UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

School of Medicine

Chemokine and Chemokine Receptor Gene Expression in Human PBMCs in the Early Renal Post-Transplant Period

by

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Thesis for the degree of Doctor of Medicine

February 2005

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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CHEMOKINE AND CHEMOKINE RECEPTOR GENE EXPRESSION IN HUMAN PBMCS IN THE EARLY RENAL POST-TRANSPLANT PERIOD

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There has been increasing evidence over the last decade of the role of chemokines and their receptors in the immunological events around the time of allograft rejection, which remains an important cause of early allograft loss and a risk factor for chronic allograft nephropathy.

The aim of this work was to determine whether sequential changes in chemokine and/or chemokine receptor gene expression in the early post-transplant period of human renal allografts can be detected in PBMCs, and whether any such changes are predictive of clinical events. Also a selection of possible candidate genes that could be used as endogenous controls for gene expression in PBMCs in the transplant setting were tested, which would help to validate any results.

Blood samples from 106 renal transplant recipients and 29 donor nephrectomy patients were taken pre-operatively and then daily for 14 days. Within the two week study period 22 patients had biopsy proven acute rejection. From each blood sample the PBMCs were separated, their RNA extracted and a fixed quantity reverse transcribed to cDNA. Using real-time quantitative PCR (5' nuclease assay by TaqMan® methodology), the gene expression levels for the chemokines CCL3, CCL4, CCL5, CXCL10 and their receptors CCR1, CCR5 and CXCR3 were measured. The possible endogenous control genes tested were GAPDH, MLN51, YWHAZ, EF-1α and UbcH5B.

Changes in chemokine and chemokine receptor gene expression by sequential monitoring in PBMCs were detected in the early post-transplant period. Furthermore, different expression patterns between rejector and non-rejector groups for some genes were demonstrated and some of these changes correlated with clinical events. In particular, CCR1 and CXCL10 showed increased expression prior to rejection and returned back to baseline levels with anti-rejection therapy. The search for a suitable endogenous control gene for use in the gene expression model used in this work was unsuccessful, with significant changes in expression of all five genes tested at some time point post-transplantation.

This work has demonstrated that changes in chemokine and chemokine receptor gene expression can be detected in PBMCs in the early post-transplant period, and in particular CXCL10 and CCR1 showed changes that correlate with rejection, and therefore may have potential use in immunomonitoring and as predictive factors of rejection prior to its clinical manifestation.

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ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr Martin Howell and Mr Sami Sadek for their guidance and help throughout the duration of my work for this thesis. In particular, I am grateful to Mr Sadek for his help with all the statistical analysis and clinical teaching which has ignited an interest in transplantation, and Dr Howell for his guidance in laboratory methods and promptly reading and correcting the several drafts of this thesis.

Thanks to Paddy Paed for his practical help in the laboratory and all the staff in the Histocompatibility and Immunology laboratory at Southampton General Hospital for their help and support.

I would also like to thank the Wessex Renal Research Fund for their financial backing for this work.

Last but not least I thank Jo for her companionship and support – may Thesinger grow up to understand this work!

List of Abbreviations

ACT	immune activation
APC	antigen presenting cell
EP	endogenous pyrogen
GRB-2	growth factor receptor-bound protein-2
HLA	human leukocyte antigen
HEV	high endothelial venules
ICAM	intercellular adhesion molecule
IFN	interferon
IP	interferon inducible protein
ITK	T cell-specific protein-tyrosine kinase
LAG	lymphocyte activation gene
LPS	lipopolysaccharide
MIP	macrophage inflammatory protein
MHC	major histocompatibility complex
PCR	polymerase chain reaction
PI 3-kinase	phosphatidylinositol 3-kinase
RANTES	regulated upon activation normal T cell expressed and secreted
RT	reverse transcription / transcriptase
SCID	severe combined immunodeficiency
SCX	small inducible cytokine
SIS	small inducible secreted: SIS family of cytokines
TGF	transforming growth factor
Th	T helper
TNF	tumour necrosis factor
VCAM	vascular cell adhesion molecule

Chapter 1

Introduction

Acute rejection episodes remain an important cause of allograft failure in renal transplantation. Although the incidence of acute rejection has steadily fallen over the years with resultant higher one-year graft survival rates (44.5% 1970-79; 72.6% 1980-89; 87.3% 1990-99 – US data) [Howard et al, 2002], its impact on chronic renal allograft failure has significantly increased [Meier-Kriesche et al, 2000]. A major risk factor for chronic allograft nephropathy and long term allograft failure is acute rejection [Almond et al, 1993].

Monitoring of the immunological processes during the transplant period especially around the time of acute rejection is important to further our understanding of intragraft events. It may also lead to identification of predictive factors of allograft rejection prior to clinical manifestation. At present, allograft rejection is diagnosed by invasive biopsy that is indicated by clinical changes.

There is increasing evidence over the last decade of the role of chemokines and their receptors in the immunological events around the time of allograft rejection. In this work sequential changes in gene expression levels of the chemokines, CCL5 (RANTES), CCL3 (MIP-1α), CCL4 (MIP-1β) and CXCL10 (IP-10), and their receptors CCR1, CCR5 and CXCR3 in peripheral blood mononuclear cells were investigated in the early post-transplant period.

The method of investigation was the sequential measurement of gene specific mRNA transcripts expressed by peripheral blood mononuclear cells. Leukocytes in the peripheral blood migrate into tissues in response to inflammatory stimuli. Granulocytes and monocytes become immobilized in tissues and do not recirculate. However lymphocytes recirculate from blood through tissue, into lymph, and return to blood.

Lymphocytes acquire a predilection, based on the environment in which they first encounter foreign antigen, to home to or recirculate through that same enviroment [Picker et al, 1992; Mackay et al, 1992]. Thus changes in gene expression within lymphocytes in peripheral blood may well be a consequence of the immunological events within a transplant allograft. Previous work in this laboratory using the same methods has demonstrated significant changes in cytokine gene expression over the early post-transplant period, which correlated with clinical events including rejection [Tan et al, 2001; Gibbs et al, 2001].

Firstly, in this introduction, the complex immunological processes involved in acute allograft rejection will be described, starting with the role of antigen presentation, costimulatory signals, cytokines and adhesion molecules. Then the evidence to date of the involvement of chemokines and their receptors will be considered, in allograft acceptance/rejection, focusing specifically on the individual receptors and ligands studied in this project. This is followed by a brief discussion of immunomonitoring and finally of the quantitative RT-PCR Taqman methodology used. The specific aims of this project will then be outlined, which are related to determining whether changes in chemokine ligand/receptor gene expression can be detected in peripheral blood mononuclear cells, and if so do they reflect the immunological processes within a transplanted renal allograft.

1.1 ACUTE ALLOGRAFT REJECTION

There are three main patterns of rejection, namely hyperacute, acute and chronic. Hyperacute rejection occurs when the recipient has been presensitised to donor antigen and has circulating anti-HLA and non-HLA antibodies against the donor tissue which causes allograft rejection within hours of transplantation. This involves complement activation and neutrophil infiltration into the allograft.

Chronic rejection is a slow progressive deterioration in allograft function involving possibly antibody-mediated and cell-mediated processes. There are other aetiological factors influencing this such as the nephrotoxicity of the calcineurin inhibitor immunosuppressive drugs used to control acute rejection processes and CMV infection. Chronic rejection is often more appropriately given the name chronic allograft nephropathy, due to the involvement of both immune and non-immune processes. Acute allograft rejection involves priming of naïve recipient T-lymphocytes, and T-lymphocytes already present in the graft, by donor antigen presenting cells leading to lymphocyte infiltration of the allograft. This lymphocyte infiltration is mediated through the release of cytokines, chemokines, and cell to cell interactions, and includes CD4+ helper T cells, CD8+ cytotoxic T cells, antibody-forming B cells, and other pro-inflammatory leukocytes. Their effector mechanisms lead to destruction of the allograft if not treated. When this occurs, it is commonly within the first few weeks of transplantation and is the focus of this work.

1.1.1 Antigen Presentation

Peptides from foreign proteins, such as alloantigen from allografts, are processed intracellularly and presented by specialized antigen presenting cells (APC), namely dendritic cells , macrophages and B cells. The peptide antigens become bound to major histocompatibility complex (MHC) proteins, and transported to the cell surface of the APC for presentation to T cells. Interaction between the T cell receptor and MHC-peptide antigen complex leads to T cell activation. Class I MHC molecules (i.e. HLA-A, HLA-B and HLA-C) are recognized by CD8+ cytotoxic T cells, and Class II MHC molecules (i.e. HLA-DR, HLA-DP, and HLA-DQ) are recognized by CD4+ T cells.

There are two mechanisms by which alloantigen from an allograft is recognized, namely direct and indirect allorecognition. Direct allorecognition occurs when the recipient T lymphocytes recognise donor MHC with recipient or donor alloantigen presented on the surface of donor APC's (Figure.1.1). Indirect recognition occurs

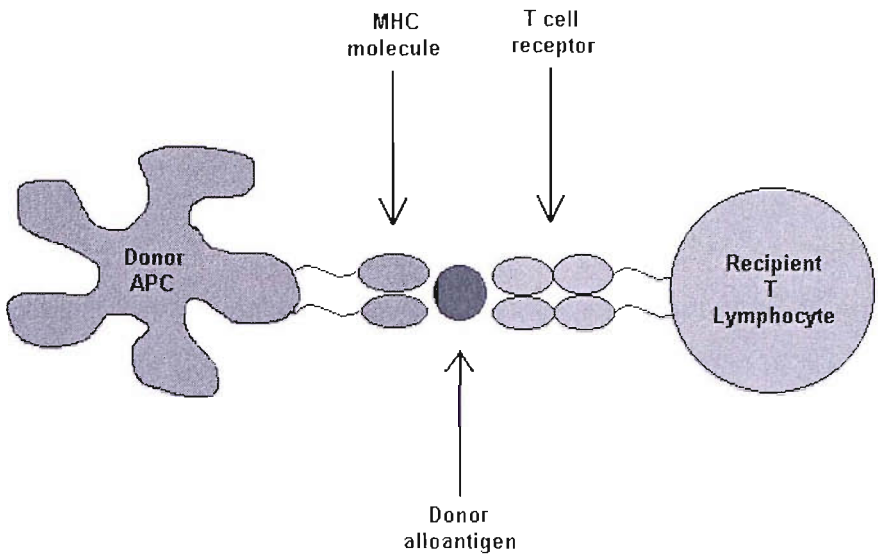


Figure.1.1 Direct antigen presentation

(APC-antigen presenting cell)

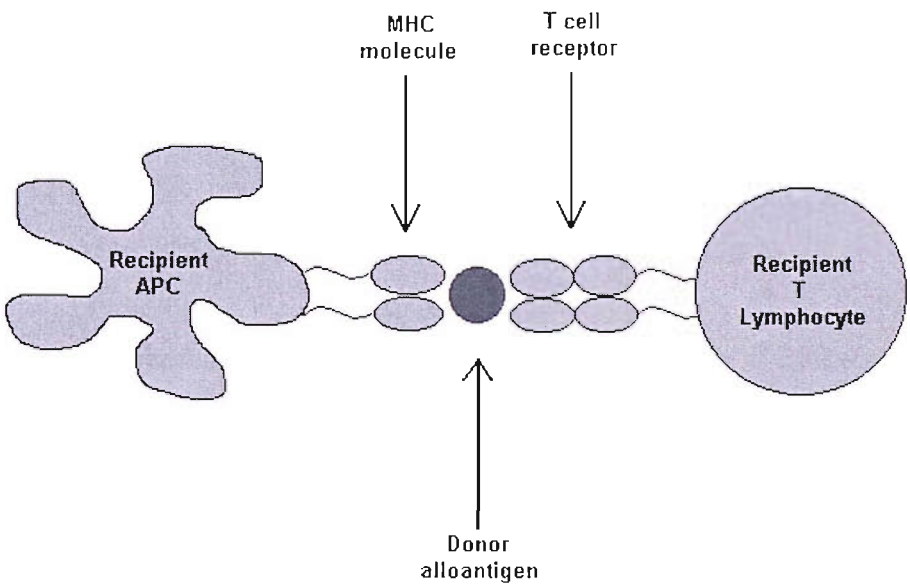


Figure.1.2 Indirect antigen presentation

(APC-antigen presenting cell)

when the recipient T lymphocytes recognize recipient MHC with donor alloantigen presented on the surface of recipient APC's (Figure.1.2).

1.1.2 T-cell Activation / Costimulatory Signals

The primary requirement for T-cell activation is interaction between its antigen – specific T-cell receptor (TCR) and antigen within an MHC protein of an APC, i.e recognition of 'non-self ', or aberrantly expressed self antigen. However, other co-stimulatory signals are required, since without them activation is incomplete and the T-cell becomes unresponsive to further antigenic stimulation, a state called anergy [Schwartz et al, 1990].

