0.999
	CYSC (µg/mL)	6.86 (3.95-11.87)	6.05 (3.62-11.87)	0.678
1 vs 4 freeze-thaw cycles -80°C	ACY-1 (ng/mL)	9.71 (3.16-85.78)	11.17 (2.70-65.84)	0.841
	sTNFR1 (ng/mL)	35.95 (17.93-54.85)	32.17 (16.37-62.16)	0.6215
	YKL-40 (ng/mL)	942.67 (126.95-3276.0)	925.11 (37.06-3006.30)	0.475
	CYSC (µg/mL)	7.27 (4.46-11.87)	6.46 (3.29-11.87)	0.058

3.2.3 Long term stability

To assess the stability of the sample during frozen storage serum samples were stored for specified periods of time prior to analysis. The blood was collected in Greiner Z serum clot activator tubes and processed within 2 hours of venepuncture before being stored at -80^{o}C for 2 weeks, 2 months, 6 months and 12 months.

Table 3.10 Long term stability study investigating the impact that long term storage at -80^{o}C has on the integrity of samples

Investigation	Blood tubes	Processing	Storage	Analysis
Stability over time	Greiner Z serum clot activator	Process within 1-2 hours post venepuncture. Centrifuge at 2000g for 10 minutes at 20^{o}C	-80^{o}C	2 weeks (+/- 3 days)
				2 months (+/- 1 wk)
				6 months (+/- 2 wks)
				12 months (+/-1mth)

Due to the time frame of the long term stability and the onset of the COVID-19 pandemic this study element has been delayed and results are not yet available for inclusion.

3.3 Standard curve considerations

When investigating the dilutional linearity of biomarkers it was necessary to assess the quality of the nine-point standard curves in order to be confident in the result from a diluted sample. Ideally the standard curve would be linear with the difference in light intensity reading as a clear and consistent difference in biomarker concentration. ACY-1 had a standard curve of this description and an example can be seen in Figure 3.4. FABP1, IL-8 and CYSC had curves of a similar quality and DDIMER, MIP1A, sTNFR2 and NGAL showed slight but not significant plateauing at the top and bottom of the curves.

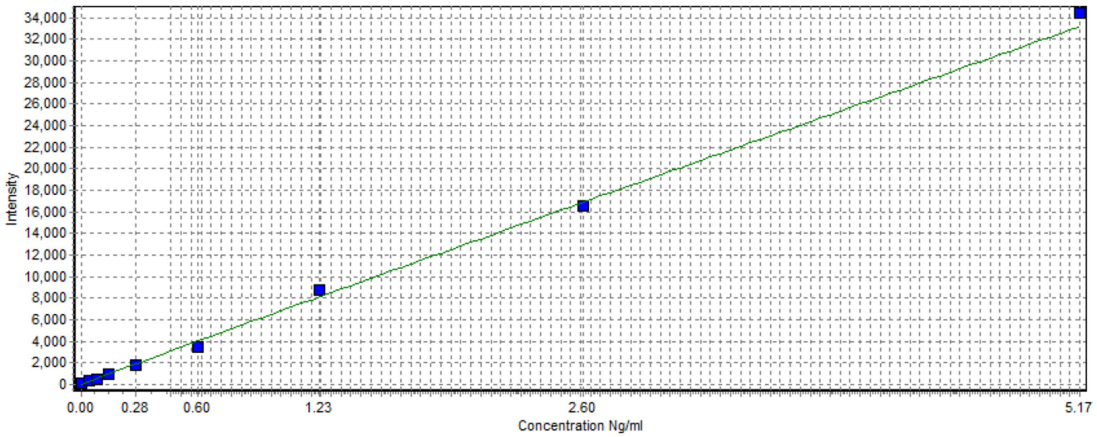


Figure 3.4 Representative example of ACY-1 standard curve generated during an ACY-1 assay using the kit-provided calibrators (Cat:EV4321, Lot:0396). Line of best fit is straight and consistent across the whole curve with difference in light intensity making a clear difference in read concentration. There are no areas of flattening where the reading would be questionable.

Some markers had less ideal curves with areas of plateauing at the top and bottom ends where a small difference in light intensity could make a large difference in detected biomarker concentration. Samples with concentrations in these areas of the curves tended to have significantly increased %CVs compared to samples that fell in the middle, straighter regions of the curves. EGF, sTNFR1, C3DA, CRP and YKL-40 all had notable flattened regions in their calibration curves. Figure 3.5 shows a visual representation of a YLK-40 calibration curve for as an example showing the less than ideal shape of the curve.

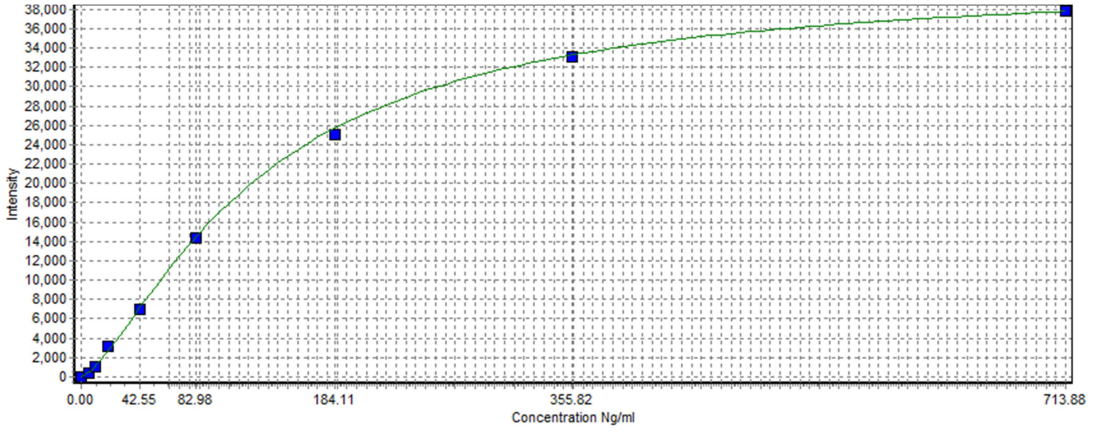


Figure 3.5 Representative example of YKL-40 calibration curve generated during a YKL-40 assay using the kit-provided calibrators (Cat:EV4253, Lot:0976). Flattening can be seen beginning at Cal7, above which a smaller difference in light intensity could be read as a large difference in biomarker concentration leading to poor %CVs between replicates.

3.4 Precision Profiles

The percent coefficient of variation (%CV) was calculated as follows:

$$CV\% = \left(\frac{Standard\ deviation\ between\ replicates}{Mean\ between\ replicates}\right) x\ 100$$

The %CV for all replicates for all samples was collated and plotted against the biomarker concentration to give a visual representation of precision across the standard curves.

Precision profiles visualise the variability of different regions of the standard curve and at what concentrations results may be less precise due to the shape of the standard curve. For example, many high %CVs in an area where a small difference in light signal would make a large difference in concentration reading (i.e. in a more horizontal portion of the standard curve) could be attributed to the assay performance as opposed to a single high %CV in a usually reliable concentration range would be analyst error. It is also can be affected by how close to the assay limits of quantification the concentration is. Data in these regions are more difficult to compare with confidence if they occur in these flatter regions of the curve.

In the vast majority of instances the CVs increased at the bottom end of the standard curve as expected. Based on this samples were reanalysed when the replicate CV was >20% except in the bottom 10% of the standard curve where higher CVs were expected and therefore a tolerance of >20% was recognised and data treated with caution in this lower region close to the lower limit of quantification (LLOQ). Figure 3.6 is a precision profile for ACY-1 measurements that demonstrates the clustering of higher %CVs towards the lower end of the assay range.

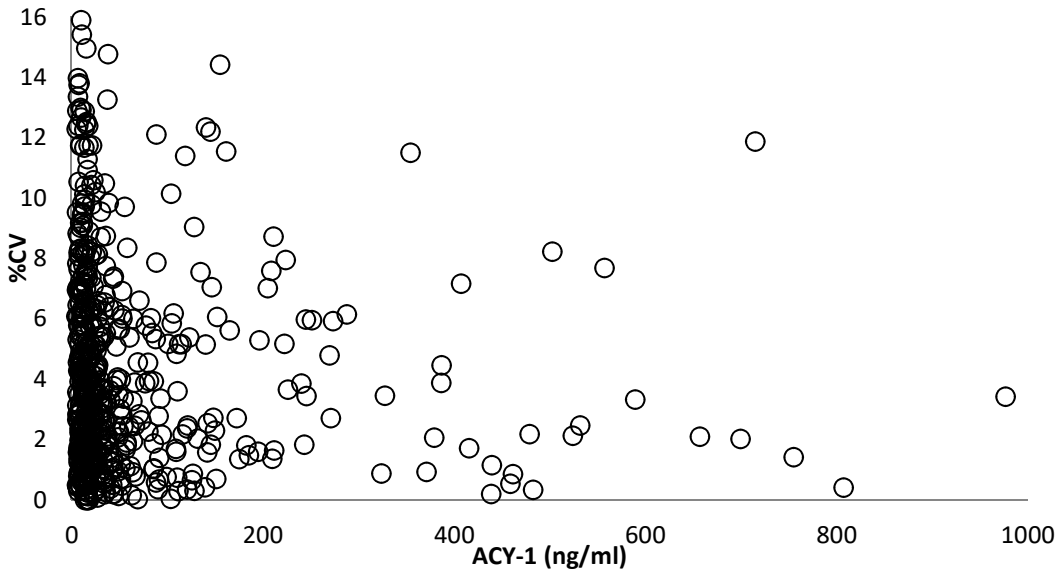


Figure 3.6 ACY-1 precision profile. %CV is particularly low for ACY-1, however it can be seen that higher %CVs tend to cluster towards the bottom end of the assay range.

Ideally samples would be diluted in order that the out of range biomarker would read in the middle of the standard curve where it is most robust to minimise %CVs however due to variability in the concentrations of the biomarkers this was not always possible.

3.5 **Dilutional Linearity**

A significant number of samples had a biomarker concentration above the range of the assay when analysed at the normal recommended dilution. This was particularly seen in the case of sTNFR1, CRP and CYSC (Table 3.11). Therefore an investigation was necessary into whether biomarkers would dilute linearly compared to the standard curve in order to be able to repeat analysis to obtain a reliable value.

Table 3.11 Samples returning a value out of assay range either above the upper limit of quantification (>ULOQ) or below the lower limit of quantification (<LLOQ)

Assay	Biomarker	>ULOQ	<LLOQ
ACY-1	ACY-1	7	4
CKD1	EGF	0	18
	FABP1	1	0
	DDIMER	2	0
	MIP1A	0	147
	sTNFR1	477	0
	sTNFR2	0	5
	IL-8	4	18
CKD2	C3DA	0	3
	CRP	132	8
	NGAL	0	0
	CYSC	318	0

Randox laboratories performed dilutional linearity comparisons. In the case of CKD1, sTNFR1 diluted in a linear fashion but the remainder did not appear to. However this had no particular impact as sTNFR1 was the biomarker mostly out of range.

As the samples for the CKD1 assay were analysed neat as standard practice, the dilutions for samples out of range were performed using the diluent supplied for the routine dilution of the CKD2 kit (ref: EV4171) as it was assumed that the diluent would be suitable for any assay. However Randox replaced this with a new more suitably optimised diluent (ref: EV4171B) specifically formulated for use in the CKD1 kit. Additionally, a new conjugate mix omitting the sTNFR1 detection reagents (lot: 15150EV) was supplied for use when generating data for all other biomarkers assayed neat.

Following these developments each sample was assayed twice in parallel: once diluted using the specially formulated CKD1 diluent and original kit conjugate to generate data for sTNFR1 (ignoring data for all other biomarkers that were not validated for use with the new diluent) and once using neat samples and sTNFR1-omitted conjugate for all other biomarkers.

The comparability between the CKD1 and CKD2 diluents was still assessed to determine whether it was necessary to repeat samples previously diluted with CKD2 diluent. They were found to be comparable enough that it was not necessary to repeat.

For CKD2 all but CYSC were linear up to a dilution of 600x (a further threefold dilution from standard). CYSC did not dilute reliably and an issue with the higher end of the calibration curve meant that correlation between Leeds CYSC (as originally measured on the Siemens platform in the Leeds Hospital NHS routine clinical chemistry labs) and Evidence Investigator CYSC data was poor. This resulted in the decision to use the CYSC data generated in the Leeds lab. For data that did not exceed the limits of quantification of the Randox assay the correlation between Evidence Investigator CYSC and Leeds Siemens CYSC was good (Figure 3.7) with a Spearman's rank correlation coefficient of 0.90.

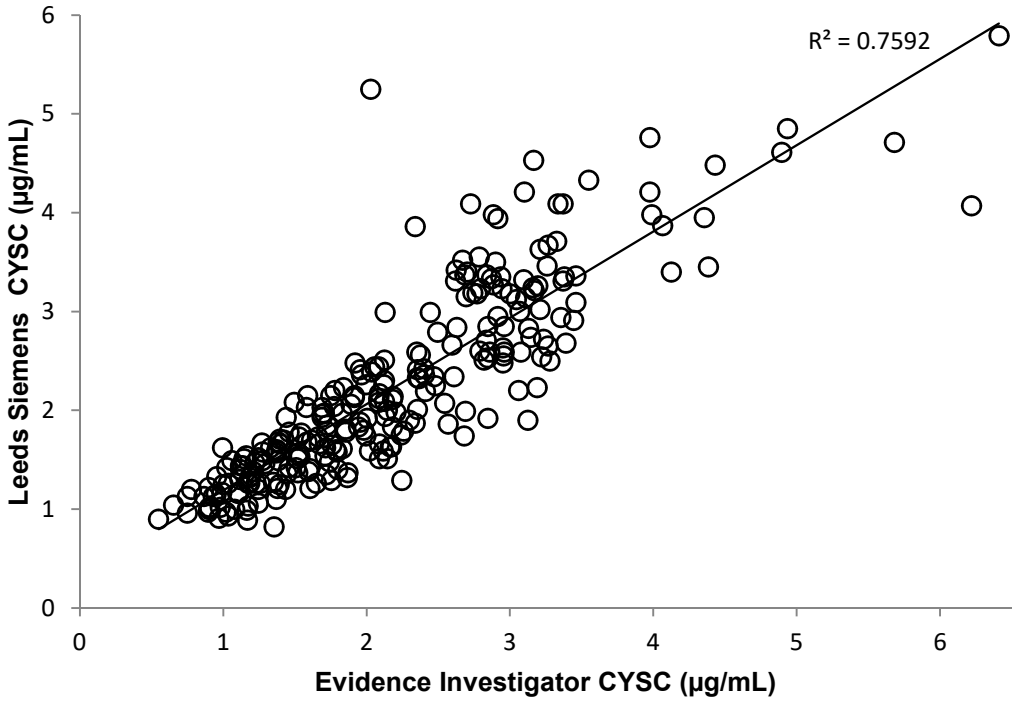


Figure 3.7 Correlation between Leeds Siemens CYSC data and Randox Evidence Investigator CYSC with >ULOQ results removed. Spearman's rank correlation coefficient = 0.90

3.6 **Assay Precision**

Randox assigned an acceptable CV limit of 20% between replicates across the analytes but in practice we found 15% to be acceptable for ACY-1. This target was achieved on first analysis for the majority of samples with the exception of 323 results from the 7116 total results (593 samples x 12 analytes). In order to conserve sample reserves, repeat analysis for unacceptable CVs was only performed if the sample was taken at either day 1 or day 3 post-transplant and therefore included in the primary statistical analysis (day 2 samples had been included in the original analysis to examine kinetics in a small number of patients). Repeat analysis was also not considered in cases where the concentration of the biomarker was in the lowest 10% of the assay's working range as precision profiles showed an increase in %CV in this area. The number of replicates with a CV of >20% that remained in the final data set can be found in Table 3.12 and the majority of these fell into the bottom 10% of their respective standard curve.

A large proportion of the poor CV numbers came from MIP1A results and once the alternative conjugate omitting sTNFR1 conjugation antibody (Chapter 3.5) was used the prevalence of high MIP1A CVs was reduced. The exact reason for this is unknown, however it is theorised that samples containing sTNFR1 concentrations >ULOQ were generating light signals so great that there was some "spill over" from the sTNFR1 discrete test region that was being picked up in the MIP1A region causing inconsistent readings and large %CVs. By removing sTNFR1 from the conjugate and therefore the sTNFR1 signal, the theorised spill over did not occur and MIP1A results had much less variance.