There are a number of T cell surface proteins that are required for its activation. The CD4 and CD8 proteins that bind to the APC Class II and Class I MHC molecules respectively cause intra-cellular signaling via two Src-family kinases [Rudd et al, 1994]. The leukocyte integrin, lymphocyte function-associated antigen-1 (LFA-1, previously known as CD11a and CD18), binds to ICAM-1 (formerly known as CD54) and ICAM-2 on T cell activation. Antibodies to LFA-1 inhibit the activation of both naïve and armed effector T cells [Dustin et al, 1989]. Other T cell surface proteins such as CD2 and CD5, which interact with CD58 (also known as LFA-3) and CD72 respectively on the APC, may also play a role in T cell activation.

One of the most important co-stimulatory signals is between the B7 proteins (B7.1 (CD80) and B7.2 (CD86)) on the APC and the CD28 receptor on naïve T cells and CTLA-4 on activated T cells. Their interaction has a different intracellular transduction mechanism (via the signaling proteins PI 3-kinase, GRB-2 and ITK) to the other T cell/APC interactions and is resistant to inhibition by the immunosuppressive drugs, Cyclosporin and Tacrolimus [Rudd et al, 1996].
Dendritic cells are the most potent APC in providing these costimulation signals to naïve T cells [Steinman et al, 1991].

1.1.3 Cytokines

Following antigen presentation in the presence of the required co-stimulatory signals, activated T cells produce soluble proteins called cytokines, which act locally in an autocrine and possibly paracrine fashion. A cascade of cytokines is produced that influence immune and inflammatory processes. Interleukin-2 (IL-2) is recognized as being one of the most important, driving T cell proliferation and differentiation.

Naïve CD4+ cells can differentiate upon activation into either Th1 or Th2 cells, which differ in their cytokine production and their function (Figure 1.3). Cytokine exposure influences development into Th1 or Th2 cells. IL-12 induces Th1 differentiation, which produces IL-2, IFN-γ, and TNF-β; IL-4 induces Th2 differentiation which produces IL-4, IL-5, IL-6 and IL-10. The Th1 phenotype is strongly associated with cell mediated immunity, and therefore acute allograft rejection, and the Th2 phenotype with humoral immunity. IFN-γ (Th1 product) inhibits Th2 differentiation while IL-4 and IL-10 (Th2 products) block Th1 differentiation. It has been suggested that this interplay between Th1 and Th2 via their cytokines plays an important role in the mechanisms underlying rejection and tolerance in transplantation [Nickerson et al, 1994]. This dichotomy into Th1 and Th2 is not absolute, as there are other categories, for example Th0 and Th3 with their own cytokine expression profiles. CD8+ T cells can also be differentiated into Tc1 and Tc2.

A complete understanding of the roles cytokines play in the immunological processes of allograft rejection is limited due to the redundancy within the system, and the pleiotrophic nature of cytokines.

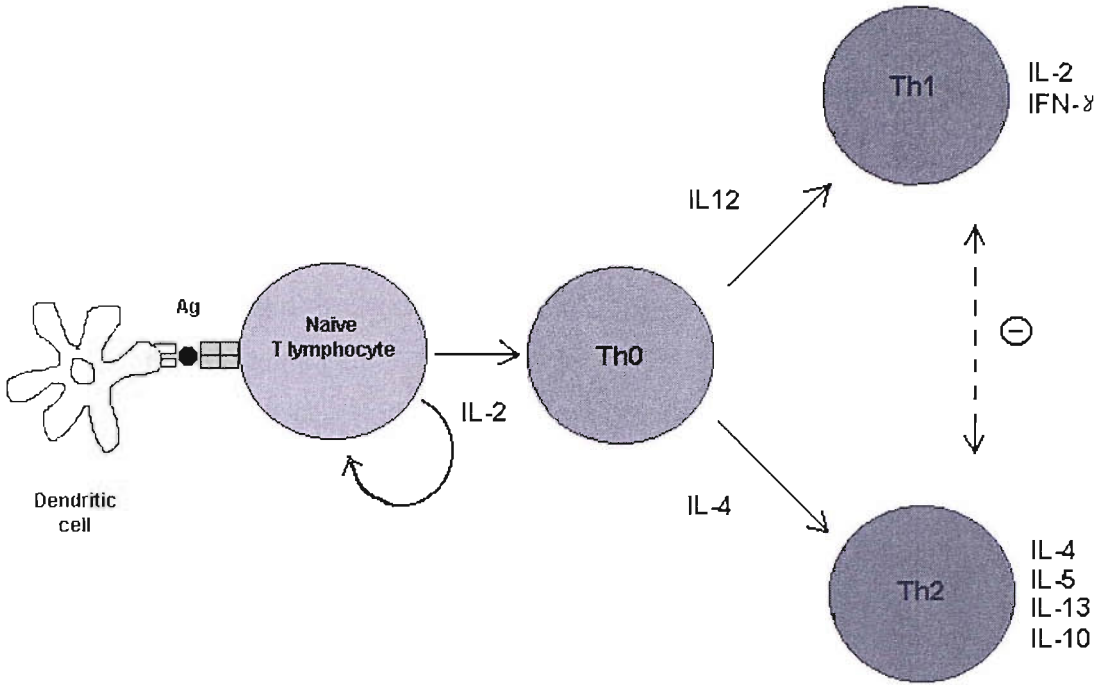


Figure.1.3. Differentiation of T cells into Th1 and Th2 cells, with different profiles of cytokine production

1.1.2 Immune Cell Migration / Adhesion Molecules

The immunological response of allograft rejection requires immune cell migration. Immature dendritic cells that have migrated from the bone marrow into the allograft via the blood, mature with exposure to alloantigen via indirect antigen presentation, and migrate downstream to the draining lymph nodes. Here they are mainly found in the T cell areas of the lymph node (also known as interdigitating reticular cells) where they activate the antigen specific naïve T cells. Naïve T cells circulate continuously from the blood stream to the lymph via the high endothelial venules (HEV) of lymph tissue to the T cell follicles coming in contact with antigen presenting cells in the lymphoid tissue and then return to blood. Once naïve T cells have recognized their specific

antigen on an antigen presenting cell they cease to migrate, undergo clonal proliferation, and mature into armed effector T cells. This takes several days and they then leave the lymphoid organ to re-enter the bloodstream so that they can migrate to the sites of inflammation and the source of the original antigen.

This migration requires T cell / endothelial cell recognition by expression of adhesion molecules. Up until a decade ago the main players were thought to be the selectins and integrins expressed by T cells recognized by the addressins and ICAM's expressed by endothelial cells respectively [Picker et al, 1992]. With the more recent characterization of chemokines and their receptors, these have been found to have an important role in lymphocyte migration and homing, as well as dendritic and other immune cells. This is discussed in more detail in sections 1.3 and 1.4 below, following section 1.2 on cytokine gene expression analysis previously undertaken in this laboratory .

1.2 PREVIOUS WORK IN THIS LABORATORY

Initial studies of the feasibility of sequential monitoring of peripheral blood immune markers began in this laboratory in 1995. Tan et al set out to establish whether sequential monitoring of peripheral T cell cytokine gene expression can correlate with, and reflect, the clinical immunological status of renal transplant patients. They used RT-PCR (Reverse Transcription-Polymerase Chain Reaction) combined with an ELISA method of detecting the PCR products. This was used to semi-quantitatively detect sequential changes in interleukin (IL-) 2, IL-4, IL-5, IL-10, IL-13 and IFNγ mRNA expression, and the expression of Granzyme B (GrB) and fas ligand (fas L), which are cytotoxic T lymphocyte (CTL) activation markers. Significant changes were detected, with detection of increased levels of mRNA transcripts of the Th2 cytokines IL-4, IL-5, and IL-13 at the time of rejection, and a decrease following successful anti-rejection therapy. Th1 cytokines IL-2 and IFNγ showed a reduction in their gene expression during the first week following transplantation in rejectors, and immediately following

anti-rejection therapy. This work showed that sequential analysis of cytokine gene expression in peripheral T cells may have potential in immunomonitoring in the early post-transplant period [Tan et al, 2001].

Subsequent work was performed using a quantitative real time ('TaqMan') PCR method for determining cytokine gene expression in T cells, at first, by re-analysis of the same T cell mRNA samples used by Tan et al, and then applied to PBMCs. The cytokine genes investigated were IL-4, IL-10, transforming growth factor (TGF) β1, tumour necrosis factor (TNF) α, and possible house keeping genes (also called endogenous control genes) β actin, β_2 microglobulin and transferrin. The earlier results using RT-PCR ELISA for IL-4 detection were confirmed as changes in IL-4 expression were detected prior to rejection. This was also found with TNFα [Gibbs et al, 2001]. The three possible housekeeping genes were found to be inappropriate for use in the transplant setting, since significant changes in their expression occurred either post-transplant, at the time of rejection or both [Gibbs et al, 2003].

Thus, from the above work IL-4, IL-5, IL-13 and TNF-α, may be candidates for immunomonitoring in transplantation. This work has been directed towards examination of chemokines and their receptors, with the aim of adding to this profile of potentially useful markers for immunomonitoring. A panel of potential endogenous control genes has also been examined, in order to develop a more robust and quantitative methodology.

1.3 CHEMOKINES AND CHEMOKINE RECEPTORS

Two essential components of acute rejection are the recruitment of leukocytes into the allograft from the circulation and migration of dendritic cells from the allograft into secondary lymphoid tissue [Lakkis et al, 2000]. The mechanism of this recruitment and migration are poorly understood but involves chemotactic factors produced by both the graft and inflammatory cells which attract and retain leukocytes within that graft. Chemokines (chemoattractant cytokines) and their receptors play a major role in this process, being expressed locally by the graft, leukocytes and dendritic cells.

1.3.1 Chemokines

Chemokines are small (6-14kDa), secreted proteins of which more than forty have been well characterized to date. They are basic proteins that have an affinity for heparin. Other terms used in the past for chemokines are intercrines, SIS (Small Inducible Secreted) and SCY (Small Cytokine). Chemokines have been discovered over the past fifteen years by different pathways, ranging from biological and biochemical identification to direct cDNA cloning.

Chemokine molecules are structurally similar having three β-pleated sheets, a C-terminal α helix, and four cysteine residues with a disulphide bond between the first and third, and second and fourth. An example of the primary structure of a chemokine protein is shown in Figure 1.4. The chemokine superfamily is divided into four families (outlined below) according to the position of these four cysteine residues in highly conserved positions. They can also be categorized into inducible and constitutive chemokines. Inducible chemokines, such as CCL2, CCL3, CCL4 and CCL5, are regulated by proinflammatory stimuli such as IL-1,TNF-α and LPS (lipopolysaccharide). They regulate innate and adaptive immune responses. The constitutive chemokines are involved in homeostatic activity, and are important in

lymphocyte and dendritic cell migration during immune surveillance [Fahy et al, 2001; Kunstfeld et al, 1998; Hancock et al, 2001; Fairchild et al, 1997; Kapoor et al, 2000]. Examples of constitutive chemokines are CCL21 and CCL19 (expressed by lymphatic endothelium and HEV respectively), both are important in guiding lymphocytes and dendritic cells to lymphoid organs [Gunn et al, 1998].

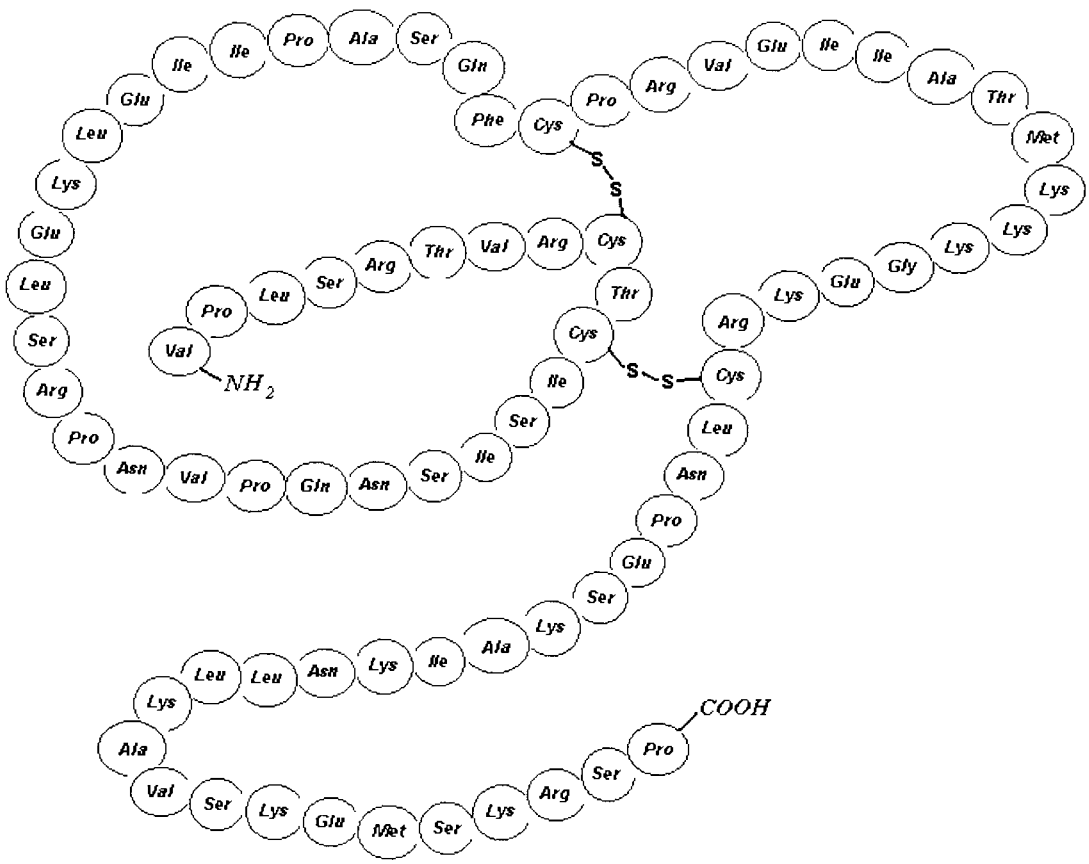


Figure.1.4. Primary Structure of CXCL10 Protein

Post translational modification of chemokines occurs by CD26. CD26, which was originally a marker for activated memory T lymphocytes [Morimoto et al, 1998], is a dipeptidyl-peptidase IV and is expressed in several cell types including epithelial and endothelial cells . Some chemokines are processed to a truncated form by CD26 cleaving dipeptides from the NH2 terminus with a proline or alanine at the penultimate

site. The effect on the biological activity is variable with some chemokine activity being unaffected whilst in others it leads to altered receptor binding and signaling. An example of this is truncated CCL5 which is a receptor antagonist for CCR1 and CCR3 but shows increased affinity for CCR5 [De Meester et al, 1999].

1.3.1.i α Chemokines

The α chemokines (CXC) have an amino acid (x) between the pair of cysteine residues nearest the N-terminus of the protein. In humans, the α chemokine genes are mostly found clustered on chromosome 4q21.1. CXCL12, CXCL14, and CXCL16 genes are found on chromosomes 10, 5, and 17 respectively. α chemokines generally attract neutrophils. They are produced as precursor molecules with cleavage of a signal sequence of 17 to 34 amino acids to mature proteins of 70 to 103 amino acids. These mature proteins are then secreted except for CXCL16 which is a membrane bound protein [Matloubian et al, 2000].

Subfamilies are now recognized within the CXC family, the ELR and non-ELR CXC subfamilies, which have functional differences. An "ELR" CXC chemokine contains a glutamate-leucine-arginine amino acid motif between the N-terminus and the first cysteine. They bind with high affinity to CXCR1 and/or CXCR2 receptor and show potent chemotactic activity against neutrophils eg. CXCL8 (IL-8), and some have angiogenic effects [Yoshida et al, 1997]. Non-ELR CXC chemokines attract activated T-lymphocytes [Taub et al, 1995] and have angiostatic effects [Streiter et al, 1995]. Thus, expression of these angiogenic/static chemokines may influence the microvasculature within an allograft. The α chemokines are listed in Table 1.1.

Name	Synonyms	Receptor	ELR	Chromosome
CXCL1	*groα , MGSA , NAP-3 SCYB1*	CXCR2	+	4q21.1
CXCL2	*groβ , MIP-2α , SCYB2*	CXCR2	+	4q21.1
CXCL3	*groγ , SCYB3 , MIP-2β*	CXCR2	+	4q21.1
CXCL4	*PF- 4 , SCYB4*		–	4q21.1
CXCL5	*ENA-78 , SCYB5*	CXCR1 CXCR2	+	4q21.1
CXCL6	*GCP-2 , SCYB6*	CXCR1 CXCR2	+	4q21.1
CXCL7	*PBP , CTAP- III , β-TG NAP-2 , SCYB7 , low affinity platelet factor-4*	CXCR2	+	4q21.1
CXCL8	*IL-8 , MDNCF , NAP-1, GCP-1 , SCYB8*	CXCR1 CXCR2	+	4q21.1
CXCL9	*Mig , SCYB9*	CXCR3	–	4q21.1
CXCL10	*IP-10 , SCYB10*	CXCR3	–	4q21.1
CXCL11	*I-TAC , IP-9 , βR1 SCYB11 , H174*	CXCR3	–	4q21.1
CXCL12	*SDF-1α , SDF-1β, PBSF SCYB12*	CXCR4	–	10q11.21
CXCL13	*BLC , BCA-1 , SCYB13*	CXCR5	–	4q21.1
CXCL14	*BRAK , BMAC SCYB14*		–	5q31.1
CXCL16		CXCR6	–	17p13

Table.1.1 CXC / α Chemokines

Gro – growth related oncogene ; *Scy* – small cytokine ; *SDF* – Stromal cell derived factor ; *PBSF* – Pro-B cell growth-stimulating factor ; *I-TAC* – Interferon-inducible T cell and chemoattractant ; *Mig* – momokine induced by interferon γ ; *MDNCF* – Monocyte-derived neutrophil chemotactic factor ; *NAP* – Neutrophil activating protein ; *TG* – Thromboglobulin ; *BLC* – B lymphocyte chemoattractant ; *CTAP* – Connective tissue activating peptide ; *PF* – Platelet factor ; *BCA* – B cell attracting chemokine ; *MGSA* – melanoma growth-stimulating activity ; *ENA* – Endothelial-derived neutrophil attractant ; *GCP* – Granulocyte chemotactic protein ; *PBP* – Platelet Basic Protein ; *BRAK* – breast and kidney ; *BMAC* – B cell and monocyte-activating chemokine.

1.3.1.ii β Chemokines

The β chemokine group (CC) has no amino acid between the cysteine residues, and its genes encoding these chemokines are mostly found clustered on chromosome 17q11.2 (for exceptions see β/CC chemokine table 3), mainly exerting their effects on mononuclear cells and dendritic cells. The β chemokines are listed in Table 1.2.

Name	Synonyms	Receptor	C/I	Chromosome
CCL1	*I-309*	CCR8	I	17q11.2
CCL2	*MCP-1, MCAF TDCF*	CCR2	I	17q11.2
CCL3	*MIP-1α, LD78α GO519-1*	CCR1 CCR5	I	17q12
CCL4	*MIP-1β, ACT-2 SIS-γ LAG-1*	CCR5	I	17q12
CCL5	*RANTES, SIS δ*	CCR1 CCR3 CCR5	I	17q12
CCL7	*MCP-3, NC28, FIC*	CCR1 CCR2 CCR3	I	17q11.2
CCL8	*MCP-2, HC14*	CCR3 CCR5	I	17q11.2
CCL11	*Eotaxin*	CCR3	I	17q11.2
CCL13	*1 MCP-4, Ckβ10, NCC-1*	CCR2 CCR3	I	17q11.2
CCL14	*HCC-1, NCC-2, Ckβ1*	CCR1 CCR5	C	17q12
CCL15	*HCC-2, Lkn-1, MIP-1δ MIP-5*	CCR1 CCR3	C	17q12
CCL16	*HCC-4, LEC, LCC-1, NCC-4*	CCR1 CCR2	C	17q12
CCL17	*TARC, STCP-1*	CCR4	C/I	16q13
CCL18	*DC-CK1, PARC, AMAC-1 , MIP-4*		C	17q12
CCL19	*MIP-3β, ELC, exodus-3*	CCR7	C/I	9p13.3
CCL20	*MIP-3α, ARC, exodus-1*	CCR6	C/I	2q36.6
CCL21	*6Ckine, SLC, exodus-2*	CCR7	C	9p13.3
CCL22	*MDC, STCP-1*	CCR4	C/I	16q13
CCL23	*MPIF-1, Ckβ8, Ckβ8-1 MIP-3*	CCR1	I	17q12
CCL24	*Eotaxin-2, MPIF-2*	CCR3	I	7q11.23
CCL25	*TECK*	CCR9	C	19p13.3
CCL26	*Eotaxin-3, MIP-4α*	CCR3	I	7q11.23
CCL27	*CTACK, Eskine*	CCR10	C	9p13.3
CCL28	*MEC*	CCR10 CCR3	I	5p12

Table. 1.2 CC / β Chemokines

C – Constitutive Chemokine ; **I** – Inflammatory/Inducible Chemokine ; MCP-Monocyte chemoattractant protein ; MIP-Macrophage inflammatory protein ; RANTES-Regulated upon activatio, normal T cell expressed and secreted ; TARC-Thymus and activation-regulated chemokine ; DC-CK-Dendritic cell derived chemokine ; PARC-Pulmonary and activation regulated chemokine ; SLC-Secondary lymphoid tissue chemokine ; MDC-Macrophage derived chemokine ; TECK-Thymus expressed chemokine ; HCC-Haemofiltrate CC chemokine ; MPIF-Myeloid progenitor inhibitory factor.

1.3.1.iii γ Chemokines

The γ (C) family (Table 1.3) has only one cysteine residue near the N-terminus and so far lymphotactin α and β are the only family members. Lymphotactin attracts T lymphocytes and its gene maps to human chromosome 1q23 [Kelner et al, 1994; Kennedy et al, 1995].

Name	Synonyms	Receptor	Receptor Expression	Chromosome
XCL1	Lymphotactin α ,SCM-1α, ATAC	XCR1	NK, T	1q23
XCL2	Lymphotactin β, SCM-1β, ATAC			1q23-q25

Table. 1.3 C / γ Chemokines

1.3.1.iv δ Chemokines

The δ (CX3C) chemokine family (Table1.4) is made up of fractalkine (also called neurotactin) which has different properties to the other chemokine families [Bazan et al, 1997]. It has three amino acid residues between the two cysteines and is an integral membrane protein. The CX3C gene maps to chromosome 16. The δ (CX3C) chemokine family functions as an adhesion molecule, possibly aiding dendritic cell / T cell interactions, and a chemoattractant for lymphocytes [Bazan et al, 1997; Papadopoulos et al, 1999; Kanazawa et al, 1999].

Name	Synonyms	Receptor	Receptor Expression	Chromosome
CX3CL1	Fractalkine, CX3C ligand	CX3CR1	NK, T, Mo	16q13

Table. 1.4 CX3C / δ Chemokine

1.3.2 Chemokine Receptors

Chemokines mediate their effect through seven trans-membrane spanning G-protein coupled, Bordetella pertussis toxin sensitive receptors (Figure 1.5). They are similar to other seven-transmembrane-spanning G-protein receptors but have some defining features [Baggiolioni et al, 1997].

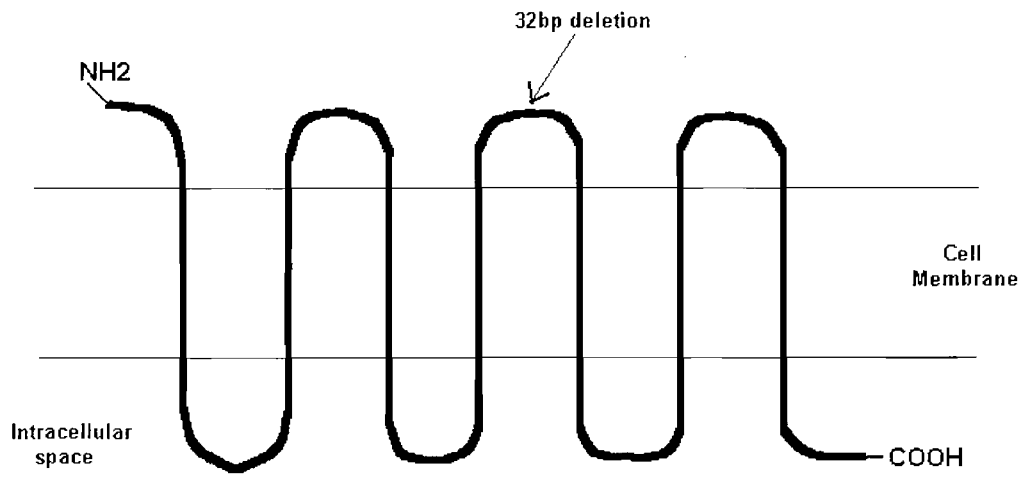


Figure. 1.5 Schematic representation of CCR5 receptor protein configuration showing position of 32bp deletion variant

The CXC chemokine receptors have in the range of 36-77% identical amino acid sequences and the CC chemokine receptors 46-89% [Baggiolioni et al, 1997]. The ligands of the CXC and CC chemokine receptors, with the cells on which they are expressed and location of their genes are shown in Tables 1.5 and 1.6 respectively. Chemokine receptors are not specific for one particular chemokine, several chemokines can bind more than one receptor. For example, CCL5 binds to CCR5, CCR1 and CCR3, and CCL3 binds to CCR5 and CCR1. CC chemokines do not bind CXC receptors and vice versa. However the Duffy antigen receptor for chemokines

Name	Ligand	Leukocyte expression	Chromosome
CXCR1	CXCL5, CXCL6, CXCL8	Mo, Neut	2q34-q35
CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8	Mo, Neut, Eo	2q34-q35
CXCR3	CXCL9, CXCL10, CXCL11	B, T	Xq13
CXCR4	CXCL12	Thy, B, T, iDC, mDC, Mo, Neut, Plts	2p
CXCR5	CXCL13	B , T	11
CXCR6	CXCL16	NK, B, T, iDC, mDC	3p21.3