Table 3.12 Intra-replicate precision in final data set (n=593 for each analyte)

Array	Analyte	Samples with an intra-replicate CV >20% (>15% for ACY-1)
ACY-1	ACY-1	2
CKD1	EGF	2
	FABP1	29
	DDIMER	1
	MIP1A	66
	sTNFR1	18
	sTNFR2	28
	IL-8	15
CKD2	C3DA	15
	CRP	43
	NGAL	9
	CYSC	21

By calculating the %CV from the control samples across all runs it was possible to determine inter-assay precision. In the majority of cases it was under 10%.

Table 3.13 Inter-assay precision calculated from the control samples across all runs

Assay	Biomarker (units)	Assay range	Mean concentration			%CV			Number of assays
			Control 1	Control 2	Control 3	Control 1	Control 2	Control 3	
ACY-1	ACY-1 (ng/mL)	0-1000	17.41	73.32	364.19	11.89	10.63	13.14	39
CKD1	EGF (pg/mL)	0-400	38.38	146.26	261.00	9.03	9.07	13.24	73
	FABP1 (ng/mL)	0-400	9.97	47.30	193.07	8.33	8.11	7.80	
	DDIMER (ng/mL)	0-1000	30.63	129.12	472.49	8.50	5.94	5.61	
	MIP1A (pg/mL)	0-1500	28.24	118.03	511.56	5.56	4.52	7.61	
	sTNFR1 (ng/mL)	0-10	0.49	1.79	4.65	9.06	6.75	9.27	
	sTNFR2 (ng/mL)	0-20	0.56	1.94	7.84	5.16	5.97	6.43	
	IL-8 (pg/mL)	0-600	22.40	98.76	427.94	6.87	5.15	12.44	
CKD2	C3DA (µg/mL)	0-100	5.43	10.77	18.75	10.82	12.90	13.50	54
	CRP (µg/mL)	0-60	1.82	3.53	10.61	5.16	9.01	12.37	
	NGAL (µg/mL)	0-2	0.12	0.45	1.01	10.20	9.69	13.14	
	CYSC (µg/mL)	0-5	0.15	0.47	1.72	8.30	6.40	8.57	
YKL-40	YKL-40 (ng/mL)	0-714	10.07	20.78	83.89	15.40	11.38	17.97	4

4 Phase 1

4.1 Background

In Phase 1 a panel of serum biomarkers was evaluated in an initial cohort (379 samples from 241 renal-transplant patients) to determine their use in both the prediction of DGF post-transplant and long-term prognostic outcome of patients. Using the data generated in Phase 1 a combination of biomarkers was selected to comprise the final Renal Transplant Array which will go on to be independently validated using a separate cohort of samples in Phase 2.

For each patient and based on the original study[68] a day 1 or 2 post-transplant sample was used for the early diagnostic statistical analysis (n=173 patients) and a day 1-3 post-transplant result was used for long-term prognostic analysis (n=237 patients).

4.2 Results

4.2.1 Descriptive statistics

The clinical and demographic characteristics of patients and biomarker concentrations are summarised in Table 4.1. with a detailed breakdown of biomarker concentrations with respect to various clinical and demographic characteristics available in (Table 8.1) of the appendix.

The median age of patients who develop DGF is slightly higher than those who do not (52.6yrs and 44.3yrs respectively). Of the overall cohort the majority of patients are male (66.5%) and around the same proportion of each gender develop DGF with 31.0% of women and 29.5% of men developing DGF. The small sample size of ethnicities makes it impossible to determine with certainty whether there are any differences in rates of DGF by ethnicity.

The majority of graft donors are deceased with 22.5% of grafts coming from live donors compared to 77.5% from deceased. Of these deceased donors DBD grafts account for 64.2% to 35.8% DCD grafts.

HLA mismatch is fairly evenly split irrespective of the degree of mismatch or whether the patients developed DGF or not.

Both warm and cold ischaemic times are greater in DGF patients than non-DGF but the duration of WIT is significantly longer in DGF patients comparative to CIT, with CIT being only 3 minutes longer in DGF patients but 10 minutes in WIT.

As expected, creatinine reduction ratio is much greater in non-DGF patients than DGF patients. Conversely the opposite trend is seen in all significant biomarker levels with all statistically significant biomarkers (ACY-1, FABP1, sTNFR1&2, CRP, NGAL, CYSC and SCr) having an increased measurement in DGF patients, sometimes drastically so such as ACY-1 (Non-DGF median: 24.54ng/mL, range: 6.5 - 657.2ng/mL; DGF median: 128.69ng/mL, range: 11.1 - 4437.7 ng/mL).

The Shapiro-Wilk test for normality was used to assess the distribution of each biomarker and all returned a p value of below 0.001 indicating that they are not normally distributed and as a result non-parametric statistical analyses were used when assessing the data.

Table 4.1 Clinical and demographic variables for Biopassport cohort 1 broken down by DGF status. For continuous variables values are displayed as "median (range)" with a p-value from the Wilcoxon rank-sum test. For categorical variables values displayed represent n(%) with a p-value from chi-square test. Creatinine reduction ratio defined as: (Day0 Scr-Day7 SCr)/Day0 sCr[101]. (Statistical analysis undertaken by Michelle Wilson)

Characteristic	Level	Non-DGF n=121 samples	DGF n=52 samples	p-value
Age at transplant (years)	-	44.3 (16.6-78.4)	52.6 (23-75.7)	<0.01
Gender	Male	81 (66.9)	34 (65.4)	0.32
	Female	40 (33.1)	18 (34.6)	
Ethnicity	White	96 (79.3)	37 (71.2)	0.26
	Asian	12 (9.9)	10 (19.2)	
	Black	2 (1.7)	2 (3.8)	
	Other	11 (9.1)	3 (5.8)	
Transplant type	LD	37 (30.6)	2 (3.8)	<0.01
	DBD	62 (51.2)	24 (46.2)	
	DCD	22 (18.2)	26 (50)	
Total HLA mismatch	0-2	71 (58.7)	22 (42.3)	0.06
	3+	50 (41.3)	30 (57.7)	
CIT (hr:min)	-	14:4 (0:57-30:6)	15:9 (1:57-26:0)	<0.01
WIT (mins)	-	30.0 (7.80-84.0)	40.0 (10.8-110)	<0.01
CRR	-	0.78 (-0.17-0.90)	0.06 (-0.76-0.48)	<0.01
ACY-1 (ng/ml)	-	24.54 (6.5, 657.2)	128.69 (11.1, 4437.7)	<0.001
EGF (pg/mL)	-	87.18 (12.1, 222.5)	92.47 (23.7, 167.6)	0.83
FABP1 (ng/mL)	-	4.31 (0.55, 35.11)	10.52 (2.99, 33.10)	<0.001
D-Dimer (ng/mL)	-	77.66 (13.5, 4663.1)	77.20 (12.7, 913.8)	0.894
MIP1A (pg/mL)	-	4.12 (3.7, 301.6)	6.42 (3.7, 56.8)	0.005
sTNFR1 (ng/mL)	-	13.85 (3.2, 61.0)	28.68 (14.9, 60.7)	<0.001
sTNFR2 (ng/mL)	-	0.90 (0.07, 6.19)	1.63 (0.56, 3.09)	<0.001
IL-8 (pg/mL)	-	11.3 (3.0, 5488.0)	15.6 (4.8, 1010.3)	0.002
C3DA (µg/mL)	-	3.9 (0.5, 35.8)	3.9 (1.4, 11.1)	0.739
CRP (µg/mL)	-	21.5 (1.6, 219.8)	45.7 (2.1, 177.8)	<0.001
NGAL (µg/mL)	-	0.37 (0.12, 1.25)	0.68 (0.32, 1.50)	<0.001
CYSC (µg/mL)	-	2.36 (0.55, 5.68)	3.60 (2.34, 6.22)	<0.001
Original Leeds ACY-1 (ng/mL)	-	35.5 (15.6, 864.1)	259.7 (15.6, 7324.5)	<0.001
Leeds CysC (mg/L)	-	2.25 (0.90, 5.49)	4.25 (2.56, 5.66)	<0.001
SCr (µmol/L)	-	504.0 (160.0, 1059.0)	683.0 (358.0, 1374.0)	<0.001
Midkine (pg/mL)		168.0 (90.0-19005.0)	3104.5 (719.0-24785.0)	<0.001

Table 8.1 in the appendix shows that IL-8, C3DA and sCr differed significantly by gender, with IL-8 being higher in female patients and C3DA and sCr being higher in males. The majority of biomarkers had no significant differences with regards to ethnicity, the exceptions being CRP and sCr which were both significantly higher in black patients compared to any other ethnicity. Almost all biomarkers were affected by ischaemic time but CIT appears to be more significant than WIT, while HLA mismatch appears to have little association with the biomarker levels.

Figure 4.1 to Figure 4.6 shows the spread of data for ACY-1, sTNFR1, sTNFR2, CYSC, YKL-40 and Midkine as these ultimately came out as significant in the statistical analysis. The median biomarker concentration is marked for each patient group and in all cases are noticeably higher in DGF compared to non-DGF patients.

The same biomarkers had a significant difference based on transplant type, with DCD donors usually having much higher biomarker readings, followed by DBD and then LD with the lowest. ACY-1 had previously been shown to be significantly higher in DCD grafts[68] and the same outcome was seen in this case.

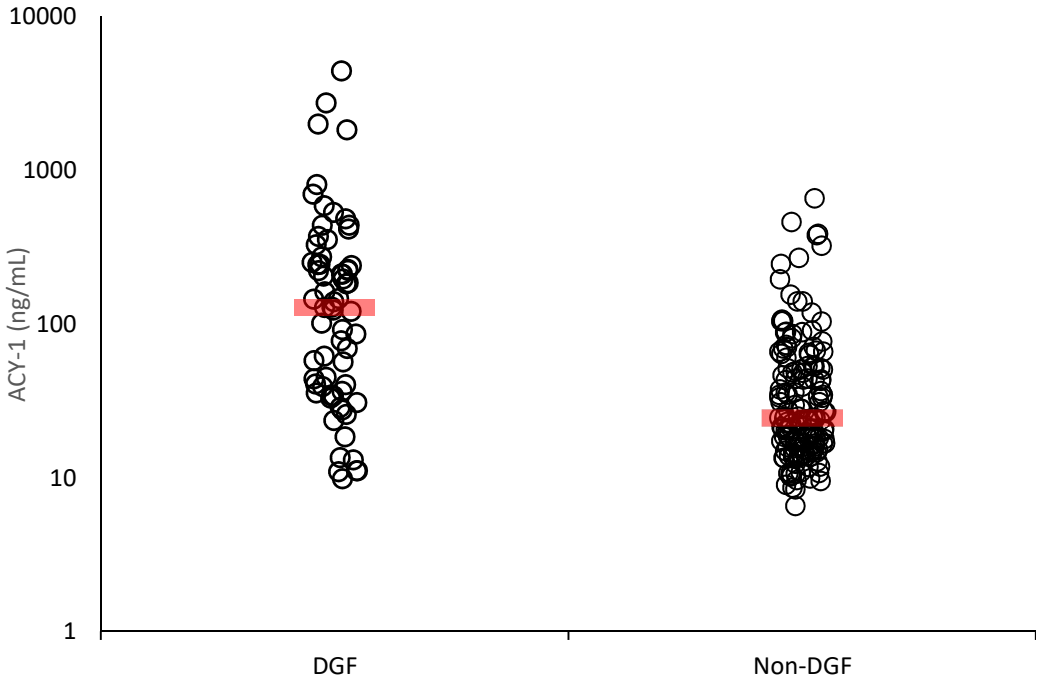


Figure 4.1 Dot plot visualising the spread of ACY-1 concentrations in day 1-3 samples divided by DGF status. Red line indicates median value (128.69ng/mL and 24.32ng/mL respectively).

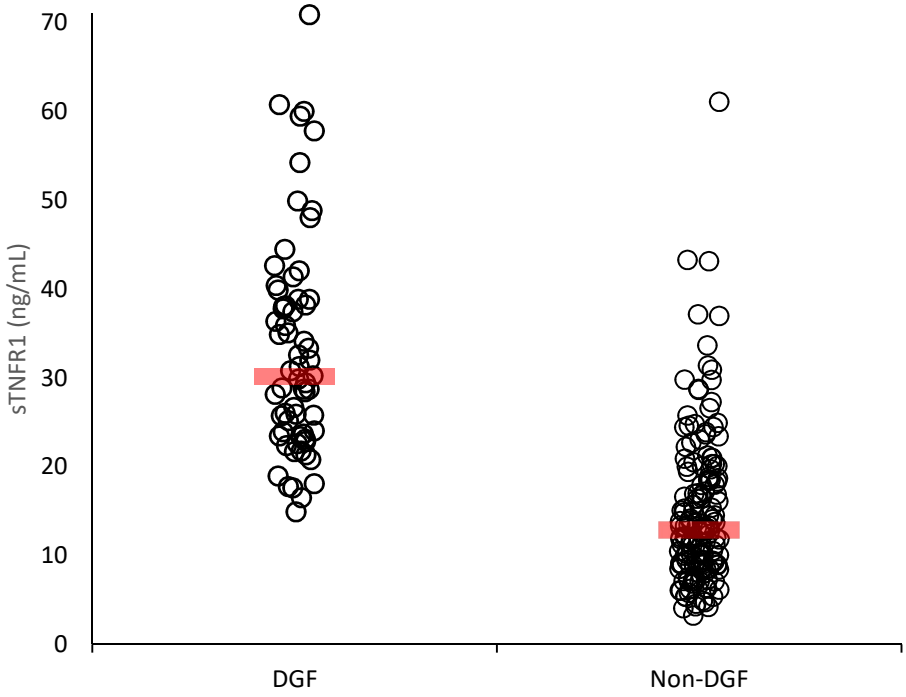


Figure 4.2 Dot plot visualising the spread of sTNFR1 concentrations in day 1-3 samples divided by DGF **status.** Red line indicates median value (30.21ng/mL and 12.89ng/mL respectively).

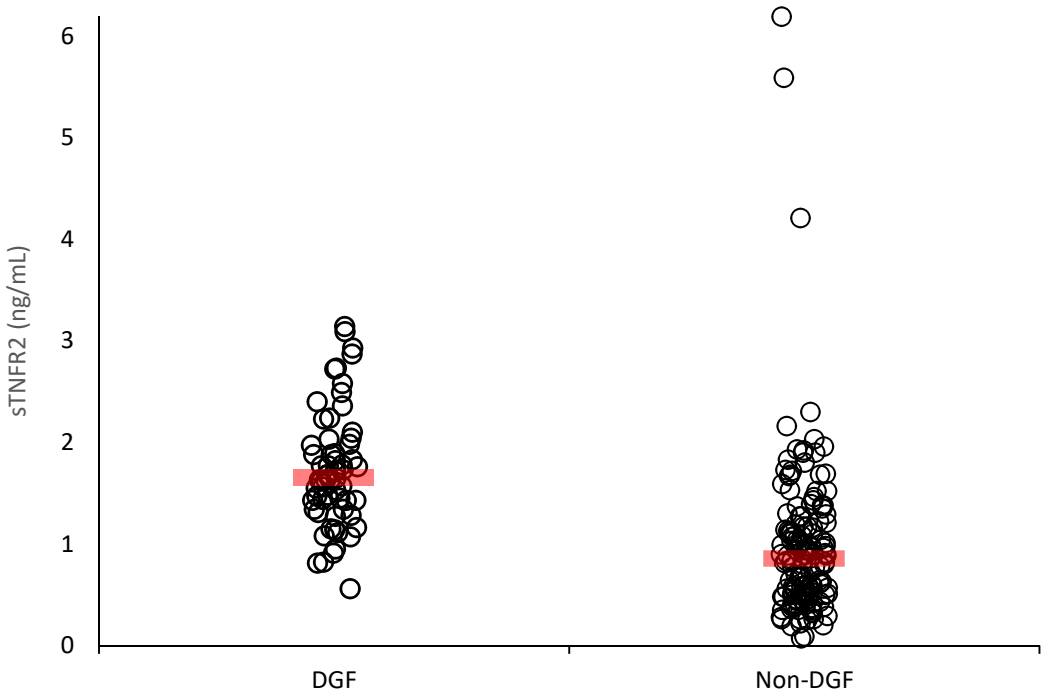


Figure 4.3 Dot plot visualising the spread of sTNFR2 concentrations in day 1-3 samples divided by DGF **status.** Red line indicates median value (1.65ng/mL and 0.855ng/mL respectively).