Table.1.5 CXC / α Chemokine Receptors
T – T Lymphocyte ; B – B Lymphocyte ; Th – T helper lymphocyte ; iDC – immature dendritic cell ; Tmem – Memory T lymphocyte ; Mo – Monocyte ; Neut – Neutrophil ; Eo – Eosinophil ; Thy – Thymocyte ; mDC – mature dendritic cell ; Plts – Platelets ; NK – natural killer cell

Name	Ligand	Preferential Leukocyte Expression	Chromosome
CCR1	CCL3, CCL5, CCL7, CCL8, CCL14, CCL15, CCL16, CCL23	T_{h1}, T_{h2}, iDC, NK, Mo, Ba, Eo, Neu	3p21
CCR2	CCL2, CCL7, CCL8, CCL13, CCL16	T_{h1}, T_{h2}, Ba, Mo, NK	3p21
CCR3	CCL5, CCL7, CCL11, CCL13, CCL15, CCL24, CCL26	T_{h2}, Eo, Ba	3p21
CCR4	CCL17, CCL22	T_{h2}, Ba, NK, T_{mem}, Tc, T_{hy}	3p22
CCR5	CCL3, CCL4, CCL5, CCL8, CCL14	T_{h1}, iDC, Mo, Tc, T_{hy}	3p21
CCR6	CCL20	T_{mem}, iDC, B	6q27
CCR7	CCL19, CCL21	T_{naiv}, B, Mdc, T_{hy}	17q12-21
CCR8	CCL1	T_{h2}, Mo, T_{hy}	3p22-p23
CCR9	CCL25	T_{mem}, B, T_{hy}	3p21.3-22
CCR10	CCL27, CCL28	T_{mem}	17q21.1-q21.3
CCR11	CCL19, CCL21, CCL25	iDC, T	3p22

Table. 1.6 CC / β Chemokine Receptors
T – T lymphocyte ; B – B lymphocyte ; Th – T helper lymphocyte ; T_{mem} – Memory T lymphocyte ; T_{naiv} – Naïve T lymphocyte ; iDC – immature dendritic cell ; mDC – mature dendritic cell ; NK – natural killer cell ; Ba – Basophil ; Eo – Eosinophil ; Mo – Monocyte ; T_{hy} – Thymocyte

(DARC), which is the recognition structure for the malarial parasite Plasmodium vivax found on red blood cells, will bind both of these chemokine families. It does not confer any signal on ligand binding and is thought to possibly act to 'mop-up' circulating chemokines to keep their levels low. In general, the proinflammatory chemokine receptors tend to be more promiscuous with their ligand binding whereas the receptors involved in normal leucocyte trafficking have relatively few ligands.
Chemokine receptors are widely expressed on lymphocytes and other immune cells as well as by non haematopoietic tissue such as human mesangial cells, endothelial cells, epithelial cells, microglial cells and neuronal cells [Baggiolioni et al, 1997; Murphy et al, 2000; Rossi et al, 2000; Keane et al, 1999; Nelson et al, 2001]. Following ligand binding, chemokine receptors generally undergo internalization, phosphorylation and activation of Gα protein subunits. The subsequent signal transduction cascade leads to activation of phospholipase and generation of inositol (1,4,5)-triphosphate and diacylglycerol [Murphy et al, 2000; Segerer et al, 2000]. This leads to a transient rise in intracellular calcium and activation of protein kinase C bringing about a kinase signal transduction cascade.

1.3.3 Effects of chemokines and their receptors

Chemokines are involved in inflammatory cell migration but also influence the development and proliferation of some of these inflammatory cells. They have also been shown to play an important role in viral infections and tumour activity. These actions are discussed below in more detail.

1.3.3.i Role in Cell Adhesion

Leukocytes are recruited into tissue from the circulation by adhesion molecule interactions between themselves and the vessel endothelium using the adhesion molecules integrin and intracellular adhesion molecules (ICAM) respectively. An initial interaction occurs between the leukocyte and selectin family of adhesion proteins

[Adams et al, 1994; Tedder et al, 1995], which slows the leukocyte, bringing it in contact with chemokines within the endothelial glycocalyx. Chemokines are immobilized on proteoglycans in the endothelial glycocalyx [Tanaka et al, 1993; Rot et al, 1996] which are produced by endothelial, epithelial and activated leukocytes. The chemokines bind to their specific G-protein coupled receptors on the leukocytes activating them. This process of activation leads to increased integrin expression [Vaddi et al, 1994] and the leukocyte becoming adherent to the endothelium. Thus, chemokines produced by inflammatory cells and endothelial cells within the vicinity of an inflammatory reaction are immobilized on the surface of the endothelium, allowing exposure to circulating leukocytes. The subsequent chemokine/receptor interaction on the leukocyte brings about its recruitment into the tissue across the endothelium and then it migrates to the area of inflammation by haptotaxis under the influence of chemokines. A diagramatic representation of T lymphocyte migration is shown in Figure 1.6.

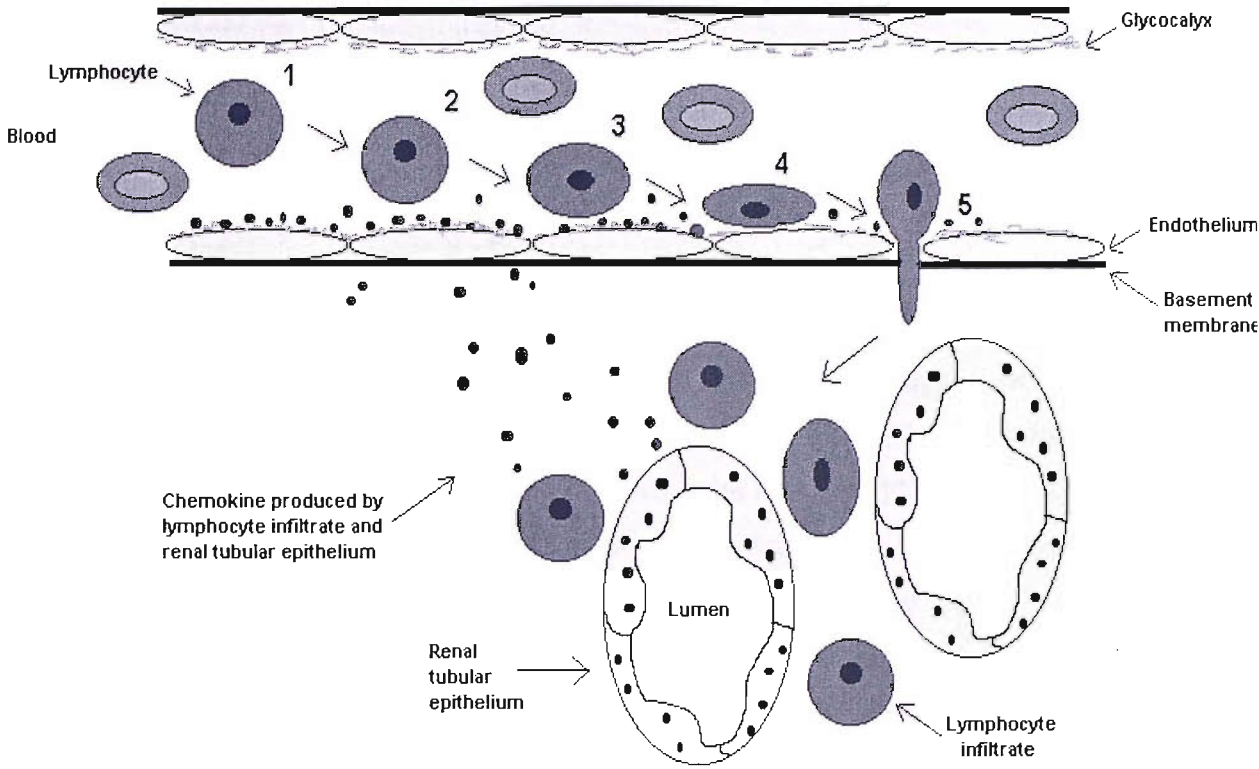


Figure.1.6 Diagramatic representation of circulating T lymphocyte migration to inflammatory area of renal allograft rejection.

1. Circulating blood lymphocyte.

2. Tethering of lymphocyte to vessel endothelium by selectin-carbohydrate interaction.

3. Rolling of lymphocyte on endothelium brings it in close proximity to chemokines immobilized within the endothelial glycocalyx and produced at the site of inflammation by activated lymphocytes, vessel endothelial and tubular epithelial cells.

4. Chemokine/lymphocyte receptor interaction leads to up regulation of integrins and lymphocyte becomes firmly adherent to endothelium.

5. Diapedesis of lymphocyte through endothelium and basement membrane to area of inflammation.

1.3.3.ii Role in Dendritic Cell Migration

Dendritic cells are important antigen-presenting cells found in peripheral tissues, lymph nodes and solid organs. They are thought to originate from precursors in the bone marrow and migrate via the blood to tissues. A diagramatic representation of dendritic cell and T lymphocyte migration is shown in Figure 1.7. Chemokines released in transplanted allografts play an important role in recruiting immature dendritic cells from the blood into a graft. Here they can acquire antigen, mature and drain to lymphoid tissue where they activate naïve B and T cells. Their importance in allograft rejection is demonstrated by cardiac allografts that could be accepted indefinitely in recipient mice that lack secondary lymphoid tissue. This suggests the immune response to an allograft requires dendritic cell trafficking to secondary nodes [Lakkis et al, 2000]. They express a number of chemokine receptors, some preferentially in dendritic cells, and also produce chemokines. Immature dendritic cells express the inducible proinflammatory chemokine receptors CCR1,CCR2,CCR5 and CXCR1, which allow them to migrate to inflamed tissue [Murphy et al, 2000; Sallusto et al, 2000; Sozzani et al, 2000]. With exposure to alloantigen and maturation there is a decrease in expression of these receptors, and an increase in CCR4, CXCR4 and CCR7 expression, the homeostatic constitutive receptors. These receptors help direct the mature dendritic cell to secondary lymphoid tissue [Murphy et al, 2000; Sallusto et al, 2000; Sozzani et al, 2000; Forster et al, 1999]. Activation of naïve T cells by these dendritic cells decreases T cell expression of CXCR4 and CCR7 and increases CCR5, CCR3, CXCR3 and CCR8 expression. This facilitates migration of the activated T cells to the sites of inflammation. There is a correlation between expression of chemokine receptor patterns and migration to specific anatomical sites [Campbell et al, 2000].

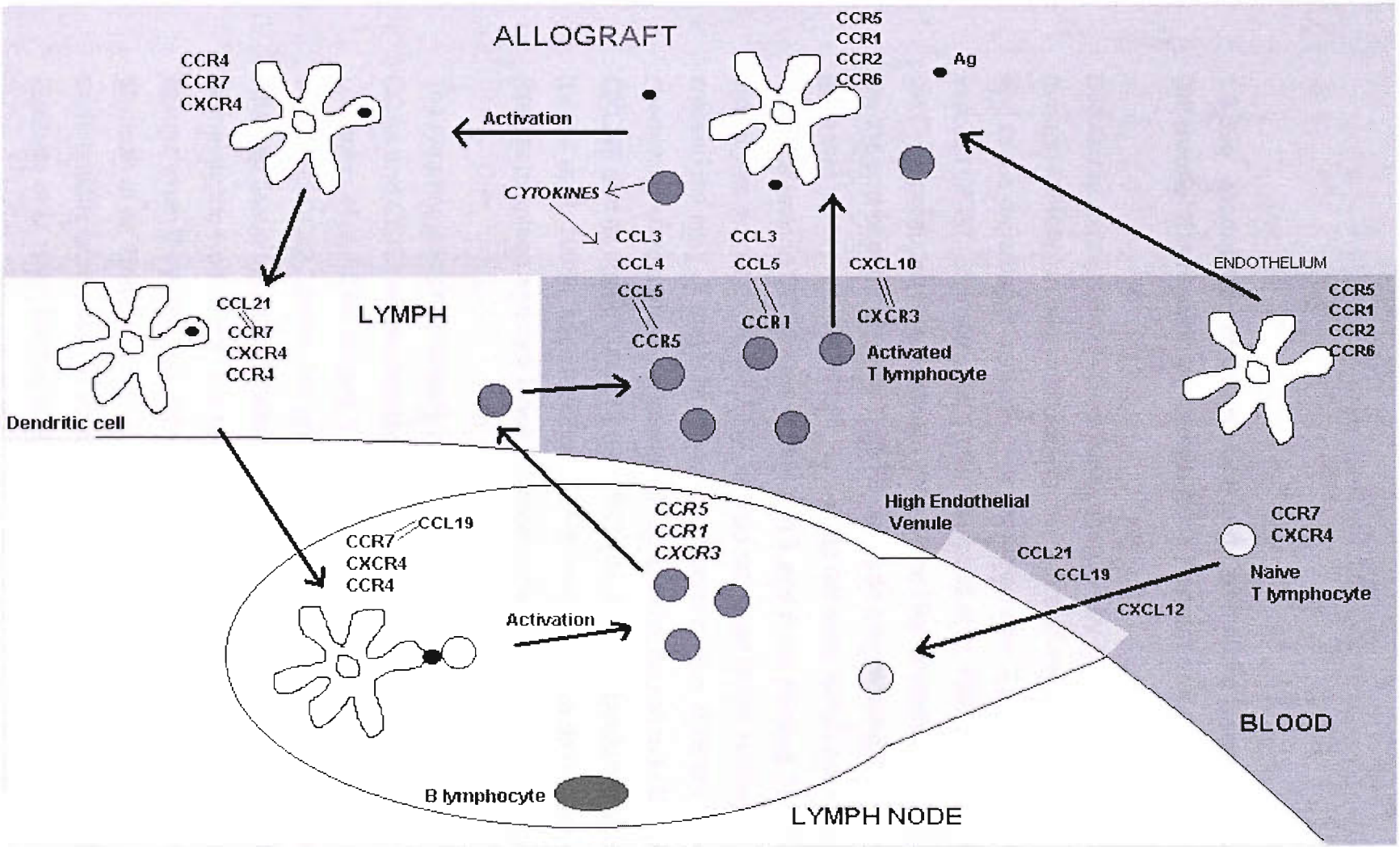


Figure 1.7 Diagramatic representation of dendritic cell and T lymphocyte migration, and chemokine expression

1.3.3.iii Modulation of Chemokine and Chemokine Receptor Expression and Differential Expression by Lymphocyte Populations

Differential expression of chemokine receptors by different T and B cell populations, through stimulation by cytokines and their state of activation, influences their migratory and tissue recruitment patterns. For example naïve T and B lymphocytes selectively express CCR7 [Sallusto et al, 1998; Campbell et al, 1998]. A ligand of CCR7, CCL21 (SLC), is produced by endothelial cells of the High Endothelial Venules (HEV) which are the port of entry of naïve T and B cells into lymph nodes [Gunn et al, 1998]. In mice that fail to produce CCL21, T cells do not enter lymph nodes [Gunn et al, 1999] and mice lacking CCR7 have a defect in T and B cell homing. Neutrophils and monocytes do not express CCR7, and do not enter lymph nodes via the HEV. Having crossed the HEV, T and B lymphocytes migrate to their different sites by specific chemokine chemotaxis. An important receptor for migration to B cell follicles is CXCR5 and its ligand CXCL13 (BLC/BCA-1), which is produced by stromal cells [Legler et al, 1998]. Mice lacking CXCR5 have defective development of B cell follicles in spleen and lack inguinal lymph nodes [Forster et al, 1996].

Th1 lymphocytes preferentially express CXCR3, CCR5 and CCR1, whereas in vitro CCR4 and CCR3 are associated with Th2 cell differentiation [Bonecchi et al, 1998; Loetscher et al, 1998] (Figure 1.8). This is in keeping with the fact that CCR5 ligands, CCL5 and CCL3, attract Th1 and not Th2 cells. Thus chemokines produced by inflamed tissue influence whether Th1 versus Th2 cells infiltrate the tissue and ultimately the type of immune response. For example, eotaxin is produced by mucosal tissues when they undergo allergic inflammation [Jose et al, 1994; Li et al, 1999; Maclean et al, 1996; Ganzalo et al, 1996]. The receptor for eotaxin is CCR3, which is preferentially expressed on eosinophils [Ponath et al, 1996] basophils and Th2 cells [Sallusto et al 1997; Gerber et al, 1997] allowing them to co-localise at sites of allergic inflammation.

In rheumatoid arthritis, which is a Th1 type inflammatory reaction, virtually all the lesion infiltrating T cells express CCR5 and CXCR3. The cytokines produced by Th1 cells (eg. IFN-γ) upregulate Th1 attracting chemokines whilst antagonizing Th2 attracting chemokines. CXCL10, the ligand for CXCR3, is induced by IFN-γ and expressed in Th1 lesions [Kaplan et al, 1987; Luster et al, 1987].

Chemokine receptors can be modulated on T lymphocytes by cytokines and TCR triggering [Loetscher et al, 1996; Sallusto et al, 1999]. Sallusto et al showed that within hours following TCR stimulation, the chemokine receptors CCR1, CCR2, CCR3, CCR5, CCR6 and CXCR3 were down regulated at protein and mRNA levels, and CCR7, CCR4, CCR8 and CXCR5 upregulated. This receptor modulation changes their migratory capacity allowing the antigen activated T cells to migrate from the tissue to the draining lymph nodes. The CCR1, CCR5, and CXCR3 inflammatory chemokine receptors direct T cells from the blood into inflamed tissues before encountering antigen. The tissue cells as well as the infiltrating lymphocytes in areas of inflammation secrete chemokines that attract lymphocytes from the blood.

Activated lymphocytes express chemokines as well as chemokine receptors. CCL3, CCL4, XCL (lymphotactin), CXCL8 (IL-8) and CCL5 have been shown to be up-regulated by activation of lymphocytes at either the protein or mRNA level. Upregulation of some of these chemokines has been shown to be augmented by co-stimulation of CD3 and CD28 [Riley et al, 1997].

Chemokine receptors can be modulated by the Th1 inflammatory and Th2 anti-inflammatory cytokines. IL-2 can induce both CCR1 and CCR2 in T-lymphocytes and NK cells but cannot induce chemokine production [Loetscher et al, 1996; Polentarutti et al, 1997]. TNF and IL-1 down regulate CCR2 in human monocytes [Sica et al, 1997]. The anti-inflammatory (Th-2) cytokine IL-10 can up-regulate CCR1, CCR2 and CCR5 in human monocytes. IFN-α up-regulates CCR1 and CCR3 in human neutrophils [Bonecchi et al, 1999]. These studies indicate that locally produced

cytokines may regulate the composition of leuykocyte infiltrate by influencing chemokine receptor expression.

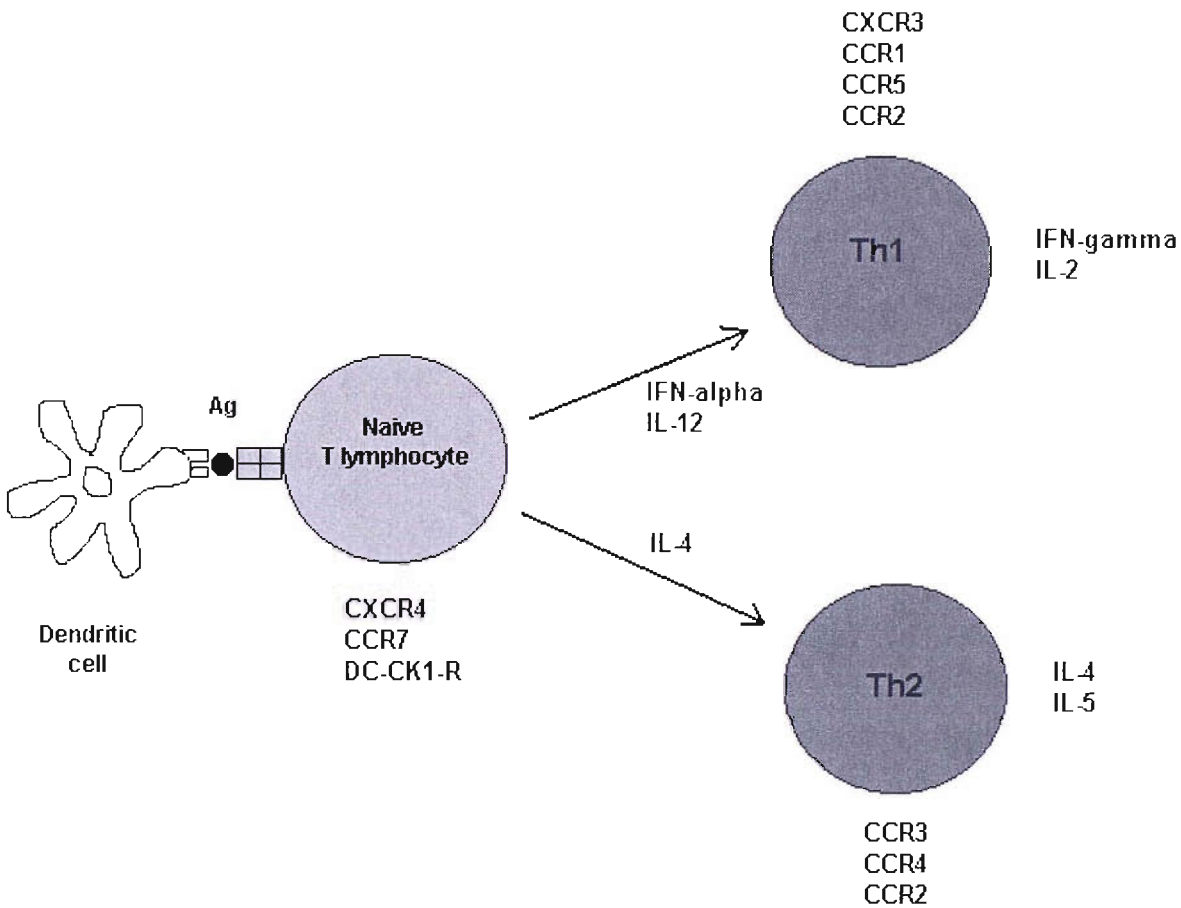


Figure.1.8 T cell Polarisation and Chemokine Receptor Expression

1.3.3.iv Role in Lymphocyte Activation, Differentiation and Proliferation

In addition to their chemotactic effect, chemokines have a role in lymphocyte differentiation, activation and proliferation [Taub et al, 1996; Bacon et al, 1995]. Taub et al showed that the β chemokines CCL3, CCL4, CCL5 and CCL2 (MCP-1) have a costimulatory role in human TCR-mediated activation. They showed in vitro that costimulation with one of these β-chemokines and monoclonal antibodies (mAb) to CD3 led to T-cell proliferation, as with various cytokines. This co-stimulatory activity was found to function at least in part by induction of CD25 expression and IL-2 production. CCL3 and CCL2 are less potent in inducing a proliferative response than CCL5 and CCL4. They further showed that these chemokines may induce an increase in intracellular calcium to promote this T-cell activation.

1.3.3.v Role in Viral Infection

Chemokine receptors play a role in entry of HIV-1 virus into CD4+ lymphocytes. It has been shown that CCR5 is a co-receptor for M-tropic HIV-1 and CXCR4 a co-receptor for the T-tropic strain of HIV-1 [Bleul et al, 1996; Oberlin et al, 1996]. There is a 32bp deletion in the human CCR5 gene (CCR5Δ32) in 1% of Caucasian individuals producing a mutant receptor that is subsequently not expressed on the cell surface which confers resistance to infection by HIV-1 [Samson et al, 1996]. In addition a study by Fischereder et al showed prolongation of human renal allograft survival in patients that were CCR5Δ32 homozygotes [Fischereder et al, 2001].

Some viruses encode chemokines, chemokine receptors and chemokine inhibitors. Human cytomegalovirus contains the US28 gene which encodes a functional receptor for CCL2, CCL3, CCL4 and CCL5 [Gao et al, 1994] and is involved in smooth muscle cell migration [Streblow et al, 1999]. Strains of cytomegalovirus also encode chemokine like molecules, eg. vCXC-1, which binds to human CCR2 inducing calcium mobilization, chemotaxis and degranulation of human neutrophils [Penfold et al, 1999].

Human herpesvirus 8 (Kaposi's sarcoma herpes virus) encodes two β chemokine-like proteins vMIP-I and vMIP-II (viral macrophage inflammatory protein) [Boshoff et al, 1997]. These chemokines are attractants of Th2 lymphocytes, interacting with CCR3, CCR4 and CCR8 receptors. This may be a strategy to subvert immunity from effective Th1 defence mechanisms to Th2 responses.

1.3.3.vi Role in Lymphocyte Development

There is evidence to suggest that chemokines and their receptors play a role in lymphocyte development as this involves lymphocyte movement through different tissues. Specific chemokines are expressed by lymphoid tissues - eg. CCL17 (TARC), CCL18 (DC-CK-1) - that attract lymphocytes bearing the appropriate receptor. Animal models that lack a specific chemokine receptor have shown poor development of particular lymphoid tissues [Legler et al, 1998]. Maturation of haematopoietic progenitors into mature T lymphocytes within the thymus seems to require differential expression of chemokines and their receptors. The receptor CCR9 is thought to have a role in retaining thymocytes in the thymus until completion of their maturation process. Cortical and medullary thymocytes respond to CCL25 (TECK), a CCR9 ligand, which they lose late in maturation before leaving the thymus with upregulation of L-selectin [Vicarrri et al, 1997; Zaballos et al, 1999; Campbell et al, 1999].

1.3.3.vii Chemokine Expression in Tumours

Most tumours produce CC and CXC chemokines. There is evidence that in human tumours, CCL2 and CCL5, amongst others in the CC family, are major determinants of macrophage and lymphocyte infiltration in carcinoma of the ovary, breast, cervix and melanoma [Negus et al, 1995; Luciani et al, 1998; Valkovic et al, 1998; Luboshits et al,

1999]. These cell infiltrates on the one hand may help reject the tumour or on the other provide growth factors promoting tumour progression.
Chemokine receptors are expressed by tumour cells, as well as the infiltrating leukocytes. Chemokine agonists induce migration and proliferation of these tumour cells. It may be that tumour cells use chemokine gradients to metastasise around the body. Breast carcinoma cells and melanoma cells express CCR7 and CXCR4 and may be important for migration to lymphoid organs [Muller et al, 2001].