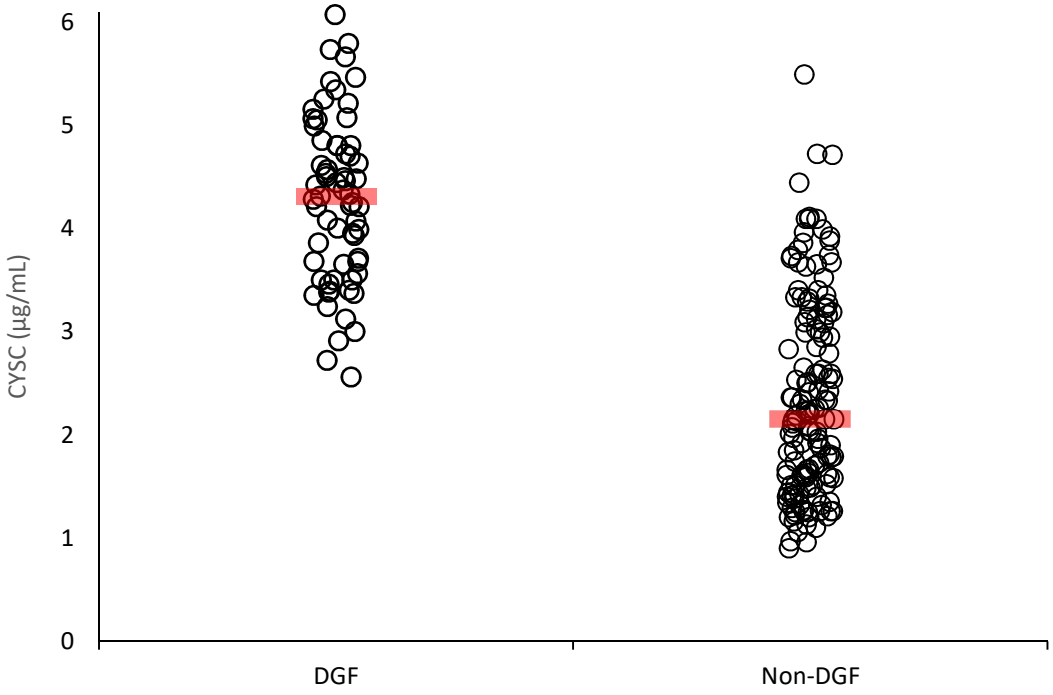


Figure 4.4 Dot plot visualising the spread of CYSC concentrations in day 1-3 samples divided by DGF **status.** Red line indicates median value (4.31µg/mL and 2.15µg/mL respectively).

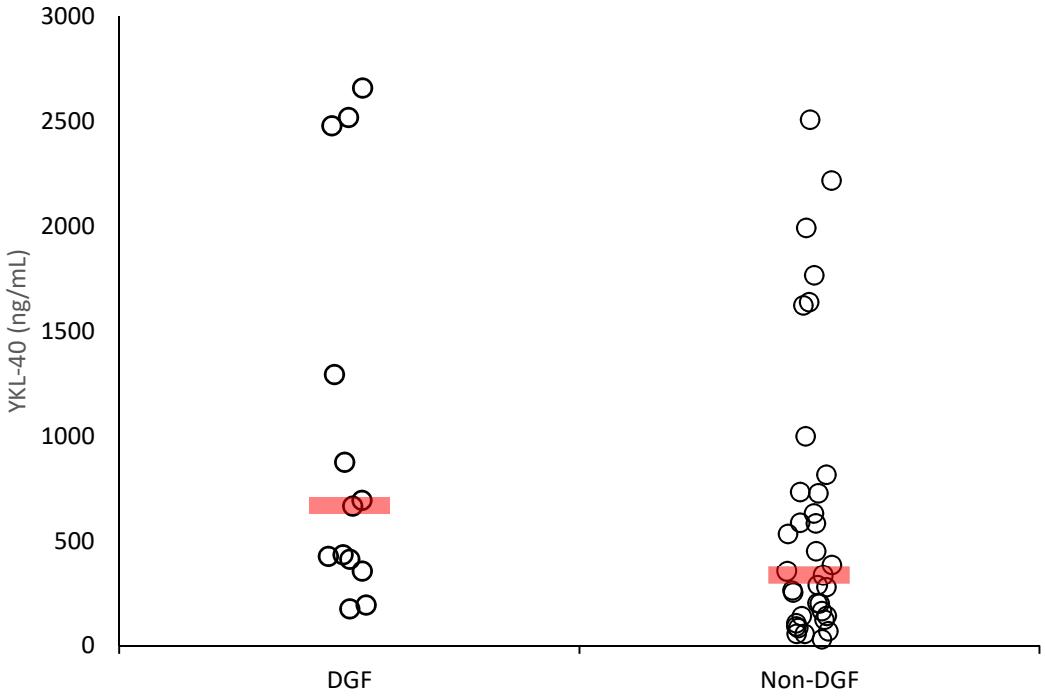


Figure 4.5 Dot plot visualising the spread of YKL-40 concentrations in day 1-3 samples divided by DGF **status.** Red line indicates median value (664.7ng/mL and 334.3ng/mL respectively).

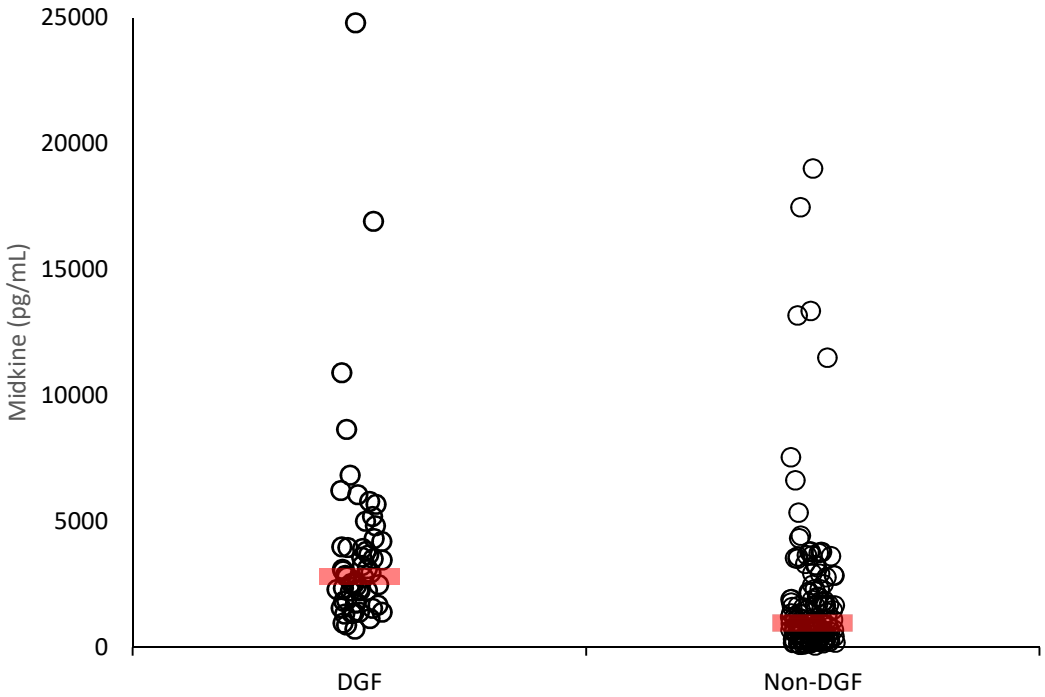


Figure 4.6 Dot plot visualising the spread of Midkine concentrations in day 1-3 samples divided by DGF **status.** Red line indicates median value (2820.5ng/mL and 963.5ng/mL respectively).

4.2.2 Biomarker diagnostic ability

4.2.2.1 Earlier diagnosis with individual biomarkers

In a univariate regression (Table 4.2) significant characteristic predictors of DGF included transplant type, age, HLA mismatch, CIT and WIT. Biomarkers that presented as significant predictors included ACY-1, FABP1, sTNFR1, sTNFR2, CRP, NGAL, CYSC (both Leeds and biochip) and Cr. Additionally, Midkine was evident in much higher concentrations in DGF than non-DGF patients, with a median of nearly three-fold greater in DGF vs non-DGF patients.

Table 4.2 Phase 1 univariate logistic regression with DGF as outcome. (Statistical analysis undertaken by Michelle Wilson)

Predictor	Level	Odds ratio	95% CI	p-value
Gender	Male	1.00		
	Female	0.87	(0.48, 1.55)	0.639
Age at transplant	-	1.03	(1.01, 1.05)	0.004
Transplant Type	DBD	0.36	(0.24, 0.52)	<0.001
	DCD	3.07	(1.64, 5.82)	<0.001
	LD	0.12	(0.02, 0.42)	0.005
CIT (mins)	-	1.00	(1.00,1.00)	0.002
WIT (mins)	-	1.04	(1.02, 1.06)	<0.001
Total HLA mismatch	0-2	1.00	(0.19, 0.42)	<0.001
	3+	2.07	(1.18, 3.67)	0.012
Initial steroid use	No	1.00		
	Yes	1.50	(0.68, 3.21)	0.300
ACY-1 (ng/ml)	-	1.01	(1.00, 1.01)	<0.001
EGF (pg/mL)	-	1.00	(0.99, 1.01)	0.925
FABP1 (ng/mL)	-	1.14	(1.08, 1.22)	<0.001
D-Dimer (ng/mL)	-	1.00	(1.00, 1.00)	0.708
MIP1A (pg/mL)	-	1.00	(0.97, 1.01)	0.781
sTNFR1 (ng/mL)	-	1.15	(1.10, 1.21)	<0.001
sTNFR2 (ng/mL)	-	4.34	(2.37, 8.65)	<0.001
IL-8 (pg/mL)	-	1.00	(1.00, 1.00)	0.785
C3DA (µg/mL)	-	0.98	(0.86, 1.08)	0.721
CRP (µg/mL)	-	1.02	(1.01, 1.03)	0.002
NGAL (µg/mL)	-	199.18	(24.07, 738.29)	<0.001
CYSC (µg/mL)	-	10.67	(4.90, 29.20)	<0.001
Original Leeds ACY-1 (ng/mL)	-	1.00	(1.00, 1.01)	<0.001
Leeds CysC (mg/L)	-	6.78	(3.94, 13.26)	<0.001
SCr (µmol/L)	-	1.00	(1.00, 1.01)	<0.001
Midkine (pg/mL)	-	1.00	(1.00, 1.00)	0.029

ROC analysis (Table 4.3) was performed with DGF as the outcome and individual biomarkers as the predictor variables with cut points selected by maximising the Youden Index to calculate sensitivity, specificity and predictive values per biomarker. ACY-1, FABP1, sTNFR1, sTNFR2, NGAL, Leeds CYSC and sCr showed high AUCs of greater than 0.75. A value close to 1 is determined to be good discrimination with 0.5 and below being inadequate. Some biomarkers with a low AUC had greater sensitivity or specificity such as MIP1A with a specificity of 74% or DDIMER and C3DA with sensitivities of 73%. Midkine alone had a lower AUC than ACY-1 and CYSC but had higher sensitivity and NPV.

Table 4.3 ROC analysis of individual biomarkers.(Statistical analysis undertaken by Michelle Wilson)

Biomarker	Cutpoint	AUC (95% CI)	Sensitivity% (95% CI)	Specificity% (95% CI)	PPV (95% CI)	NPV (95% CI)	DGF (n)	Non-DGF (n)
ACY-1	>91.22	0.79 (0.71, 0.87)	61.00 (47.00, 74.00)	89.00 (84.00, 95.00)	70.00 (57.00, 84.00)	84.00 (78.00, 91.00)	51	121
EGF	>100.81	0.51 (0.40, 0.62)	43.00 (28.00, 57.00)	70.00 (62.00, 79.00)	36.00 (24.00, 49.00)	75.00 (67.00, 84.00)	47	118
FABP1	>7.05	0.80 (0.73, 0.87)	72.00 (60.00, 85.00)	76.00 (69.00, 84.00)	55.00 (42.00, 67.00)	87.00 (81.00, 94.00)	47	118
DDIMER	<117.08	0.49 (0.40, 0.59)	73.00 (60.00, 85.00)	36.00 (27.00, 44.00)	32.00 (23.00, 40.00)	76.00 (65.00, 88.00)	48	118
MIP1A	>6.12	0.64 (0.54, 0.72)	56.00 (42.00, 70.00)	74.00 (66.00, 82.00)	47.00 (34.00, 60.00)	80.00 (73.00, 88.00)	48	115
sTNFR1	>21.27	0.88 (0.82, 0.92)	86.00 (76.00, 96.00)	80.00 (73.00, 87.00)	64.00 (52.00, 75.00)	93.00 (88.00, 98.00)	49	121
sTNFR2	>1.31	0.83 (0.76, 0.89)	83.00 (72.00, 94.00)	76.00 (69.00, 84.00)	58.00 (46.00, 70.00)	92.00 (86.00, 97.00)	47	118
IL-8	>13.06	0.65 (0.56, 0.74)	67.00 (53.00, 80.00)	60.00 (51.00, 69.00)	41.00 (30.00, 51.00)	82.00 (73.00, 90.00)	48	118
C3DA	>3.13	0.52 (0.42, 0.60)	73.00 (60.00, 85.00)	39.00 (30.00, 48.00)	33.00 (25.00, 42.00)	77.00 (66.00, 88.00)	51	121
CRP	>28.5	0.72 (0.63, 0.80)	75.00 (63.00, 86.00)	64.00 (56.00, 73.00)	48.00 (37.00, 58.00)	85.00 (78.00, 93.00)	51	118
NGAL	>0.52	0.82 (0.76, 0.88)	78.00 (67.00, 90.00)	75.00 (68.00, 83.00)	57.00 (46.00, 69.00)	89.00 (83.00, 95.00)	51	121
Leeds CYSC	>3.34	0.91 (0.86, 0.95)	88.00 (79.00, 97.00)	80.00 (72.00, 87.00)	65.00 (54.00, 77.00)	94.00 (89.00, 99.00)	49	114
SCr	>457.5	0.75 (0.68, 0.82)	96.00 (91.00, 100.00)	47.00 (38.00, 56.00)	45.00 (36.00, 55.00)	96.00 (91.00, 100.00)	52	113
Midkine	>1998	0.76 (0.68-0.83)	92.00 (83.00, 100.00)	56.00 (46.00, 65.00)	41.00 (31.00, 52.00)	95.00 (90.00, 100.00)	37	108

4.2.2.2 Earlier diagnosis with biomarker combinations

Variable selection methods were used to explore the best combination of biomarkers for diagnostic utility. The variables found to be significant in univariate analysis were carried forward and used in a multivariable logistic regression with DGF as the outcome. Each biomarker previously found to be significant were added sequentially as a predictor variable. Penalised LASSO regression was used to investigate combinations of biomarkers under the following inclusions:

I. All biomarkers
II. Biomarkers with AUCs greater than 0.75
III. Biomarkers with AUCs greater than 0.75 and a sensitivity or specificity greater than 0.70

In all three criteria, ACY-1, sTNFR1, sTNFR2 and Leeds CYSC were selected by the regression. Figure 4.8 shows ROC curves for these biomarkers, both separately and combined. There was little difference seen (Table 4.4) between the AUC, sensitivity, specificity or predictive values for the combinations .sTNFR1 and sTNFR2 were well correlated (Spearman's rank correlation coefficient= 0.78 Figure 4.7) and models including them separately or in combination were similar.

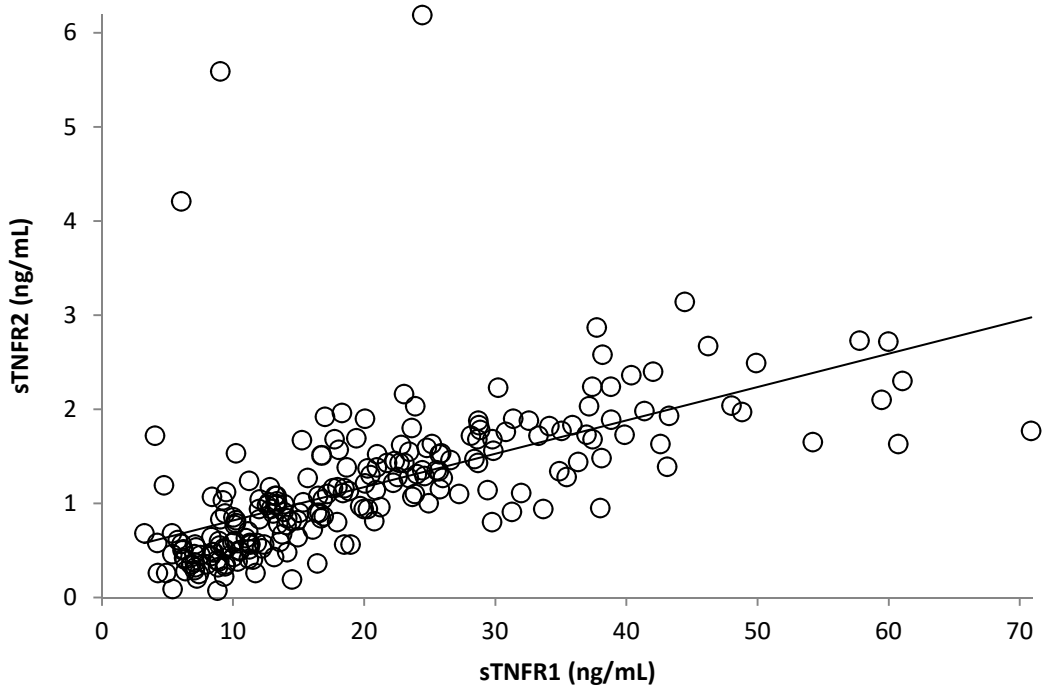


Figure 4.7 Scatter plot showing correlation between sTNFR1 and sTNFR2.

Table 4.4 ROC analysis of biomarker combinations. (Statistical analysis undertaken by Michelle Wilson)

Biomarker	Cutpoint	AUC (95% CI)	Sensitivity% (95% CI)	Specificity% (95% CI)	PPV (95% CI)	NPV (95% CI)	DGF (n)	Non-DGF (n)
ACY-1 + CYSC	>0.19	0.94 (0.90, 0.97)	98.00 (94.00, 100.00)	78.00 (70.00, 86.00)	65.00 (54.00, 76.00)	99.00 (97.00, 100.00)	48	114
ACY-1 + sTNFR1 + CYSC	>0.19	0.93 (0.89, 0.97)	98.00 (94.00, 100.00)	78.00 (70.00, 86.00)	64.00 (53.00, 76.00)	99.00 (97.00, 100.00)	46	114
ACY-1 + sTNFR2 + CYSC	>0.16	0.94 (0.91, 0.97)	98.00 (93.00, 100.00)	77.00 (70.00, 85.00)	63.00 (52.00, 75.00)	99.00 (97.00, 100.00)	44	111
ACY-1 + sTNFR1 + sTNFR2 + CYSC	>0.17	0.94 (0.90, 0.97)	98.00 (93.00, 100.00)	79.00 (72.00, 87.00)	64.00 (52.00,76.00)	99.00 (97.00, 100.00)	42	111

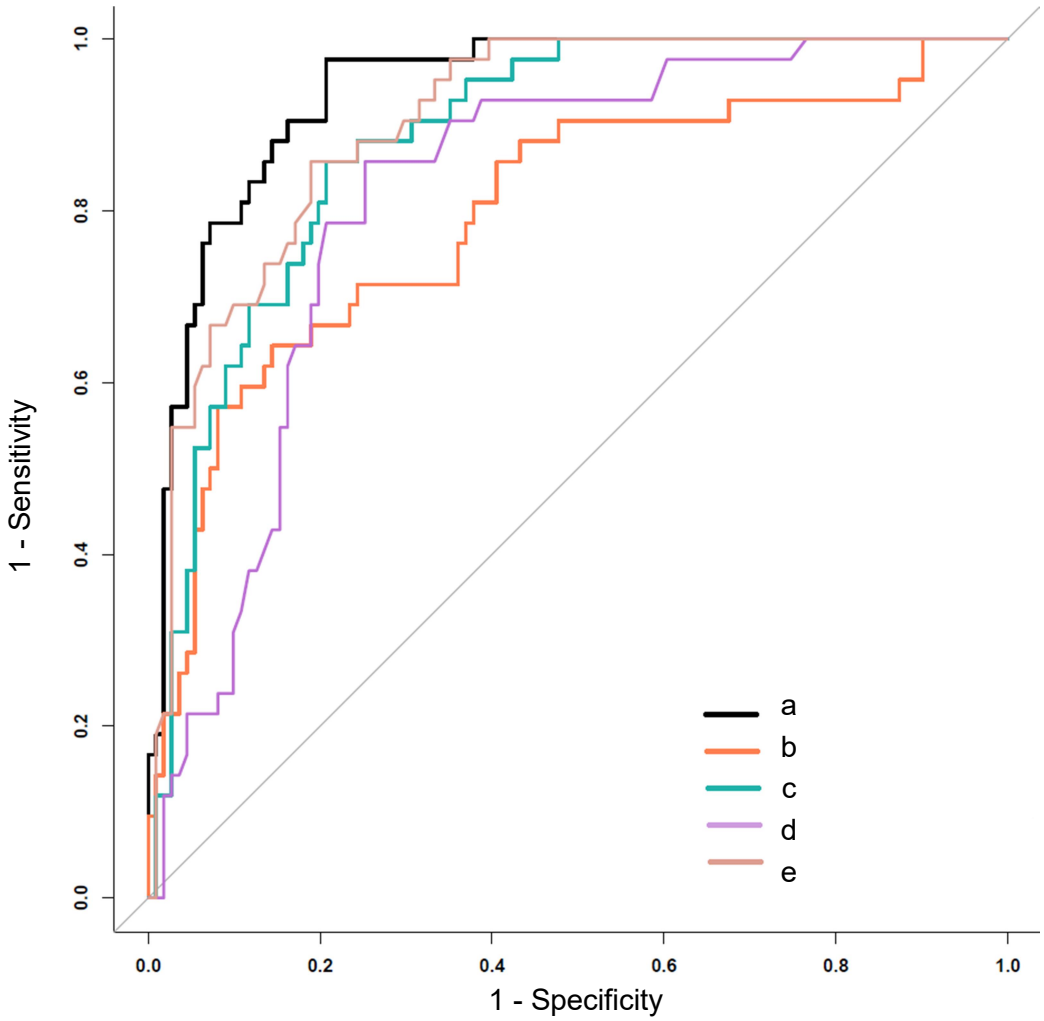


Figure 4.8 ROC curves for ACY-1, sTNFR1, sTNFR2 and CYSC. **a**) ACY-1 + sTNFR1 + sTNFR2 + CYSC. AUC=0.94,95% CI: 0.90, 0.97. **b**) ACY-1. AUC=0.79, 95% CI: 0.71, 0.87. **c**) sTNFR1. AUC=0.88, 95% CI: 0.82, 0.92. **d**) sTNFR2. AUC=0.83, 95% CI: 0.76, 0.89. **e**) CYSC. AUC=0.91, 95% CI: 0.86, 0.95. (Statistical analysis undertaken by Michelle Wilson)

A number of multivariable selection methods were used to incorporate clinical and demographic variables in the model to assess which markers were independently significant. Fractional polynomial, penalised LASSO and stepwise regressions were used on ACY-1, FABP1, sTNFR1, sTNFR2, CRP, Leeds CYSC and sCr with the patient variables of age at transplant, transplant type, CIT, WIT and total HLA mismatch. In every regression ACY-1 was an outcome and additionally sTNFR1, Leeds CYSC and sCr were consistently selected which supports the outcome of the penalised regression with only the biomarkers. Midkine was analysed in combination with CYSC and ACY-1, and when combined with both the AUC improved

Table 4.5 Multivariable logistic regression including clinical and demographic variables. Markers added sequentially. (Statistical analysis undertaken by Michelle Wilson)

Variable	Units	ACY-1			FABP1			sTNFR1			sTNFR2		
		OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value
Age	-	1.03	[1.00;1.06]	0.050	1.02	[1.00;1.05]	0.083	1.01	[0.98;1.05]	0.346	1.02	[0.99;1.05]	0.156
Transplant Type	DBD	1.00	-	-	1.00	-	-	1.00	-	-	1.00	-	-
	DCD	0.86	[0.27;2.80]	0.808	1.77	[0.65;4.82]	0.263	1.34	[0.44;4.12]	0.605	1.77	[0.66;4.76]	0.256
	LD	0.24	[0.02;2.45]	0.230	0.21	[0.02;2.27]	0.200	1.38	[0.11;17.33]	0.805	0.21	[0.02;2.63]	0.229
CIT	-	1.00	[1.00;1.00]	0.955	1.00	[1.00;1.00]	0.807	1.00	[1.00;1.00]	0.333	1.00	[1.00;1.00]	0.991
WIT	-	1.03	[1.00;1.05]	0.048	1.02	[1.00;1.05]	0.080	1.03	[1.00;1.06]	0.021	1.02	[1.00;1.05]	0.068
Total HLA mismatch	0-2	1.00	-	-	1.00	-	-	1.00	-	-	1.00	-	-
	3+	1.61	[0.68;3.82]	0.276	1.28	[0.53;3.09]	0.578	2.05	[0.77;5.46]	0.149	1.41	[0.59;3.38]	0.437
Marker	-	1.01	[1.00;1.01]	0.006	1.08	[1.01;1.15]	0.020	1.14	[1.08;1.20]	<0.001	2.74	[1.43;5.23]	0.002
Variable	**Units**	**CRP**			**NGAL**			**Leeds CYSC**			**SCr**		
		OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value
Age	-	1.03	[1.00;1.05]	0.056	1.03	[1.00;1.06]	0.044	1.01	[0.98;1.05]	0.520	1.04	[1.01;1.06]	0.016
Transplant Type	DBD	1.00	-	-	1.00	-	-	1.00	-	-	1.00	-	-
	DCD	1.44	[0.51;4.06]	0.491	1.62	[0.56;4.68]	0.368	3.25	[0.93;11.45]	0.066	1.48	[0.52;4.18]	0.462
	LD	0.22	[0.02;2.15]	0.193	1.03	[0.09;11.78]	0.978	1.56	[0.03;94.46]	0.832	0.37	[0.04;3.86]	0.406
CIT	-	1.00	[1.00;1.00]	0.911	1.00	[1.00;1.00]	0.327	1.00	[1.00;1.00]	0.520	1.00	[1.00;1.00]	0.714
WIT	-	1.03	[1.01;1.06]	0.013	1.03	[1.00;1.05]	0.022	1.04	[1.01;1.07]	0.022	1.03	[1.01;1.06]	0.010
Total HLA mismatch	0-2	1.00	-	-	1.00	-	-	1.00	-	-	1.00	-	-
	3+	1.77	[0.76;4.13]	0.187	1.30	[0.53;3.18]	0.561	1.26	[0.42;3.76]	0.680	1.69	[0.69;4.13]	0.254
Marker	-	1.01	[1.00;1.03]	0.038	55.16	[8.81;345.32]	<0.001	8.28	[3.89;17.62]	<0.001	1.00	[1.00;1.01]	<0.001

4.2.3 Biomarker prognostic utility

In order to assess the prognostic utility of the biomarkers univariate Cox proportional hazard models were used to explore the association with dialysis-free survival and overall survival.

Dialysis-free survival was taken as the time period from transplant date to the date that the patient permanently returned to dialysis. In cases where patients didn't return to dialysis, the last known dialysis-free date was used. Temporary dialysis in the first week after transplant to treat DGF does not count as a return to dialysis in this context.

Overall survival was determined to be the time period from date of transplant to date of death. Patients still living were cut off at the date last known to still be alive.

Dialysis-free survival curves were plotted using Kaplan-Meier curves (Figure 4.10) for both DGF and non-DGF patients and for the patient cohort as a whole (Figure 4.9). Statistical significance (p-value) between curves was assessed using the logrank test. Patients with live donor grafts tend to have longer dialysis-free survival periods than grafts from deceased donors, the exception to this being in the LD patients that developed DGF who both remained dialysis free for less than 12 months however the sample size is too small to draw conclusion from.

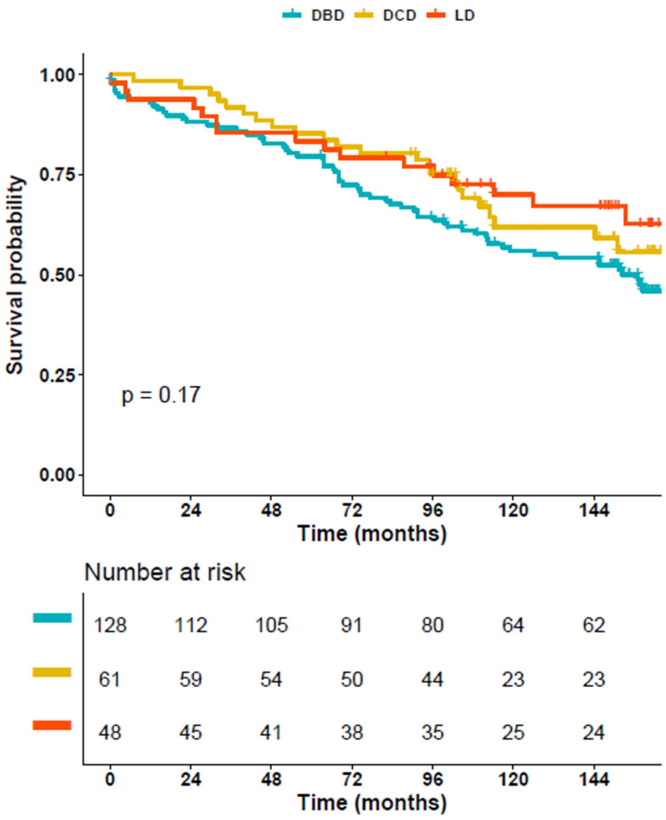


Figure 4.9 Kaplan-Meier dialysis free survival curve for all patients differentiated by graft donor type (Statistical analysis undertaken by Michelle Wilson)

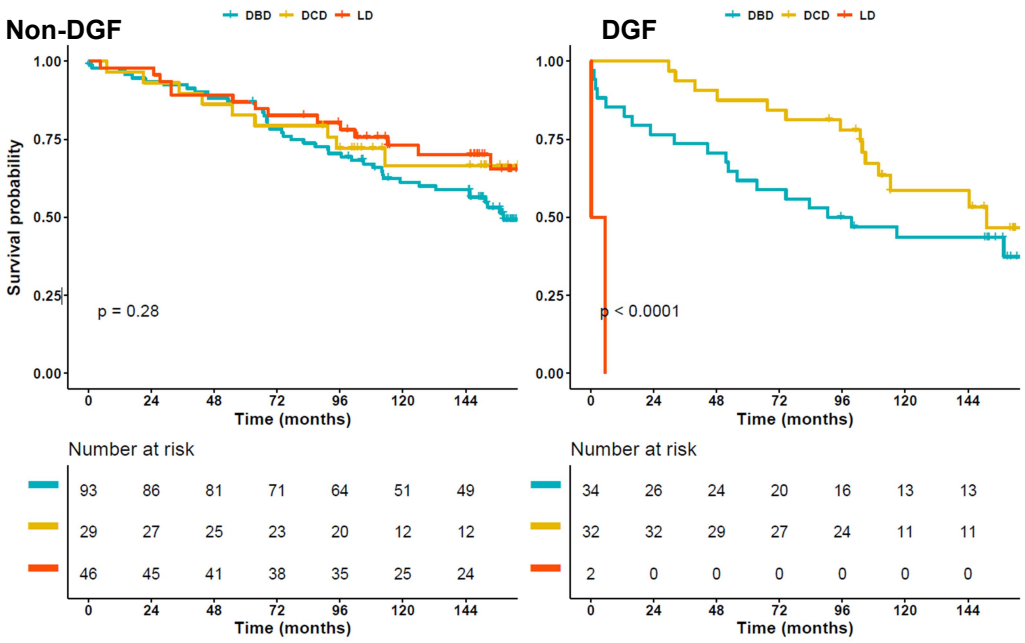


Figure 4.10 Kaplan-Meier dialysis-free survival curves in DGF and non-DGF patients differentiated by graft donor type (Statistical analysis undertaken by Michelle Wilson)

Biochip ACY-1, EGF, DDIMER, sTNFR1, sTNFR2 and Leeds CYSC were associated with significant differences in the outcome of DGF patients. In all cases except ACY-1 lower biomarker concentrations were associated with improved dialysis-free survival.