Another influence of chemokines on malignancy is their effect on neovascularisation of the tumour. Most ELR+ CXC chemokines are chemotactic for endothelial cells and are angiogenic, whereas the non-ELR chemokines can inhibit these angiogenic effects. The balance between these produced by the tumour and infiltrating leukocytes may determine the level of vascularisation and hence tumour progression [Streiter et al, 1995].

1.4 LITERATURE REVIEW OF THE CHEMOKINES AND CHEMOKINE RECEPTORS STUDIED IN THIS PROJECT AND THEIR ROLE IN TRANSPLANT IMMUNOLOGY

Studies have demonstrated a spatial and temporal correlation between chemokine production and leukocyte infiltration into allografts in both animal models and humans [Pattison et al, 1994; Adams et al, 1996; Cockwell et al, 1997]. Certain chemokine receptor antagonists have been shown to attenuate leukocyte infiltration into allografts and reduce the incidence of rejection [Grone et al, 1999]. There are also animal knockout models that lack some functional chemokine receptor, showing reduced incidence of allograft rejection [Gao et al, 2000].

Each chemokine and chemokine receptor investigated in this project will be described below. Each was selected due to the evidence that they play a role in lymphocyte trafficking and early recruitment into a rejecting allograft. There is evidence that other

chemokines and chemokine receptors play a role in transplant immunology eg. XCL and CCL2, but were not included in this project due to limited time and cDNA quantity. None was excluded from the study based on published evidence, but rather lack of published evidence for a role in transplant immunology.

1.4.1 CCL3 (MIP-1α)

CCL3 (also known as MIP-1α, SCYA3, SIS-alpha, EP and LD78) belongs to the β chemokine family and is a 69 amino acid acidic protein with a 7.8 kDa molecular mass. The CCL3 gene is located at 17q11-q21, and shows a 55% homology to CCL4 (MIP-1β) at the amino acid level. In 1987, Wolpe et al purified a murine heparin-binding protein secreted by macrophages in response to LPS stimulation, and was referred to as MIP-1 [Wolpe et al,1987]. Sherry et al resolved MIP-1 to be two components, MIP-1α (CCL3) and MIP-1β (CCL4), which are highly homologous [Sherry et al, 1988]. The genes for human CCL3 and CCL4 were identified by Irving et al in 1990, and were found to be closely linked in the human genome, separated by 14kb [Irving et al, 1990].

The receptors for CCL3 are CCR1 and CCR5. CCL3 has been shown to be chemotactic for human monocytes in vitro [Wang et al, 1993] and murine dendritic cells [Foti et al, 1999]. It has been demonstrated to be a more potent chemoattractant for cytotoxic as well as CD4+ T cells than CCL4, and also has a chemoattractant effect on B cells. CCL3 attracts naïve and memory T cells (CD45) whereas CCL4 has a preferential effect on naïve (CD45RA) T cells [Schall et al, 1993]. In vivo, using a human skin grafted to SCID mouse model, Kunstfeld et al showed that the injection of human CCL3 into the graft attracted significant numbers of human T cells from the peritoneal cavity into the allograft. This also occurred with CCL2, but minimally with CCL5 and CXCL10 [Kunstfeld et al, 1998].
In addition to its chemoattractant effects CCL3 may have a role in T cell activation and proliferation. Taub et al demonstrated that CCL3 is capable of costimulating T cell

proliferation and IL-2 production in the presence of CD3 ligation in vitro. However its costimulatory activity was less potent than CCL4 and CCL5, and significantly less than the cytokines IL-1 and IL-7 [Taub et al, 1996].

CCL3 has been shown to be produced by macrophages, activated lymphocytes, mast cells, and endothelial cells [Adams et al, 1996; Schall, 1991; Zipfel et al, 1989; Lukacs et al, 1996]. It is an important chemoattractant of immune cells to sites of inflammation in vivo. In a mouse allergic airway model, Lukacs et al demonstrated CCL3 production by mast cells with subsequent eosinophil accumulation [Lukacs et al, 1996].

CCL3 has been demonstrated to possibly have a role in allograft rejection, by recruitment of T cells into the graft. In a murine allogeneic skin graft model Kondo et al demonstrated, using Northern blot analysis, early expression of CCL3 and CCL4 in the rejection process (day 3). Levels of CCL3 and CCL4 then declined with increased CCL5 and CXCL10 expression late in the rejection process (day 9) [Kondo et al, 1996]. A different pattern was seen in a heterotopic heart transplant model in mice using Northern blot analysis [Fairchild et al, 1997]. In this study, intra-allograft expression of CXCL10 was prominent early after transplantation (day 3) and maintained through to day 8. CCL3, CCL4 and CCL5 by contrast were detected at low levels by day 3 but high levels by day 8 (complete rejection occurring between days 8 and 13). When immunosuppressed (graft survival approx. 60 days), CCL3 and CCL4 were undetectable whereas CCL5 and CXCL10 expression was high. The differences in chemokine expression patterns between these two studies may be due to the fact that skin allografts must be neovascularised by the host resulting in prolonged ischaemia, and skin and heart allografts differ in the intensity of the rejection process. Belperio et al, using a rat lung transplant model, showed that CCL3 protein was significantly elevated in lung allografts compared with syngeneic controls. However, CCL5 protein levels were at least 170 fold greater than CCL3 levels [Belperio et al, 2000]. Using Northern blot analysis, Grau et al demonstrated increased levels of CCL3 mRNA in rat renal allografts during acute rejection [Grau et al, 2000].

In human liver allografts both CCL3 protein and mRNA have been shown to be strongly expressed by infiltrating leukocytes and sinusoidal endothelium during acute rejection. Levels of CCL3 protein and mRNA were reduced after successful corticosteroid treatment of the acute rejection, but persisted in patients progressing to chronic rejection [Adams et al, 1996].

In human renal allografts Robertson et al demonstrated, by immunocytochemistry, increased levels of CCL3 protein in Banff grade 2 compared to grade 1 rejection. CCL3 was also demonstrated to be expressed in normal renal tissue but at lower levels than tissue from rejecting allografts [Robertson et al, 2000]. Segerer et al using an RNA protection assay showed increased levels of CCL3 mRNA in human renal allograft biopsies with rejection, than in normal renal tissue [Segerer et al, 2001]. Conversely, Oliveira et al found no significant difference in CCL3 protein expression from fine-needle aspiration biopsy cultures, between renal allografts with and without acute rejection [Oliveira et al, 1997]. However, unlike the previous two studies they made no comparison with normal renal tissue, but compared CCL3 levels to allograft tissue 7 days post-transplant. Increased CCL3 mRNA expression levels have been demonstrated immediately following allograft transplantation [Adams et al, 1996] and this may explain why Oliveiras' group found no difference. Also Oliveira et al cultured the cells from the fine-needle aspirates for 48 hours which may have influenced cellular CCL3 expression, however they did find increased levels in chronic allograft rejection biopsies.

There is evidence therefore, that CCL3 produced by graft infiltrating leukocytes and graft endothelium, may play a significant role in regulating T cell recruitment into allografts in the rejection process.

1.4.2 CCL4 (MIP-1β)

CCL4 (also known as MIP-1β, SCY A4, ACT 2 and LAG 1) belongs to the β chemokine family, and is a 69 amino acid acidic protein of 7.8kDa. The CCL4 gene is located at 17q21-q23. CCL4 is highly homologous to CCL3, and its protein and gene have been

characterized along with CCL3 as previously described (section 1.5.4). Its receptor is CCR5.

CCL4 is a chemoattractant for monocytes, T lymphocytes and dendritic cells. Compared with CCL3, it is a less potent chemoattractant, but is more specific for certain lymphocyte subpopulations, for example CD4+ T lymphocytes with some preference for the naïve CD45RA phenotype [Wang et al, 1993; Schall et al, 1993; Foti et al, 1999].
As with CCL3, CCL4 may have a role in T cell activation and proliferation as its been shown to have costimulatory activity in the presence of CD3 ligation [Taub et al, 1996]. Macrophages, activated lymphocytes, renal tubular epithelial cells and endothelial cells have been demonstrated to produce CCL4 [Schall et al, 1991; Zipfel et al, 1989; Robertson et al, 2000; Adams et al, 1996].

There is evidence for a role of CCL4 in allograft rejection. As mentioned in section 4.4.1 CCL4 is expressed eary in rejection in a murine allogeneic skin graft model but late in rejection in a heterotropic heart transplant model in mice [Kondo et al, 1996; Fairchild et al, 1997]. With long-term acceptance due to immunosuppression in the latter study, CCL4 was undetectable.
In human liver transplantation Adams et al demonstrated that CCL4 protein and mRNA were expressed at increased levels by infiltrating leukocytes and graft endothelium during rejection. CCL4 rather than CCL3 protein was particularly detected on the vascular and sinusoidal endothelium, being coexpressed with the T cell β1-integrin receptor, vascular cell adhesion molecule-1 (VCAM). In the same study, CCL4 and CCL3 mRNA was found in biopsies taken at the end of the transplant operation, suggesting an early induction of chemokines possibly in response to graft reperfusion [Adams et al, 1996]. This early induction of chemokines has also been demonstrated in a murine cardiac graft model by Morita et al. They showed in both isografts and allografts that CCL4 mRNA tissue expression began to appear at 3 hours post transplant rising to a peak at 24 hours and returning to background levels at 48 hours. CCL3 mRNA did not appear until 12-18 hours post transplant reaching a peak at 24-

48 hours. Treatment with antibodies which reduced early expression of these chemokines led to prolonged allograft survival [Morita et al, 2001]. These studies indicate a possible role of CCL3 and CCL4 in the early inflammatory events of transplant reperfusion and subsequent development of allograft rejection
In a rat lung transplant model Beperio et al found lower CCL4 protein levels in the rejecting allografts compared with those in syngeneic contols, in contrast to elevated CCL3 and CCL5 protein levels [Belperio et al, 2000]. However, in human renal allografts Segerer et al showed an increase in CCL4 mRNA and Robertson et al showed an increase in CCL4 protein during acute rejection [Segerer et al, 2001; Robertson et al, 2000]. In the latter study CCL4 and CCL2 were shown to be expressed at significantly higher levels in Banff grade 2 compared to grade 1 rejection. This was not seen with CCL5 and CCL3.

1.4.3 CCL5 (RANTES)

CCL5 (also called RANTES, SIS-delta, SCYA5 and EoCP-1) is an 8KDa protein, whose gene maps to human chromosome 17q 11-12 in the vicinity of other β chemokine genes. The two major receptors of CCL5 for mononuclear chemotaxis are CCR1 and CCR5, but it also binds to CCR3, CCR9 and DARC [Uguccioni et al, 1995; Proudfoot et al, 1995; Loestcher et al, 1996; Hadida et al, 1998]. It has been demonstrated that CCL5 is produced by a variety of cells, including NK cells, T-lymphocytes [Conlon et al, 1995], macrophages [Devergne et al, 1994], endothelium [Thienel et al, 1999], platelets [Kameyoshi et al, 1992], fibroblasts [Monti et al,1996; Brouty-Boye et al, 2000] smooth muscle cells [Jordan et al, 1997], and epithelial cells [Robertson el, 2000].

CCL5 is the most studied of the chemokines as regards its functional influence on T-lymphocytes and signal-transduction mechanisms. It has been shown to stimulate biphasic calcium mobilization in lymphocytes. An initial transient peak mediated by a G-protein-coupled pathway and more sustained calcium influx dependent on protein

tyrosine kinases. The transient calcium peak is associated with chemotaxis and the more sustained peak with calcium channel opening, IL-2 receptor expression, cytokine release and T-cell proliferation [Bacon et al,1995]. Other chemokines such as CCL3 and CXCL8 that are chemotactic only induce the initial transient rise in Ca^{2+} [Bacon et al, 1995]. The consequences of these signaling mechanisms in T-lymphocytes include up-regulation of adhesion molecules [Taub et al, 1995; Schall et al, 1990] cytokine release [Turner et al, 1995; del Pozo et al, 1995], uropod formation [Brezinschek et al, 1995], T-cell proliferation [Taub et al, 1996] and inhibition of M-tropic HIV entry [Cocchi et al, 1995] . CCL5 stimulates a variety of effects on T-lymphocytes and may be does this through different signaling pathways.