For non-DGF patients, neither ACY-1 (biochip or original ELISA) held any significance but for DDIMER, CRP, Leeds CYSC and sCr lower concentrations were correlated with significantly better dialysis-free survival. For NGAL patients with a higher concentration had better outcomes than those with a lower concentration.

The overall survival of DGF patients was significantly associated with ACY-1, EGF, DDIMER, sTNFR1, sTNFR2, and borderline Leeds CYSC. Similar to dialysis-free survival, overall survival was better in patients with lower biomarker concentrations. For non-DGF patients, biomarkers of significance were DDIMER, CRP, Leeds CYSC and sCr. Again, Kaplan-Meier overall survival curves were plotted for DGF and non-DGF patients for both transplant type and each individual biomarker.

To assess the independent utility of individual biomarkers when clinical and demographic variables were also included the same multivariable models as described in section 4.2.2.2were used. Biomarkers included in the selection models were ACY-1, EGF, sTNFR1, sTNFR2 and Leeds CYSC with patient variables of age at transplant, transplant type, CIT, WIT and total HLA mismatch.

Again ACY-1 was selected for in all methods in addition to EGF with sTNFR2 also frequently selected.

4.2.1 YKL-40 Pilot Study

The utility of YKL-40 was investigated in a small subset of 67 samples that were a representative selection of the entire cohort when accounting for DGF status, dialysis-free survival and overall survival.

Of these 67 samples, 21 fell into the unreliable flattened portion above Cal7 of the YKL-40 standard curve as discussed in Chapter 4.2.1. Nine of these fell above Cal7 and the remaining 12 were above Cal8. After exclusion, there were 46 samples left to be included in statistical modelling.

YKL-40 correlation with ACY-1 was assessed and there was no correlation apparent between the two biomarkers.

Parallels between YKL-40 and donor type and DGF status were visualised using dot-plots with out of range samples assigned a value of 3000ng/mL and a red line to indicate the median. Given the small number of samples used in this pilot study some caution must be taken when drawing conclusions.

Figure 4.11 shows how YKL-40 behaves in DGF and non-DGF patients. There were much higher YKL-40 concentrations in patients with a DCD graft than either DBD or LD, with LD the lowest and there seems to be a trend towards higher YKL-40 values and the development of DGF. The majority of patients who did not develop DGF had grafts from DBD donors and of these patients the majority have quite low levels of YKL-40.

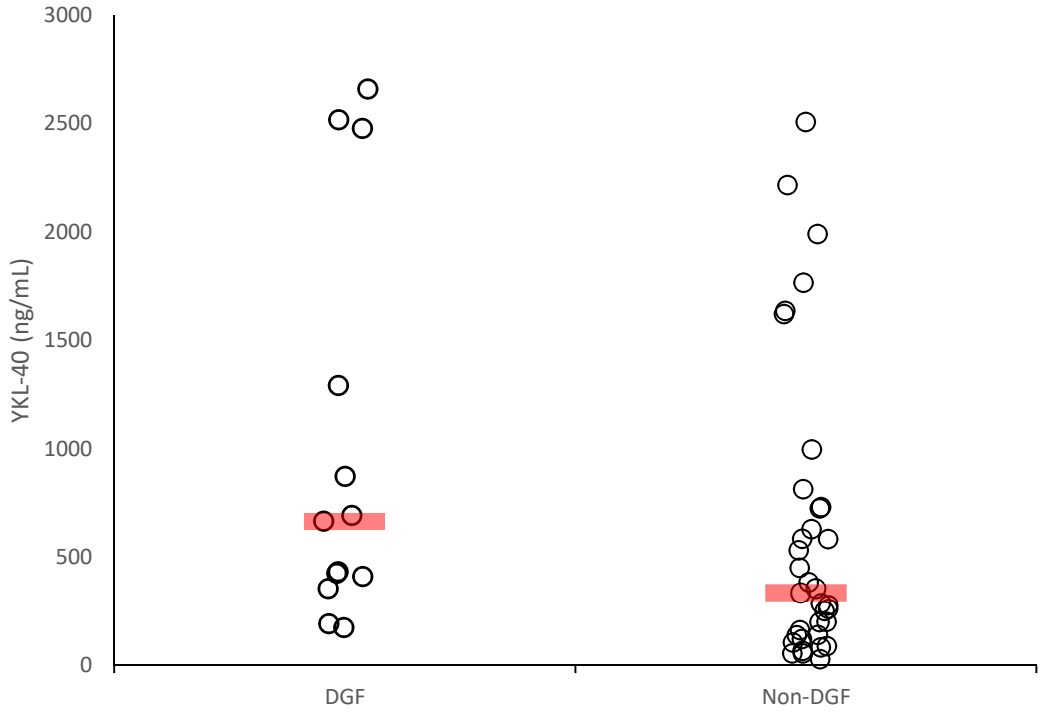


Figure 4.11 Dot plot visualising the spread of YKL-40 concentrations divided by DGF status. Red line indicates median concentration.

In Figure 4.12 the patients are divided up by DGF status and whether it was necessary that they return to dialysis after transplant. The median value was higher in patients that developed DGF and similar between those who returned to dialysis and those that did not. Patients that did not develop DGF and also didn't return to dialysis have markedly lower levels of YKL-40 with

the median close to half that of DGF patients and many more clustered at the lower end of the scale.

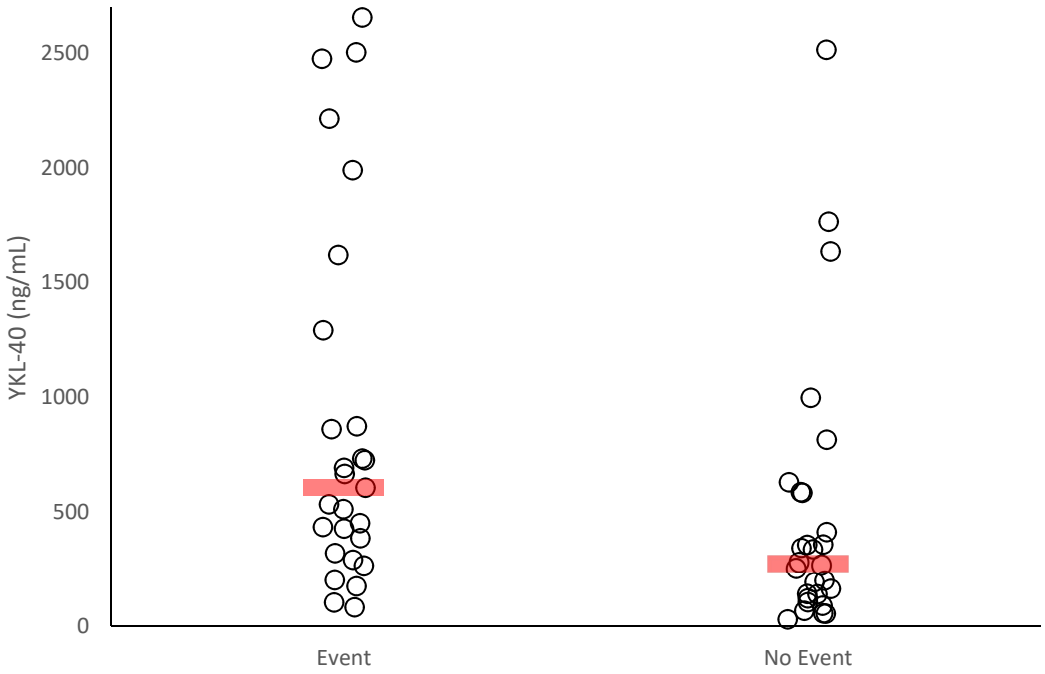


Figure 4.12 Dot plot visualising the spread of YKL-40 concentrations divided by dialysis free survival. No event means the patient remained free from dialysis and event indicates a return to dialysis. Red line indicates median concentration.

Combinations of YKL-40, Leeds CYSC and ACY-1 were used in a logistic regression to extract fitted values for ROC curves (Figure 4.13). The curves for ACY-1 and CYSC alone were similar to the overall cohort which confirmed the representative subset of samples used in this pilot study. Alone YKL-40 appeared to be of interest but had low confidence intervals which could be due to the small sample size. Despite this sensitivity and NPV were high. In the combination curves YKL-40 & CYSC were similar to CYSC alone, however when combined with ACY-1 the sensitivity and specificity improved. The combination of all three shows promise, with an AUC higher than any other individual or combination in the overall cohort and therefore the decision was made to take YKL-40 forward as one of the biomarkers for the RTA.

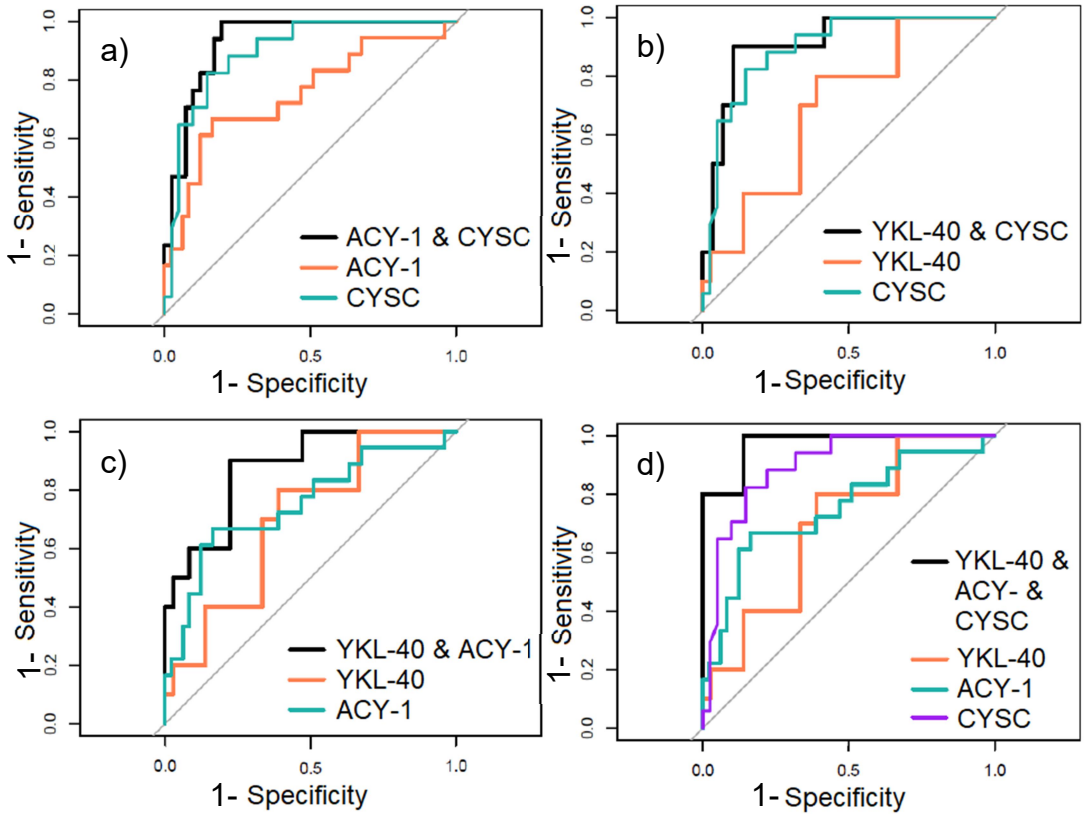


Figure 4.13 ROC analysis for a) ACY-1 & CYSC, b) YKL-40 & CYSC, c) YKL-40 & ACY-1, d) YKL-40, ACY-1 & CYSC. Analysis is of both individual biomarkers and in combination. (Statistical analysis undertaken by Michelle Wilson)

4.3 Discussion

Following the above statistical modelling biomarkers were shortlisted based on potential diagnostic/prognostic ability and accounting for logistical limitations such as the dilutional compatibility of markers on the same biochip. The selection for the Renal Transplant Array was narrowed down to ACY-1, YKL-40, sTNFR1, CYSC and Midkine.

Several of the findings in Phase 1 align with previously published data that relate clinical and demographic factors with transplant outcomes. It is already well known that live donation of graft results in DGF less often[94] than deceased and among deceased donation DCD grafts have an increased rate of DGF than DBD which is also reflected in this cohort of patients with around half of DCD recipients developing DGF compared to a quarter of DBD recipients. It has however been suggested that long term

outcomes of DCD vs DBD recipients is similar and it matters more whether the donor was extended criteria than deceased[102].

CIT has previously been established to have effect on the function of a graft[42-44] and it has also been shown that DCD kidneys perform worse after a prolonged CIT than DBD kidneys and so consideration of this needs to be taken when allocating grafts to recipients[102]. Prolonged WIT has also been shown to have an impact on early graft function[103] and the median ischaemic times in this cohort of transplant patients reflects similar trends.

Biomarkers selected by the statistical regressions for earlier diagnostic ability were ACY-1, sTNFR1, sTNFR2, Leeds CYSC, Midkine and YKL-40.

It was expected that ACY-1 would show this early diagnostic ability as it follows both with the original ACY-1[68] study and with the preliminary correlations done in Chapter 3.1. Importantly, this also indicates that the transfer from ELISA to biochip platforms has been successful.

TNF-α is a pro-inflammatory cytokine released by the kidney in response to IRI[104] that binds to receptors including membrane bound TNFR1 and TNFR2. TNFR1 is expressed on most cell types whereas TNFR2 is only expressed under inflammatory stimulation. The former has mainly been associated with apoptotic events initiated by TNF-α and the latter with cell proliferation and protection driven by TNF-α[105].

sTNFR1 and sTNFR2 are soluble forms of the membrane bound TNFR1 and TNFR2 and are formed when a cell sheds its surface receptors. By shedding these TNFR receptors the cell is less affected by TNF-α[106] which may make it resistant to the apoptotic effects of TNF-α.

Both sTNFR1 and sTNFR2 have been associated with neutralisation of TNF by competing with membrane bound TNF receptors to bind to and inactivate TNF, thereby reducing apoptosis[106,107]. The increased concentration of sTNFR1 and sTNFR2 seen in DGF patients perhaps links to the greater degree of cellular damage inflicted by more severe IRI and therefore more TNF-α being released that needs to be neutralised in order to reduce inflammation and promote healing.

sTNFR1 has also been previously linked to the development of DGF[89] and both sTNFR1 and sTNFR2 have been linked to predicting patient outcomes in advancing CKD[38] so selection by these models is a promising development. Ischaemic reperfusion injury has been strongly linked to DGF

and the mechanism of IRI causes the release of TNF-α[108]. Therefore it is reasonable to assume that sTNFR1 and 2 are also increased with the degree of IRI and therefore can be used as a tool for earlier diagnosis of DGF.

Over several years CYSC has been emerging as a potentially better biomarker for estimating GFR than the routinely used serum creatinine and was first proposed as a biomarker for renal transplantation as early as 1998[109]. CYSC is produced at a constant rate by seemingly all nucleated cells, is almost completely filtered by the kidneys and is not affected by the same biological factors as sCr (i.e. age, gender, muscle mass)[110]. When used in conjunction with sCr the biomarker combination may negate the effect of factors unrelated to GFR that skew sCr measurements[111]. It has been shown to reflect biological changes more rapidly than sCr in the early days post-transplant[112], despite overall correlating well with sCr. This early observable measure of eGFR could be a promising method for earlier diagnosis of DGF. CYSC has only had a certified reference material available since 2010[113] meaning before then assays were not standardised and potentially inaccurate. With greater confidence in assay performance there is now scope to develop CYSC into a routine clinical measure to supplement clinician information. Currently CYSC is not used in favour of sCr due to a higher cost involved and less widespread availability[114].

Midkine is a heparin-binding growth factor highly expressed in embryonic development and declining with age[115] circulating in low levels in the healthy individual. It is secreted by a number of organs including the kidneys[98] and has recently been associated with a reduced eGFR[116]. Midkine has been shown to accumulate and promote an inflammatory response immediately after IRI[117] which is a significant factor in the development of DGF and linked to inhibiting apoptotic activity[118] so may be elevated to combat the apoptosis induced by increased TNF-α after longer periods of ischaemia[119] such as those seen in renal transplantation. Midkine has also been linked to cell growth, survival and angiogenesis in cancers[120] but it could be that it is present in a similar capacity to repair damage to the kidney post-transplant. So far urinary Midkine has been noted as an early biomarker for AKI[121] but again the potential lack of urine post-transplant limits its use in the context of DGF. That Midkine found in low levels in adults unless inflammatory responses are occurring[98] in conjunction with the levels being elevated

drastically in DGF patients vs non-DGF patients suggests that it could be a useful marker in the earlier diagnosis of DGF post-transplant.

EGF is a protein that promotes cell growth and differentiation through binding to its receptor and has been linked to the proliferation of cells, including fibroblasts[122] which are essential in wound healing. It has previously been noted as a urinary biomarker of CKD progression[123] and may play a role in tissue recovery after injury which would explain its elevated levels in DGF patients.

YKL-40 is a glycoprotein involved in inflammation and tissue repair[124] expressed by a number of cell types but primarily macrophages[125] which have been suggested as the source of YKL-40 in other chronic inflammatory conditions (such as COPD)[126]. TNF-α appears to promote synthesis of YKL-40[127] so it stands to reason it could be equally implicated in renal inflammatory scenarios. Importantly, higher levels of urinary YKL-40 in the donor has been linked to improved outcomes in the recipient, including high risk recipients such as those who develop DGF[128]. From this it could be inferred that YKL-40 levels could be used to predict the outcome of post-transplant patients. The elevated YKL-40 levels from the pilot study in DCD graft patients but not in DBD patients, irrespective of DGF status, suggests that for YKL-40 the manner of donor death has more effect than the development of DGF. It could be that the prolonged lack of circulation in DCD grafts compared to DBD grafts causes a stronger inflammatory response and therefore YKL-40 levels to increase. Given that CIT has been linked to DGF, YKL-40 may also be indicative of graft issues developing.

Combining these biomarkers into a single assay will provide more information than a single biomarker, better inform clinicians and allow them to stratify patients based on risk of developing DGF and tailor long-term monitoring to specific prognoses.

4.4 Moving forward

With the prospective panel of biomarkers confirmed as ACY-1, YKL-40, sTNFR1 and CYSC, a prototype RTA went into development at Randox to be validated in Phase 2.

Although EGF and sTNFR2 were also valuable biomarkers, technical limitations prompted the decision to remove them from the panel. Dilutional incompatibility between these two biomarkers and the other five meant that a second biochip would have been necessary to analyse all seven which would double consumables and analysis time and therefore EGF and sTNFR2 were not taken forward. sTNFR1 and sTNFR2 were highly correlated and as a result the loss of sTNFR2 should not be detrimental to the overall potential of the RTA.

Again the current lack of biochip technology meant that Midkine analysis would be performed by ELISA for potential inclusion on the final commercial RTA once a biochip form was available.

5 Phase 2

5.1 Background

Following Phase 1 a prototype renal-transplant array was manufactured by Randox containing ACY-1, YKL-40, sTNFR1 and CYSC. The aim was that in Phase 2 the diagnostic and prognostic models developed in Phase 1 would be validated in an independent cohort of multi-centre samples analysed on the prototype chip. Again Midkine was analysed at Randox Laboratories using the Cellmid ELISA.

The cohort consisted of 724 samples obtained from 320 patients across ten different UK transplant centres. For each patient a day 1 or 2 post-transplant sample was used for the early diagnostic statistical analysis (n=198 patients) and a day 1-3 post-transplant result was used for long-term prognostic analysis (n=258 patients).

5.2 Results

A calibration issue with the standard curve of CYSC was identified and as a result CYSC data cannot be used for any analysis until recalibration has been done by Randox laboratories though this has not yet occurred due to indefinite delays caused by the COVID-19 pandemic.

5.2.1 Descriptive statistics

The clinical and demographic characteristics of patients in addition to median biomarker concentrations were collated and are displayed in Table 5.1.

Similar to Phase 1, the median age of patients developing DGF is greater than those that do not develop DGF although in this cohort the difference is not as marked as in Phase 1. In the Phase 2 cohort the proportion of patients developing DGF is lower than that of Phase 1 with only 18% of patients developing DGF as opposed to the 30% of patients from Phase 1 but also the proportion of live donors has increased in Phase 2, with 26% in Phase 2 compared to 22% in Phase 1.

In this cohort there were significantly more female patients with 63% of cohort 2 being female compared to the 33% of cohort 1. In Phase 1 approximately the same proportion of each gender developed DGF but in Phase 2 approximately 13.8% of men developed DGF compared with 21.0% of women. Again the small sample size of other ethnicities makes it impossible to infer any differences in transplant outcomes.

Again the majority of graft donors are deceased with 73.9% of donation occurring after death, however this is a slight reduction compared to the 77.5% of deceased donors in Phase 1. In this cohort 55% of the deceased donor grafts are from DBD donors which is markedly lower compared to the previous cohort. HLA mismatch is more noticeably different in Phase 2 with more donations occurring with an HLA mismatch of greater than 3 and a greater proportion of those patients going on to develop DGF.

CITs between cohorts are similar with the Phase 2 non-DGF cohort having a fractionally shorter median CIT of 11 minutes, however WITs are significantly longer for both DGF and non-DGF patients when compared with Phase 1. In non-DGF patients Phase 1 and 2 median WITs are 30 and 38 minutes respectively and in DGF patients the median Phase 2 WIT is 50 minutes.

Biomarker concentrations are again higher in the DGF cohort as expected but among the DGF cohort median ACY-1 concentration was much lower than in Phase 1, similar between Phases in non-DGF cohorts. In Phase 1 median ACY-1 for DGF patients was ~130ng/mL but in Phase 2 it is only ~40ng/mL. Although it was expected that the CRR would be greater in non-DGF patients than DGF patients, the differences in CRR medians and ranges between Phase 1 and 2 cohorts is extreme. In Phase 1 the median CRR was 0.78 for non-DGF patients and 0.06 for DGF patients but in Phase 2 the medians are 50 and -7.1 respectively. This may be the result of a lack of day 0 creatinine measurements in many Phase 2 patients where day 1 measurements were used instead which may have led to the significant differences in range and higher CRR medians.

Figure 5.1 to Figure 5.5 are dot plots showing the distribution of the biomarkers within the second cohort. Figure 5.1, Figure 5.3 and Figure 5.5 are on a log10 scale to allow for easier visualisation. These figures corroborate the findings of Phase 1, with biomarker median values being higher in patients that developed DGF than those that did not.

Table 5.1 Clinical and demographic variables for Biopassport cohort 2 broken down by DGF status. For continuous variables values are displayed as "median (range)" with a p-value from the Wilcoxon rank-sum test. For categorical variables values displayed represent n(%) with a p-value from chi-square test. (Statistical analysis undertaken by Michelle Wilson)

Characteristic	Level	Non-DGF n=210 patients	DGF n=47 patients	p-value
Age at transplant (years)	-	52 (19-80)	56 (24-78)	0.01
Gender	Male	81 (38.6)	13 (27.7)	0.32
	Female	128 (61)	34 (72.3)	
	Missing	1 (0.5)	0 (0)	
Ethnicity	White	189 (90)	41 (87.2)	0.52
	Asian	12 (5.7)	3 (6.4)	
	Black	2 (1)	2 (4.3)	
	Other	6 (2.9)	1 (2.1)	
	Missing	1 (0.5)	0 (0)	
Transplant type	LD	66 (31.4)	1 (2.1)	<0.01
	DBD	85 (40.5)	20 (42.6)	
	DCD	58 (27.6)	25 (53.2)	
	Missing	1 (0.5)	1 (2.1)	
Total HLA mismatch	0-2	83 (39.5)	10 (21.3)	0.06
	3+	125 (59.5)	36 (76.6)	
	Missing	2 (1)	1 (2.1)	
CIT (hr:min)	-	11:8 (0:42-26:6)	15:8 (3:55-25:8)	<0.01
WIT (mins)	-	38 (0-225)	50 (0-112)	<0.01
CRR	-	50 (-81.9-87.1)	-7.1 (-168-71.7)	<0.01
ACY-1 (ng/ml)	-	23.11 (2.63-1480.20)	40.36 (3.09-2201.45)	<0.001
sTNFR1 (ng/mL)	-	12.85 (3.54-95.85)	31.85 (5.04-100.35)	<0.001
YKL-40 (ng/mL)	-	220.30 (8.88-14813.85)	428.39 (22.77-13605.53)	<0.001
CYSC (µg/mL)	-	4.30 (1.31-10.63)	6.31 (1.50-11.25)	<0.001
Midkine (pg/mL)	-	909.38 (55.00-52500.00)	342.11 (1965.00-52500.00)	<0.001

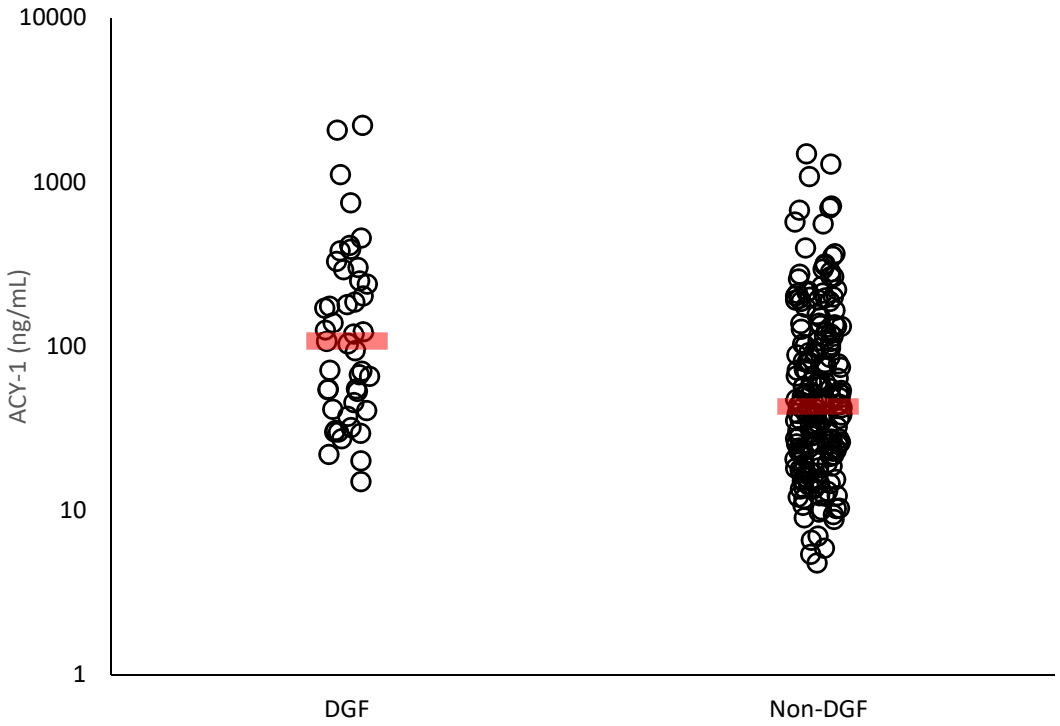


Figure 5.1 Dot plot visualising the spread of ACY-1 concentrations in day 1-3 samples divided by DGF status. Red line indicates median value (106.54ng/mL and 42.57ng/mL respectively).

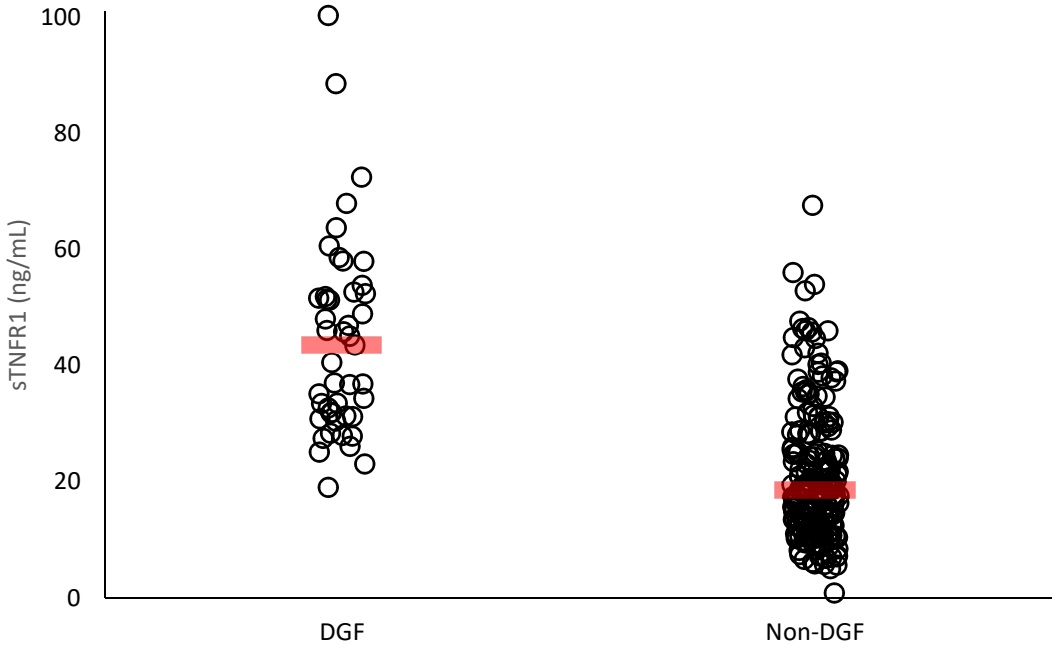


Figure 5.2 Dot plot visualising the spread of sTNFR1 concentrations in day 1-3 samples divided by DGF **status.** Red line indicates median value (43.59ng/mL and 18.51ng/mL respectively).

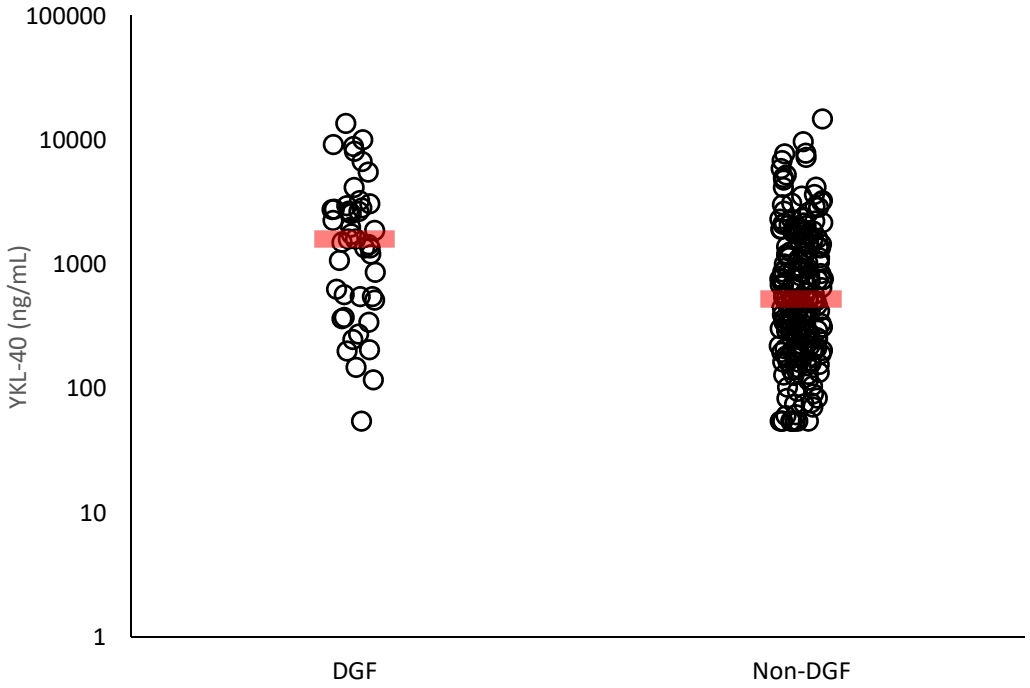


Figure 5.3 Dot plot visualising the spread of YKL-40 concentrations in day 1-3 samples divided by DGF **status.** Red line indicates median value (1594.37ng/mL and 523.72ng/mL respectively).