CCL5 is a potent lymphocyte and macrophage chemoattractant [Uguccioni et al, 1995; Schall et al, 1990] and is thought to participate in various inflammatory disease processes by mediating lymphocyte recruitment into tissues from the circulation. Studies have shown that CCL5 is expressed in delayed-type hypersensitivity reactions [Dervergne et al, 1994], necrotising glomerulonephritis [Schlondorff et al, 1997], inflammatory lung disease [Lukacs et al, 1996] and allograft rejection [Pattison et al, 1994]. Animal models of allograft rejection have identified an association between CCL5 and rejection. For example, Kondo et al looked at CCL5 mRNA expression in allogeneic skin graft models in mice. They found that maximal expression coincided with maximal rejection, i.e. maximal mononuclear cell infiltration [Kondo et al, 1996]. Using a murine cardiac allograft model Yun et al showed that CD4+ lymphocytes are not required for early (day 7 post-transplant) intra-graft CCL5 production, but are required in sustaining CCL5 production and mononuclear recruitment into the rejecting allograft [Yun et al, 2001].
Belperio et al showed, using an in vivo model of rat orthotopic lung transplantation, that an increase in CCL5 mRNA and protein correlated with recruitment of mononuclear cells into the rejecting allograft. There was also a correlation with the expression of the CCL5 receptors CCR1 and CCR5 [Belperio et al, 2000].

There is evidence to support this in the process of allograft rejection in humans. Belperio et al showed an increase in CCL5 protein in bronchoalveolar lavage specimens during acute lung allograft rejection [Belperio et al, 2000]. Robertson et al using immunohistochemistry demonstrated renal tubular epithelial cells expressed CCL5 during acute rejection [Robertson et al, 2000]. Yun et al showed, using immunocytochemistry, that CCL5 localized to graft-infiltrating mononuclear cells and vessel wall cells in human transplanted hearts with chronic rejection [Yun et al, 2001]. von Hundelshausen et al demonstrated that deposition of CCL5 by platelets could trigger shear-resistant monocyte arrest on inflamed or atherosclerotic endothelium [von Hundelshausen et al, 2001]. This may be due in part to CCL5 increasing β2 integrin expression on the surface of monocytes (G protein, calcium dependant action) leading to enhancement of their binding to endothelial cells [Vaddi et al, 1994]. Therefore CCL5 production by platelets in the vicinity of the endothelium may contribute to the rapid atherosclerosis seen in heart transplantation.

In human acute renal allograft rejection Pattison et al showed that CCL5 mRNA was detectable in infiltrating mononuclear cells and renal tubular epithelium. However CCL5 protein was also localized on the endothelial surface of peri-tubular capillaries that were largely negative for CCL5 mRNA by in situ hybridization. This suggested that the CCL5 protein deposited on the endothelium could enhance recruitment of T cells and monocytes into the rejecting graft [Pattison et al, 1994].

These studies show an important correlation between increased CCL5 expression and mononuclear cell infiltration. This has been further supported by the use of anti-CCL5 antibodies and CCL5 antagonists. Belperio et al used anti-CCL5 antibody to neutralize CCL5 in a rat lung transplant model. The antibody was shown to attenuate allograft rejection by decreasing mononuclear cell recruitment into the graft [Belperio et al, 2000]. Grone et al studied the use of the chemokine receptor antagonist Met-RANTES (Met-CCL5) in a rat renal transplant model. They showed that Met-RANTES treated rats suppressed recruitment of inflammatory cells into the renal allografts reducing rejection-associated vascular and tubular injury. It was also shown that met-

RANTES significantly augmented low dose cyclosporin treatment thereby reducing acute rejection and allograft injury [Grone et al, 1999].

1.4.4 CXCL10 (1P-10)

CXCL10 cDNA was originally cloned from IFNγ stimulated U937 cells [Luster et al, 1985] and named interferon γ induced protein 10 (IP-10). The CXCL10 gene is located at chromosome 4q21.1 and has an interferon – responsive element (ISRF) and two NFκB binding sites within 250 nucleotides of the transcription start site [Vaguri et al, 1990], which contribute to IFNγ and LPS induced CXCL10 gene transcription [Ohmori et al, 1993]. CXCL10 mRNA expression can be induced in macrophages and monocytes by IFN α/β/γ and LPS [Farber et al, 1997].
The cDNA encodes a precursor protein of 98 amino acids with a 21 amino acid signal peptide. The mature protein is about 12.4kDa but is rapidly cleaved at the carboxyl end to form a 6 to 7 kDa protein 77 amino acids in length [Luster et al, 1987] (See Fig.1.4 for primary protein structure).

CXCL10 is secreted from a variety of cells including monocytes , endothelial cells, keratinocytes and fibroblasts in response to interferon. IFN-γ induced keratinocytes secrete the most CXCL10 with endothelial cells , monocytes and fibroblasts secreting lesser amounts in that order. This was shown to be consistent with the amount of CXCL10 mRNA in these cells suggesting mRNA accumulation is an accurate reflection of the amount of CXCL10 protein secreted by cells [Luster et al, 1987]. Activated T cells also secrete CXCL10 [Taub et al, 1993].

Activities for CXCL10 in leukocytes include chemoattractant activity for monocytes , CD4+ memory T cells and NK cells but not for naïve T cells or CD8+ T cells [Taub et al, 1995; Taub et al, 1993]. Like other non-ELR chemokines CXCL10 is not active as a neutrophil chemoattractant. In addition CXCL10 induces adhesion of activated T cells to endothelial cells [Piali et al, 1998].

CXCL10 is a non-ELR chemokine and there is evidence of a role in inhibiting angiogenesis. CXCL10 inhibits CXCL8 mediated angiogenesis and evidence suggests that it inhibits tumour angiogenesis by IL-12 [Addison et al, 2000]. Transgenic mice which constitutively express CXCL10 in keratinocytes have delayed wound healing with impaired neovascularisation suggesting inhibition of neovascularisation in vivo by CXCL10 [Luster et al, 1998].

The receptor for CXCL10 is CXCR3 and is expressed mainly by memory / activated T cells and Th1 cells [Bonecchi et al, 1998; Sallusto et al, 1998]. CXCL10 is thought to regulate trafficking of Th1 cells and help elicit a Th1 response [Qin et al, 1998]. In vivo the initial descriptions of CXCL10 were in human skin during the delayed-type hypersensitivity response. Increased expression has also been demonstrated in tuberculoid leprosy and cutaneous Leishmaniasis [Kaplan et al, 1987]. Fahy et al in the hu-SCID mouse model grafted with human skin, used autologous mononuclear cells from the same donor, to demonstrate intradermal CXCL10 injection resulted in an influx of CD4+ T lymphocytes [Fahy et al, 2001]. However, these CD4+ T lymphocytes were not shown to be selectively Th1 or Th2 cytokine producing. This may be explained by the immunohistochemical method used, as in pathological conditions involving CXCL10, lymphocytes have been found to be the main cellular source of IFN-γ production.

In animal allograft models, CXCL10 has been shown to increase during allograft rejection, both at mRNA [Koga et al, 1999] and protein levels [Hancock et al, 2000]. As mentioned in section 1.4.1, in a murine allogeneic skin graft model, CXCL10 was expressed late in the rejection process [Kondo et al, 1996] whereas it was expressed early (day 2-3) in a heterotopic murine heart transplant model, and maintained through to complete rejection (day 8-13) [Fairchild et al, 1997]. Kapoor et al, also using a murine heart transplant model, showed that recipient IFN-γ was required for CXCL10 expression using IFN- γ -/- recipients, but despite lack of CXCL10 expression allograft rejection still occurred, suggesting it may not be necessary for T cell recruitment in the

rejection process [Kapoor et al, 2000]. In the same study they demonstrated CD4+, CD8+ and NK cells infiltrating both iso- and allografts by day 2 post-transplant and work that suggested CD8+ T cells mediated early (day2) expression of CXCL10 in the allografts rather than CD4+ or NK cells. Further studies by Morita et al in a murine cardiac allograft model showed early expression (day 2) of CXCL10 mRNA was attenuated by antiserum to the neutrophil chemoattractant, KC, which is the murine homologue to CXCL1 (growth-related oncogene α), administered at time of transplant. It also attenuated cellular infiltration into the allograft and graft rejection [Morita et al, 2001]. This suggests a role for neutrophils in early T cell recruitment by influencing chemokine expression, and progression to acute allograft rejection.

Mice deficient in CXCL10 are born healthy and develop normally. When used in allograft donation, acute rejection does not occur [Hancock et al, 2001]. Likewise mice deficient in the CXCL10 receptor CXCR3 accept allografts without acute rejection [Hancock et al, 2000]. In both these models the allografts showed marked reduction in CD45+, CD4+, CD8+ and CD25+ cells but not macrophages. This suggests a role in acute allograft rejection of CXCL10 and CXCR3, through inducing lymphocyte migration into the allograft. This is in contrast to the study by Kapoor et al who showed IFN-γ -/- recipient mice with a heart allograft, lacked CXCL10 expression but still rejected the graft [Kapoor et al, 2000]. It may be that the IFN- γ -/- mice produced low levels of CXCL10, which could not be detected by the Northern blot analysis technique used in the study, but still of such a level that could influence rejection.

In human lung allografts, increased levels of CXCL10 in bronchoalveolar lavage (BAL) specimens in individuals with rejection episodes have been demonstrated [Agostini et al, 2001]. The same study showed that CXCL10 was abundantly expressed by graft infiltrating macrophages and occasionally by epithelial cells in lung biopsies with evidence of rejection. The T cell infiltrates in both the BALs and lung biopsies of patients with rejection expressed CXCR3. In human cardiac allografts, rejection correlated with CXCR3 expression by T cell infiltrates and CXCL10 expression in biopsies [Melter et al, 2001]. Increased expression of CXCL10 mRNA in biopsies from

rejecting human renal allografts compared to normal renal tissue was demonstrated by Segerer et al, using an RNA protection assay [Segerer et al, 2001].
These studies indicate an important role for CXCR3 and its ligand CXCL10 in human allograft rejection.

1.4.5 Chemokine Receptor CCR1

CCR1 is a seven transmembrane G-protein coupled receptor. The gene is found on human chromosome 3p21 in a cluster with other β-chemokine receptors [Dougherty et al, 1997]. In 1993, Neote et al cloned and characterized the receptor using an "orphan receptor" cloning strategy to isolate cDNA encoding it [Neote et al, 1993]. They were able to predict it was a G-protein coupled receptor as the effects of its ligands, CCL5 and CCL3, were sensitive to pertussis toxin. They used the predicted homology between G proteins to successfully clone the CCR1 receptor, then called the MIP-1α/RANTES receptor. The G protein chemoattractant receptors for IL-8 and C5a show sequence identity to CCR1 of approximately 32% and 25% respectively.

CCR1 was the first chemokine receptor to be shown to have a functional viral homologue, US28 of human cytomegalovirus [Gao et al, 1994]. The CCR1 polypeptide is 355 amino acids in length. It is one of the most promiscuous chemokine receptors with 9 ligands identified, including the CC (β) chemokines, CCL3, CCL7 (MCP-3) [Neote et al, 1993], CCL8 (MCP-2) [Gong et al, 1997], CCL15 (MIP-5) [Youn et al, 1997; Zhang et al, 1999]], CCL14 (HCC-1) [Tsou et al, 1998], CCL23 (MPIF-1) [Nardelli et al, 1999] and CCL5 [Gao et al, 1993] which bind with similar high affinity. CCL4 and CCL2 bind with much lower affinity and are poor agonists [Neote et al, 1993].

CCR1 is expressed on a large number of leukocyte populations including mononuclear cells and neutrophils and is thought to play a part in mediating their recruitment into tissues during pathological responses. Its expression has been shown to be

influenced by cytokines; IFN-γ upregulates CCR1 expression in human neutrophils and migration to CCR1 ligands [Bonecchi et al, 1999], IL-2 induces CCR1 in T lymphocytes and NK cells [Loetscher et al, 1996; Polentarutti et al, 1997], and IL-10 upregulates CCR1 (as well as CCR2 and CCR5) in human monocytes [Sozzani et al, 1998]. These influences upon chemokine receptor expression no doubt ultimately influence the composition of leukocyte infiltration at sites of inflammation. Su et al showed that the majority of CD3+, CD4+, CD8+ and CD16+ lymphocytes are positive for CCR1 in human peripheral blood. CD45RO+ cells expressed greater amounts of CCR1 than CD45RO- cells, suggesting selective expression on the memory subtype [Su et al, 1996].

There is evidence that CCR1 plays an important part in mononuclear cell recruitment and the process of allograft rejection. In a rat lung transplant model Belperio et al showed an increase in CCR1 mRNA and protein in lung tissue corresponding to rejection associated mononuclear cell infiltration [Belperio et al, 2000]. Using mice with a targeted deletion in the CCR1 receptor (CCR1-/-) Gao et al showed a modest decrease in macrophage and T-cell recruitment comparable to normal mice treated with cyclosporin in cardiac allografts. Furthermore, if these CCR1-/- mice were treated with a short course of cyclosporin, intragraft expression of cytokines, chemokines and their receptors were suppressed and the resultant allografts lacked T-cell infiltration. This led to increased survival time of the CCR1 -/- MHC-mismatched allografts and permanent acceptance with cyclosporin treatment and no sign of chronic rejection 50-200 days after transplantation [Gao et al, 2000].

Horak et al used a CCR1 antagonist (BX471) to increase allograft survival times in rat heart transplants, and showed a synergistic effect with normally sub-therapeutic doses of cyclosporin. In vitro studies with activated microvascular endothelium showed an inhibitory effect of BX471 on mononuclear cell adhesion to the endothelium [Horak et al, 2001].