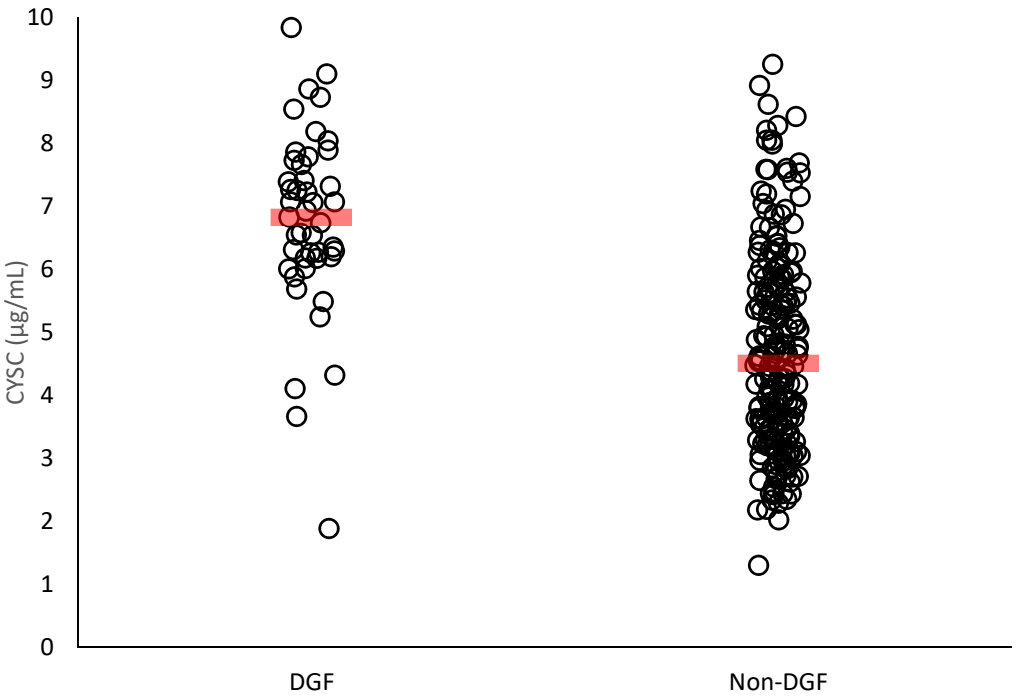


Figure 5.4 Dot plot visualising the spread of CYSC concentrations in day 1-3 samples divided by DGF **status.** Red line indicates median value (6.83µg/mL and 4.51µg/mL respectively).

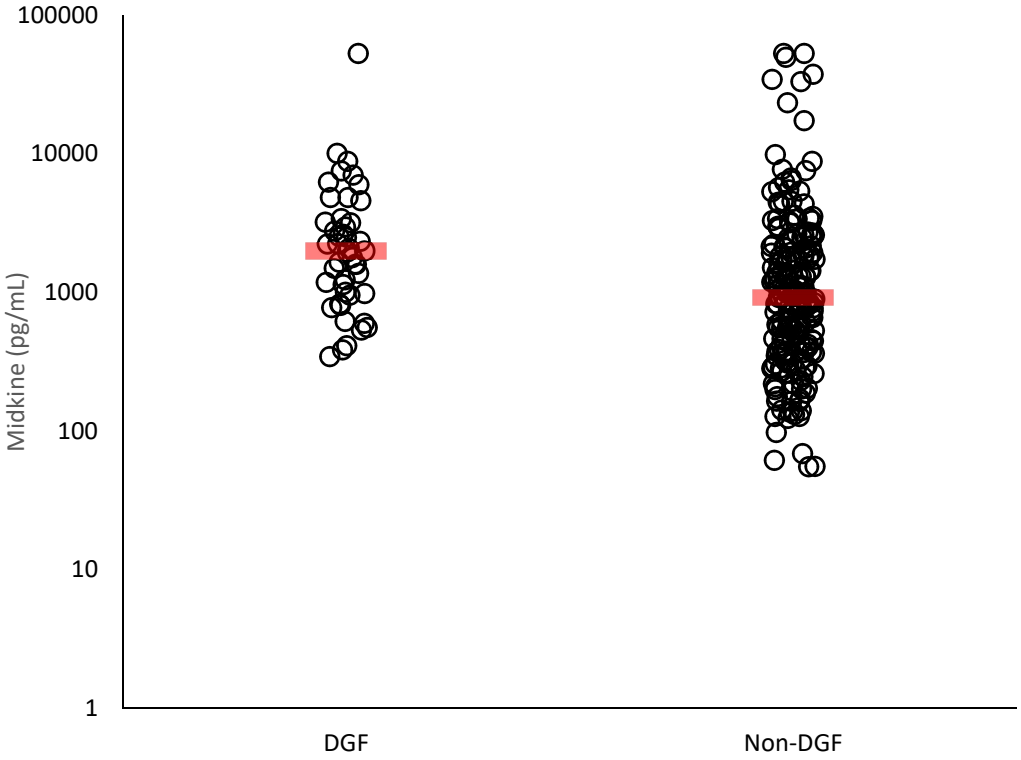


Figure 5.5 Dot plot visualising the spread of Midkine concentrations in day 1-3 samples divided by DGF **status.** Red line indicates median value (1965.00ng/mL and 909.38ng/mL respectively).

5.2.2 Diagnostic model validation

Day 1 measurements were the results of choice to use in diagnostic modelling, however if a day 1 value was not available day 2 was used instead. Of the 198 patients with day 1 or 2 measurements 36 of them developed DGF. As the sample size was smaller than expected for the DGF group, both due to a lower incidence of DGF in the cohort and the lack of day 1 or 2 samples in some patients, some statistical investigation was done into the merit of using day 3 sample results where neither day 1 nor day 2 was available. Little difference in odds ratio, 95% CI and p value were seen when Day 1, 2 or 3 measurements were used compared to when Day 1 or Day 2 measurements were used. Therefore Day 3 values were included, bringing the DGF patient cohort to 47 patients.

When the statistical modelling combining ACY-1, sTNFR1, CYSC and Midkine developed in Phase 1 was applied to Phase 2 data good discrimination between DGF and non-DGF patients was seen with an AUC of 0.82 compared to 0.93 in Phase 1. With the calibration issue for CYSC

unresolved, this modelling is preliminary. YKL-40 data was only sufficient for modelling purposes in Phase 2 where the data set was complete so the model was redeveloped to include it. Univariate logistic regression (Table 5.2) shows significant odds ratios close to 1 for all but CYSC and p values of less than 0.01 in all biomarkers except Midkine.

Table 5.2 Phase 2 univariate logistic regression with DGF as outcome. (Statistical analysis undertaken by Michelle Wilson)

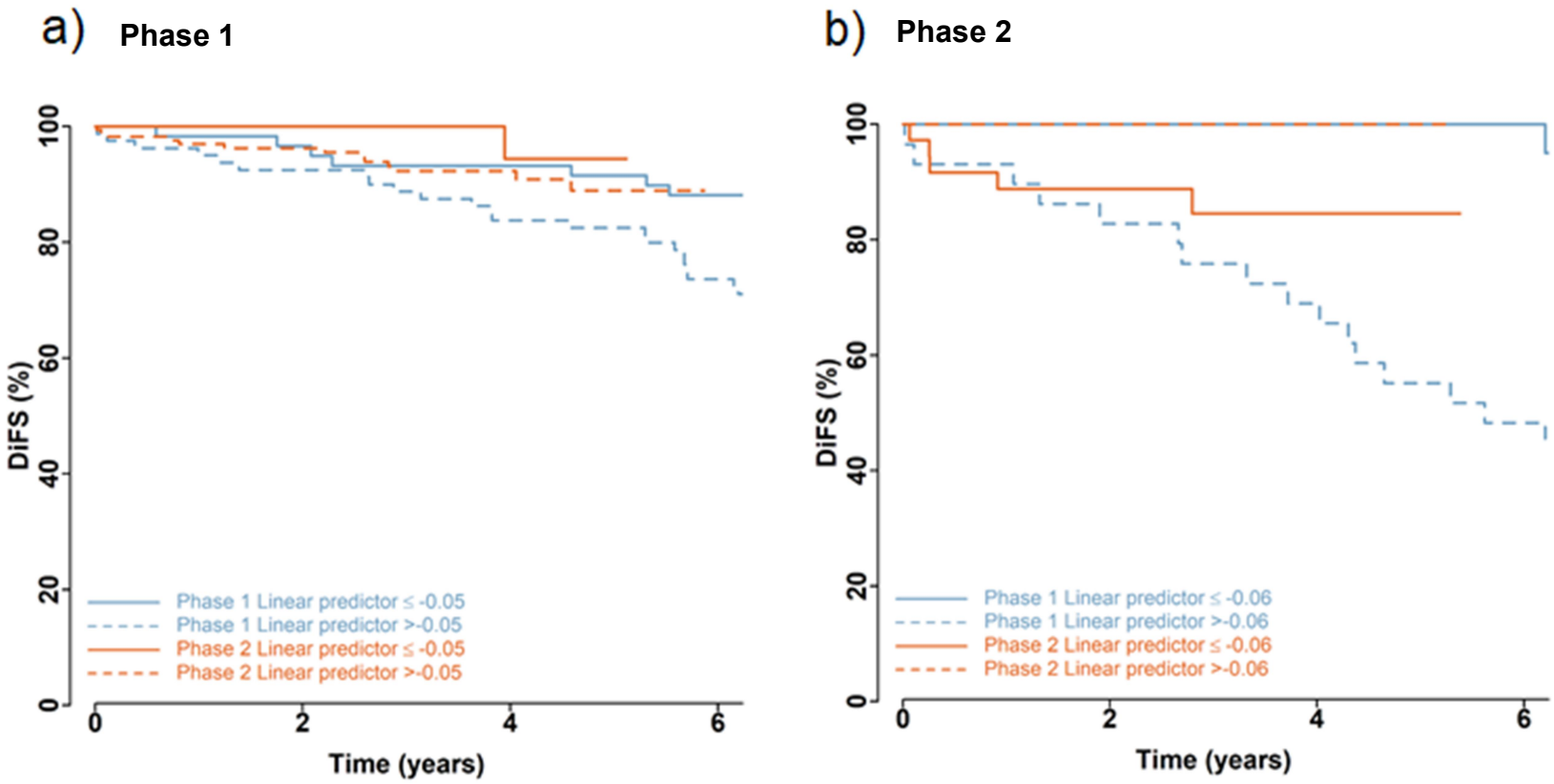
Predictor	Odds Ratio	95% CI	p value
ACY-1 (ng/mL)	1.00	(1.00, 1.00)	<0.01
sTNFR1 (ng/mL)	0.89	(0.86, 0.92)	<0.01
CYSC (µg/mL)	0.28	(0.18, 0.41)	<0.01
sCr (µmol/L)	0.99	(0.99, 1.00)	<0.01
Midkine (pg/mL)	1.00	(1.00, 1.00)	0.48
YKL-40	1.00	(1.00, 1.00)	<0.01

5.2.3 Prognostic model validation

Due to the calibration issues with CYSC prognostic model validation is preliminary at the time of writing. When prognostic models determined in Phase 1 were applied to Phase 2 data good discrimination is seen between high and low risk patients (above and below the cut point)(Figure 5.6). There is a marked improvement in the dialysis free survival time (Figure 5.7) and a shorter follow up period for patients (Phase 1: 13.1yr, Phase 2: 3.9yr) in Phase 2. However, there is some discrepancy between the predicted outcomes and the observed events which could be due to the difference in follow up data for Phase 2 coupled with fewer return-to-dialysis events.

LASSO regression was redeveloped for Phase 2 (by Michelle Wilson) to include YKL-40 in the model and selected ACY-1, sTNFR1, YKL-40 and sCr, compared to ACY-1, sTNFR1 and CYSC in Phase 1.

Figure 5.6 Kaplan-Meier survival curves showing dialysis free survival periods for patients above and below the linear predictor cut point in Phase 1 compared to Phase 2. a) Non-DGF patients, b) DGF patients. (Statistical analysis undertaken by Michelle Wilson).



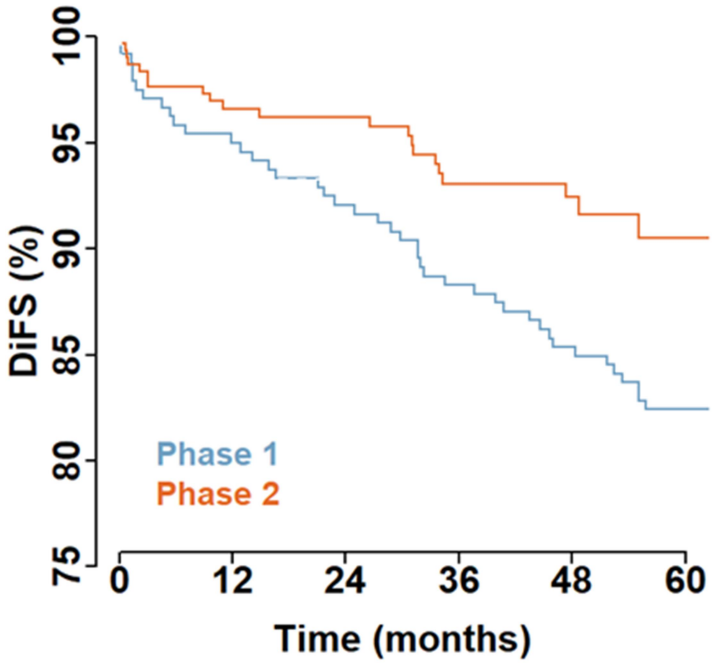


Figure 5.7 Kaplan-Meier graph comparing dialysis free survival periods in Phase 1 and Phase 2 patients (Statistical analysis undertaken by Michelle Wilson).

5.3 Discussion

The significant difference in transplant type observed between the phases may be attributed to the change in the UK as a whole over the respective time periods that the transplants took place as the Phase 2 data reflects the more current proportions of deceased donors[20]. It also may be the result of a multi-centre patient cohort used in Phase 2 as opposed to the single-centre cohort from Phase 1. The smaller proportion of patients developing DGF in this cohort of patients could be reflective of changing and improving clinical practice in the intervening years between cohort recruitment, but also the different proportion of LD:DCD:DBD donors seen in Phase 2. With lower rates of DGF in LD kidneys it follows that the overall rates of DGF seen would be lower in a cohort with a higher percentage of LD grafts.

In terms of biomarker concentrations, in DGF patients the median ACY-1 concentration is almost double (40ng/mL as opposed to 23ng/mL) with a

significantly wider range of values than those of the non-DGF patients. Similarly the median values for sTNFR1 and YKL-40 are more than double in DGF patients suggesting these biomarkers can be used to distinguish between developing DGF and not when added in conjunction with the other biomarkers.

Although the recipients in this cohort are only slightly older than those of Phase 1, there is a greater degree of HLA mismatch in Phase 2 despite a lower incidence of DGF. While this suggests that HLA mismatch has less of an impact on the graft function than other factors, it has previously been shown that HLA mismatch is more of a consideration in younger recipients than older[129].

For the early diagnostic modelling day 1 or 2 samples are the most valuable from a clinical perspective to allow earlier intervention however in reality the timing of a sample can be ambiguous since the time the transplant commences can affect how a sample is classified. For example if samples are taken during a transplant either side of midnight, they could be classed as day 0 and day 1 with only a few hours between them. It would be more accurate to time samples in terms of hours since transplant and as this data is available in this patient cohort the intention is to explore this categorisation sometime in the future.

Differences in the prognostic performance of the models when applied to Phase 2 data compared to Phase 1 data may be the result of a shorter follow-up period being available for the Phase 2 patients by virtue of their transplants occurring more recently which inherently means there are fewer return to dialysis events. Differences in the distribution of biomarkers in Phase 2 compared to Phase 1 may also be contributing to poorer performance. The model is likely to improve further once technical issues are resolved and follow up period increases.

Despite technical considerations that need to be investigated, with the exception of CYSC the biomarkers still show promise in an early diagnostic and prognostic capacity.

5.4 **Moving forward**

Due to the difference that timing of the samples taken during transplant can have with respect to whether they class as day 0 or day 1 samples, there is the intention to investigate whether changing the conventions to timing in hours rather than days will have any significant impact on the predictive ability of the RTA. In the wider BioPAsSPoRT study more detailed kinetics have been examined. At the time of writing little further progress can be made without the recalibration of CYSC standard curve.

6 Overall Conclusions

6.1 Phase 1

In Phase 1, ACY-1 and 15 serum biomarkers were evaluated through a combination of ELISA and Randox multiplex biochip to determine if any had utility in the early diagnosis of delayed graft function or had prognostic ability to determine long-term return to dialysis for renal transplant patients. A cohort of 379 samples from 241 patients were assessed statistically and a combination of ACY-1, YKL-40, sTNFR1 and CYSC were selected to be manufactured into the prototype RTA, with Midkine to be analysed separately by ELISA. It was demonstrated that the biomarkers together provided greater specificity and sensitivity (98% and 78% respectively) than alone.

ACY-1 was undetectable pre-transplant[68] and rises with low graft function, i.e. patients with immediate graft function had the lowest levels of ACY-1 and patients with DGF had the highest while live donors did not have elevated levels. Although not well understood, this suggests that something that later causes DGF to develop is responsible for the rise in ACY-1 as opposed to having a poorly functioning kidney as otherwise the pre-transplant levels in ESKD patients would be expected to be higher.

YKL-40 has been linked to anti-apoptotic signalling and in regulating vascular EGF in angiogenesis[130,131]. In kidneys that go on to develop DGF it would follow that YKL-40 would be elevated in response to the extra cellular damage caused by prolonged ischaemic time and IRI.

sTNFR1 is the soluble form of TNF-receptor-1, which allows the inflammatory cytokine TNF-α to interact with cells and ultimately leading to necrosis and apoptosis[132]. It has been suggested that in LD kidneys TNFR1 activates survival mechanisms but in deceased donor kidneys IRI causes a lack of protein synthesis causing TNFR1 instead to induce apoptosis[65] and it is well established that DGF is more prevalent in deceased donor kidneys which was also seen in the BioPAsSPoRT study.

CYSC has been proposed as an equal or better biomarker for renal function than sCr as it is produced at a constant rate and filtered completely by the kidneys, thereby indicating GFR. Therefore it is expected that its elevation

would correlate with declining graft function allowing inferences to be made about the development of DGF.

Although these biomarkers are all involved in different biological functions unrelated to one another, they can be monitored in combination to better predict DGF development post-renal transplant.

6.2 **Phase 2**

Following Phase 1 a prototype renal-transplant array was manufactured comprising of ACY-1, YKL-40, sTNFR1 and CYSC as determined in the previous statistical analysis. Midkine was also measured by ELISA for potential inclusion on the commercially available RTA biochip once Midkine has been developed for the biochip platform. Using this prototype biochip 724 samples from 320 patients were assayed to validate the early diagnostic and long-term prognostic models that were derived in Phase 1.

Due to some technical issues involving Randox's calibration of the CYSC assay and the COVID-19 pandemic that prevented these being rectified, the exact models could not be applied and hence validation of the models was not completed at the time of submission. Based on assessing the individual biomarkers, the performance of the combination models looks to be promising.

6.3 **Moving forward**

Once the final validation has been completed and assuming satisfactory performance, the plan would be to publish that data (an abstract[133] has already been presented at the 2019 British Transplant Society and NHS Blood&Transplant Congress).and patent the novel renal transplant array. A further prospective multicentre clinical trial will be designed to assess the final array, which may also include Midkine, and its potential impact when monitoring post-operative renal-transplant patients for DGF and stratifying patient care according to this novel innovation. As part of the full BioPAsSPoRT study a health economic study based on the RTA is also being conducted.

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8 Appendix

Table 8.1 Marker concentrations by patient clinical and demographic characteristics (Statistical analysis undertaken by Michelle Wilson)

Characteristic	Level	All	ACY-1 (ng/ml)			EGF (pg/mL)			FABP1 (ng/mL)		
		n=241	n	median (range) / rho	p	n	median (range) / rho	p	n	median (range) / rho	p
Age (at transplant)	(-)	47.4 (16.6-78.4)	236	0.22	**<0.01**	223	-0.047	0.49	225	0.11	0.09
Gender	Male	152 (63.1)	150	30.9 (6.54-2740)	0.34	142	86.8 (9.71-309)	0.89	142	4.4 (0.66-35.1)	0.61
	Female	89 (36.9)	86	36.3 (8.99-4440)		81	87.3 (23.7-176)		83	5.39 (0.55-99.2)	
Ethnicity	Asian	28 (11.6)	27	28 (13.1-700)	0.93	26	75.4 (23.3-158)	**0.05**	26	6.59 (1.28-30.5)	0.21
	Black	6 (2.5)	6	50.4 (13.7-246)		6	73 (12.1-96.4)		6	7.31 (1.56-14.1)	
	White	189 (78.4)	185	33.7 (8.41-4440)		173	87.2 (9.71-309)		175	4.55 (0.55-99.2)	
	Other	18 (7.5)	18	38.4 (6.54-2010)		18	103 (33.4-176)		18	4.58 (1.47-29.6)	
Transplant type	LD	48 (19.9)	48	17 (6.54-67)	**<0.01**	46	86.4 (33.4-309)	0.30	46	2.8 (0.64-13.9)	**<0.01**
	DBD	130 (53.9)	127	30.9 (8.41-2010)		120	89.1 (9.71-247)		123	5 (0.55-99.2)	
	DCD	63 (26.1)	61	155 (14.1-4440)		57	81 (12.1-176)		56	7.7 (1.56-33.1)	
HLA Mismatch	0-2	136 (56.4)	133	28 (6.54-2010)	**0.01**	125	87.9 (9.71-247)	0.64	126	4.64 (0.71-99.2)	0.14
	3+	105 (43.6)	103	40.7 (8.6-4440)		98	84.9 (12.1-309)		99	4.86 (0.55-35.1)	
CIT	(-)	918 (29-2720)	236	0.24	**<0.01**	223	0.033	0.63	225	0.22	**<0.01**
WIT	(-)	35 (7.8-110)	236	0.36	**<0.01**	223	0.025	0.71	225	0.33	**<0.01**
CRR	(-)	70.4 (-75.8-90.9)	193	-0.49	**<0.01**	180	0.049	0.52	182	-0.54	**<0.01**
DGF status	Non-DGF	171 (71.2)	168	24.3 (6.54-657)	**<0.01**	160	87.2 (12.1-309)	0.29	161	3.84 (0.55-99.2)	**<0.01**
	DGF	69 (28.7)	67	129 (9.83-4440)		62	85.2 (9.71-168)		63	9.35 (2.94-33.1)	
Graft function	IGF	99 (49.7)	99	21 (6.54-379)	**<0.01**	94	84.8 (25.5-309)	0.56	94	3.12 (0.55-21.4)	**<0.01**
	SGF	32 (16.1)	32	43.2 (10.4-657)		29	74.7 (12.1-157)		30	6.48 (1.15-99.2)	
	DGF	68 (34.2)	67	129 (9.83-4440)		62	85.2 (9.71-168)		63	9.35 (2.94-33.1)	

Table 8.1 continued

D-Dimer (ng/mL)			MIP1-alpha (pg/mL)			sTNFR1 (ng/mL)			sTNFR2 (ng/mL)		
n	median (range) / rho	p	n	median (range) / rho	p	n	median (range) / rho	p	n	median (range) / rho	p
226	0.2	**<0.01**	222	0.038	0.57	234	0.15	**0.02**	225	0.13	**0.04**
143	88.6 (20.5-914)	0.35	140	4.41 (3.73-58)	0.15	148	17 (4.21-70.9)	0.27	142	1.09 (0.09-6.19)	0.13
83	72.1 (12.7-4660)		82	5.5 (3.73-345)		86	16.3 (3.23-61)		83	0.99 (0.07-4.21)	
26	87.2 (20.5-890)	0.21	26	5.38 (3.73-117)	**0.04**	26	24.7 (4.55-48.8)	0.06	26	1.44 (0.35-3.09)	**0.03**
6	145 (63.6-409)		6	29 (3.73-345)		6	17.4 (5.79-32.6)		6	1.23 (0.56-1.88)	
176	73.9 (12.7-4660)		172	4.97 (3.73-302)		184	15.9 (3.23-70.9)		175	1 (0.07-6.19)	
18	86.6 (24-153)		18	3.73 (3.73-16.9)		18	13.3 (5.39-37.7)		18	0.81 (0.09-2.87)	
46	67 (13.5-425)	**0.03**	45	3.73 (3.73-30.3)	**0.01**	48	9.4 (3.23-30.8)	**<0.01**	46	0.585 (0.09-4.21)	**<0.01**
123	74.9 (20.5-890)		122	4.62 (3.73-345)		126	18.2 (4.04-70.9)		123	1.08 (0.07-5.59)	
57	104 (12.7-4660)		55	6.29 (3.73-302)		60	24.5 (5.36-61)		56	1.46 (0.32-6.19)	
126	78.6 (13.5-425)	0.30	124	4.3 (3.73-345)	**0.02**	132	15.2 (3.23-70.9)	0.31	126	0.995 (0.07-5.59)	0.13
100	89.8 (12.7-4660)		98	6 (3.73-302)		102	18.7 (4.21-61)		99	1.16 (0.26-6.19)	
226	-0.022	0.75	222	0.076	0.26	234	0.33	**<0.01**	225	0.25	**<0.01**
226	0.042	0.53	222	0.025	0.71	234	0.25	**<0.01**	225	0.27	**<0.01**
183	-0.069	0.35	179	-0.24	**<0.01**	191	-0.64	**<0.01**	182	-0.5	**<0.01**
161	85.1 (13.5-4660)	0.40	158	4.3 (3.73-345)	**<0.01**	168	12.8 (3.23-61)	**<0.01**	161	0.85 (0.07-6.19)	**<0.01**
64	81.6 (12.7-914)		63	6.7 (3.73-117)		65	30.2 (14.9-70.9)		63	1.65 (0.56-3.14)	
94	83.6 (13.5-4660)	0.48	91	4 (3.73-345)	**0.01**	99	10.5 (3.23-61)	**<0.01**	94	0.735 (0.09-6.19)	**<0.01**
30	98 (22.5-275)		30	4.62 (3.73-193)		32	19.3 (6.14-43.2)		30	0.985 (0.32-2.03)	
64	81.6 (12.7-914)		63	6.7 (3.73-117)		65	30.2 (14.9-70.9)		63	1.65 (0.56-3.14)	

Table 8.1 continued

IL-8 (pg/mL)			C3-des Arg (ug/mL)			CRP (ug/mL)			NGAL (ug/mL)		
n	median (range) / rho	p	n	median (range) / rho	p	n	median (range) / rho	p	n	median (range) / rho	p
226	0.067	0.32	236	-0.16	**0.01**	232	0.15	**0.02**	236	0.084	0.20
143	9.91 (3-5490)	**<0.01**	150	3.82 (0.52-35.8)	0.10	148	28.5 (1.43-220)	0.84	150	0.46 (0.11-1.25)	0.12
83	15.3 (3-1010)		86	3.67 (0.43-10.3)		84	29.1 (1.57-353)		86	0.37 (0.06-1.5)	
26	16.9 (5.39-1010)	0.29	27	3.79 (0.99-11.1)	0.29	27	32.6 (1.57-109)	0.12	27	0.59 (0.06-1.22)	**0.03**
6	15.4 (5.6-252)		6	2.44 (1.35-5.34)		6	50.4 (27.3-145)		6	0.375 (0.17-1.14)	
176	11.5 (3-5490)		185	3.82 (0.43-35.8)		181	28.8 (1.43-353)		185	0.42 (0.11-1.5)	
18	10.7 (3.97-260)		18	3.6 (1.23-7.35)		18	18.9 (5.83-156)		18	0.41 (0.15-0.88)	
46	8.78 (3-5490)	**0.01**	48	3.68 (0.52-8.9)	0.97	48	14.9 (1.43-109)	**<0.01**	48	0.295 (0.11-0.84)	**<0.01**
123	11.7 (3-5490)		127	3.81 (0.43-35.8)		125	28.7 (1.57-353)		127	0.43 (0.06-1.5)	
57	14.5 (3-5490)		61	3.87 (0.99-10.3)		59	47.7 (6.25-178)		61	0.62 (0.15-1.37)	
126	10.7 (3-5490)	0.41	133	3.72 (0.43-35.8)	0.61	130	29.5 (3.36-353)	0.52	133	0.38 (0.11-1.25)	**0.01**
100	12.7 (3-5490)		103	3.87 (0.52-16.1)		102	27.6 (1.43-145)		103	0.51 (0.06-1.5)	
226	0.13	0.06	236	0.088	0.18	232	0.22	**<0.01**	236	0.22	**<0.01**
226	0.12	0.08	236	-0.012	0.86	232	0.12	0.07	236	0.18	**0.01**
183	-0.27	**<0.01**	193	-0.051	0.48	189	-0.38	**<0.01**	193	-0.59	**<0.01**
161	10.3 (3-5490)	**<0.01**	168	3.74 (0.43-35.8)	0.41	164	23.4 (1.43-220)	**<0.01**	168	0.35 (0.06-1.25)	**<0.01**
64	15.3 (4.78-1010)		67	3.9 (1.36-11.1)		67	48.6 (2.1-353)		67	0.67 (0.32-1.5)	
94	9.16 (3-5490)	**<0.01**	99	3.55 (0.52-16.1)	0.41	97	22 (1.43-220)	**<0.01**	99	0.3 (0.11-1.25)	**<0.01**
30	12.7 (3-5490)		32	3.7 (0.43-10.2)		30	27.3 (1.57-182)		32	0.425 (0.15-1.01)	
64	15.3 (4.78-1010)		67	3.9 (1.36-11.1)		67	48.6 (2.1-353)		67	0.67 (0.32-1.5)	

Table 8.1 continued

Cystatin C (ug/mL)			SCr			Midkine		
n	median (range) / rho	p	n	median (range) / rho	p	n	median (range) / rho	p
223	0.19	**0.01**	228	-0.012	0.86	214	0.14	**0.03**
144	2.82 (0.97-6.07)	0.24	145	554 (87-1240)	**<0.01**	136	1480 (75-19000)	0.30
79	2.85 (0.9-5.73)		83	418 (98-1370)		78	1080 (90-24800)	
27	3.86 (0.9-5.66)	**0.01**	27	687 (160-1110)	**<0.01**	25	2440 (564-17500)	**0.01**
6	3.1 (1.48-4.25)		6	604 (149-1240)		6	973 (320-3960)	
174	2.59 (0.96-6.07)		178	520 (87-1370)		166	1330 (90-24800)	
16	2.37 (1.27-4.96)		17	407 (143-837)		17	1340 (75-19000)	
45	1.51 (0.9-4.72)	**<0.01**	46	345 (87-928)	**<0.01**	46	615 (90-19000)	**<0.01**
119	3.12 (1.1-6.07)		122	534 (114-1110)		117	1710 (144-24800)	
59	3.46 (1.49-5.46)		60	654 (143-1370)		51	1820 (75-16900)	
126	2.59 (0.9-6.07)	0.60	126	510 (110-1370)	0.12	123	1220 (75-8650)	0.07
97	3.02 (0.97-5.79)		102	543 (87-1240)		91	1670 (113-24800)	
223	0.42	**<0.01**	228	0.17	**0.01**	214	0.27	**<0.01**
223	0.25	**<0.01**	228	0.15	**0.03**	214	0.1	0.15
184	-0.71	**<0.01**	193	-0.43	**<0.01**	172	-0.44	**<0.01**
158	2.17 (0.9-5.49)	**<0.01**	160	424 (87-1060)	**<0.01**	157	901 (75-19000)	**<0.01**
64	4.32 (2.56-6.07)		68	691 (358-1370)		56	2820 (719-24800)	
93	1.9 (0.9-4.09)	**<0.01**	99	385 (87-1010)	**<0.01**	90	852 (113-19000)	**<0.01**
32	3.04 (1.06-5.49)		31	535 (135-1060)		30	1690 (75-17500)	
64	4.32 (2.56-6.07)		68	691 (358-1370)		56	2820 (719-24800)	