There are, however, differences in receptor expression or function in humans compared to rodents. For example CCR1 is predominantly expressed by neutrophils in mice but shows only limited neutrophil expression in humans. Also CCL5 binds to CCR1 as well as CCR4 and CCR5 in humans but does not bind to CCR1 in mice [Topham et al, 1999].
The direct evidence for a role of CCR1 in human allograft rejection/tolerance is limited. Segerer et al, using an RNA protection assay, showed a high expression of CCR1 in both normal human kidney and rejecting renal allograft, with no difference between them compared to the GAPDH house keeping gene [Segerer et al, 2001]. However, its two ligands CCL3 and CCL5 have been implicated in human allograft rejection (see section 1.4.2 and 1.4.4).

1.4.6 Chemokine Receptor CCR5

The CCR5 chemokine receptor gene was cloned in 1996, initially by Samson et al [Samson et al, 1996], and then independently the same year by Combadiere et al [Combadiere et al, 1996] and Raport et al [Raport et al, 1996]. The gene, which maps to chromosome 3p21, encodes a 355 amino acid protein with a molecular mass of 40.6 kDa. As with other chemokine receptors it is a seven transmembrane G-protein coupled receptor.

CCR5 is expressed on peripheral blood-derived dendritic cells [Granelli-Piperno et al, 1996; Rubbert et al, 1998], CD34+ haematopoietic progenitor cells [Ruiz et al, 1998] and activated/memory Th1 lymphocytes [Loetscher et al, 1998; Bleul et al, 1997]. Freshly isolated T cells express low amounts of CCR5 but this increases with prolonged stimulation by IL-2 and activating mitogens ex vivo [Bleul et al, 1997]. CCR5 expression by neurons, astrocytes, capillary endothelial cells, epithelial and fibroblasts has also been reported [Rottman et al, 1997].

Potent agonists for the CCR5 receptor include CCL3, CCL4, CCL5, CCL8 and CCL14 [Samson et al, 1996; Combadiere et al, 1996; Raport et al, 1996; Gong et al, 1997].

CCR5 has been shown to be a major HIV-1 coreceptor that controls susceptibility to HIV-1 infection and disease, with CCL3, CCL4 and CCL5 inhibiting infection of CD4+ cells by the virus [Dragic et al, 1996].

CCR5 is expressed on T cells associated with some Th-1 type inflammatory reactions. For example, immunostaining of T cells in rheumatoid arthritis synovial fluid showed that 80% of T cells expressed CCR5 compared to 15% of T cells in peripheral blood [Qin et al, 1998]. CCR5 appears to identify a subset of T cells in blood with a predilection for homing to sites of Th1 type delayed type hypersensitivity reactions.

There is evidence for the role of CCR5 expression in T cells involved in the Th1 inflammatory reactions of allograft rejection. In a rat lung allograft model, increased levels of CCR5 expression correlated with temporal recruitment of mononuclear cells and with rejection [Belperio et al, 2000]. Gao et al demonstrated that mice deficient in a functioning CCR5 receptor (CCR5-/-), and CCR5+/+ mice treated with a neutralizing mAb against CCR5, showed enhanced allograft survival when transplanted with a fully MHC – mismatched cardiac allograft. In addition, allograft recipients treated with cyclosporin that were CCR5-/-, and CCR5+/+ treated with mAb, showed long lasting allograft survival with an absence of lymphocyte graft infiltration, interstitial fibrosis or development of transplant arteriosclerosis [Gao et al, 2001].

In human liver and kidney transplants, increased expression of CCR5 in rejecting allografts have been demonstrated by immunohistochemistry [Goddard et al, 2001; Segerer et al, 1999]. Using Northern blot analysis and ribonuclease protection assay methods, increased levels of CCR5 mRNA in rejecting allografts compared to normal kidney tissue have also been demonstrated. This expression of CCR5 was restricted to infiltrating mononuclear leukocytes at sites of vascular and interstitial rejection, and corresponded to increased expression of CCL3, CCL4 and CCL5, the ligands for CCR5 [Eitner et al, 1998; Segerer et al, 2001].
The functional importance of CCR5 positive lymphocytes in human renal allograft survival has been demonstrated in patients genetically lacking a functional CCR5

receptor. About 1% of Northern Europeans have a 32 base pair deletion in the CCR5 receptor, and individuals who are homozygous for the deletion lack a functional receptor. These individuals have been shown to be highly resistant to HIV infection [Samson et al, 1996] and show significantly prolonged renal allograft half life, as compared to the heterozygous or wild-type individuals [Fischereder et al, 2001]. There is therefore, good evidence supporting an important role of the CCR5 receptor expressed on lymphocytes in human renal transplant nephropathy.

1.4.7 Chemokine Receptor CXCR3

CXCR3 is a seven transmembrane G-protein coupled receptor and the first chemokine receptor identified that is highly induced by T cell activation. The gene encoding CXCR3 maps to chromosome Xq13 and encodes a polypeptide 368 amino acids in length [Loetscher et al, 1996]. The human protein sequence is approximately 30% identical with CXCR1 and CXCR2.

CXCR3 binds three inflammatory / inducible, non-ELR CXC chemokine agonists, CXCL9, CXCL10 and CXCL11 [Loetscher et al, 1998; Cole et al, 1998; Weng et al, 1998] all of which chemoattract and induce calcium influx in activated T cells. Their order of binding affinity is CXC11>CXCL9=CXCL10.

CXCR3 is expressed on the majority of memory/activated T cells. Naïve T cells do not express CXCR3 but rather CXCR4. After human T cell activation and polarization, Th0 and Th1 cell lines express high levels of CXCR3 and Th2 low levels [Sallusto et al, 1998]. This demonstrates that CXCR3 is a Th1 cell marker. This is further shown in Th1 type inflammatory reactions such as rheumatoid arthritis, where virtually all T cells in synovial fluid express CXCR3 by immunostaining [Qin et al, 1998]. CXCR3 is also expressed on a proportion of circulating blood T cells, B cells and NK cells. The T cells expressing high levels of β1-integrins [Qin et al, 1998]. Piali et al showed that the CXCL10 and CXCL9 chemokines induced adhesion of human IL-2 stimulated T

lymphocytes to immobilized integrin ligands. They also demonstrated that CXCL10 and CXCL9 production by human umbilical vein endothelium was stimulated by IFN-γ and TNF-α. This induced IL-2 stimulated T lymphocyte adhesion to the endothelial cell surface, which was reduced with CXCR3 monoclonal antibody treatment [Piali et al, 1998].

CXCR3 appears to play a key role in T cell activation and recruitment in Th1 type inflammatory reactions. It follows that it is most likely to be important in allograft rejection, which is supported by evidence in the literature. In a murine cardiac allograft model Miura et al demonstrated an increase in CXCR3 expression coinciding with rejection using an mRNA Protection Assay method. Its ligand CXCL9 also showed increased expression, whereas CXCL10 did not [Miura et al, 2001]. In a similar model Hancock et al showed a delay in onset of rejection and therefore prolongation of allograft survival with the administration of anti CXCR3 monoclonal antibody, even if begun after onset of rejection. His team also showed, using a murine knockout model, that mice deficient in CXCR3 (CXCR3-/-) were profoundly resistant to development of acute allograft rejection. Furthermore CXCR3-/- mice given a brief subtherapeutic course of cyclosporin permanently maintained their cardiac allograft [Hancock et al, 2000].

Using an in vitro model of T cell alloactivation Goddard et al demonstrated an increased pattern of expression of functional CXCR3. They also showed increased expression of CXCR3 in circulating and graft infiltrating lymphocytes by immunohistochemistry of human liver allografts undergoing rejection. This coincided with detection of the CXCR3 ligand, CXCL10, on sinusoidal epithelium [Goddard et al, 2001]. Similar patterns of expression have been demonstrated in human lung and cardiac allograft rejection [Agostini et al, 2001; Melter et al, 2001].
Using an in vivo human skin model Fahy et al demonstrated that intradermal injection of CXCL10 induced a marked recruitment of CXCR3-positive lymphocytes in the skin [Fahy et al, 2001].

1.5 IMMUNOMONITORING

It has been a goal of clinicians to find a specific, sensitive, easily applicable, inexpensive and non-invasive way to monitor the immunological events within an allograft, which may not necessarily be by monitoring the graft itself. This would be particularly advantageous in making or pre-empting the diagnosis of rejection, as at present the definitive way of rejection diagnosis is by biopsying the allograft. This is an invasive procedure and is associated with risks to the graft and patient, particularly in endomyocardial biopsy in heart transplants.
Preventing or early diagnosis and treatment of acute allograft rejection decreases the irreversible damage to the allograft and improves its survival and ultimately, potentially, that of the patient.

The utility of immunomonitoring is not limited to the diagnosis of allograft rejection. It would also be useful if it could be applied to some measure of the overall immunosuppressive activity within a patient. There is some tailoring of immunosuppressive therapy given to transplant patients depending on their associated risk factors for rejection. For example, mycophenolate mofetil is given to patients instead of azathioprine if they are deemed higher risk in renal transplantation (ie. a previous rejection episode, high PRA (panel reactive antibody), 3-4 HLA-A/B or 2 HLA-DR mismatches). However, this tailoring of immunosuppression therapy is crude, despite monitoring of some drug levels, as the metabolism and immunosuppressive activity of these compounds varies somewhat due to age and physical makeup of the individual as well as genetic influences. Just as it is important to protect the allograft from host rejection by adequate immunosuppression, it is also important to protect the host from infections and malignancy from over-immunosuppression.

Following allograft transplantation, patients are closely monitored to determine the well-being of the patient and of the allograft. Urine output, together with serum creatinine and urea are good indicators of renal transplant function, and changes or

inadequate levels of these indicators can be caused by rejection and other factors such as poor renal blood perfusion, urinary obstruction, infection, or the nephrotoxicity of the immunosuppressive drug cyclosporin. The latter are investigated first by Doppler ultrasound scanning of the graft, urinary culture and blood cyclosporin levels, before considering a biopsy to rule out rejection.

Since transplantation began, much work has been carried out on immunomonitoring. Below is a brief resumé of some of the methods that have been applied to this end, specifically in renal transplantation, up until today, including the application of modern molecular methods for gene expression analysis.

1.5.1 Cytoimmunological Monitoring

The peripheral blood white cell count with differential count is routinely measured in the early post transplant period. A rising white cell count can reflect a rejection process within an allograft but is very non-specific and may also be due to drugs (eg, steroids), infection or surgery, for example. There have been many studies looking at subsets of human mononuclear cells from the peripheral blood that may be an indication of allograft rejection.

Examples include looking at the relative proportions of activated lymphocytes (lymphoblasts, activated lymphocytes and plasmacytoid cells) in relation to the total lymphocyte count, and certain CD receptor subsets proportions, for example, CD4/CD8 ratios and changes in CD4 and CD3CD25 subsets [Hammer et al, 1998; Wijngaard et al, 1989; Valeri et al, 1991; Tashiro et al, 1989; Takahara et al, 1989]. The results from these studies are conflicting, one problem being the difficulty of differentiation between rejection and infection. Also, the introduction of cyclosporin makes it difficult to find any prominent differences in subset counts between the resting and active phase of rejection or infection.

1.5.2 Blood/Serum Molecular Immunomonitoring

C-Reactive Protein (CRP) is routinely measured during the early post transplant period and a rise can reflect an acute rejection process. However, as CRP is sensitive to infections and tissue injury in surgery, it has a low specificity for predicting rejection [Lalla et al, 1988; Maury et al, 1984].

Workers have looked at serum levels of β2 microglobulin, which is part of the expressed HLA Class I molecule, as a predictor of acute rejection. Edwards et al showed, in a cohort of 93 renal transplant patients, that changes in serum β2 microglobulin occur earlier and are more sensitive and specific for episodes of acute rejection than are changes in serum creatinine [Edwards et al, 1983].
However, Veron et al in an equal sized cohort of renal transplant patients, obtained results in conflict with the work of Edwards et al, concluding that serum levels of β2 microglobulin are not as sensitive or specific as serum creatinine in monitoring for acute rejection [Veron et al, 1985].

Other studies have looked at serum levels of soluble CD23 [Traindl et al, 1994; Kutukeuler et al, 1995], IL-2 receptor [Noronha et al, 1990; De Boccardo et al, 1994], ICAM-1 [Stockenhuber et al, 1993], HLA Class I [Drouet et al, 1995], and amyloid A [Maury et al, 1984]. Serum levels of the cytokines IFN-α, TNF-α, IL-1, IL-2, IL-4, IL-6, IL-8 and IL-10 have also been investigated in renal transplant patients for potential use in immunomonitoring [Daniel et al, 1995; Maury et al, 1987; Noronha et al, 1990; Maury et al, 1987; Johnson et al, 1990; Budde et al, 1994; Yoshimura et al, 1991; Kutukeuler et al, 1995]. These potential indicators for rejection show relatively low sensitivity, specificity or both particularly when trying to differentiate between cyclosporin toxicity or infection and rejection. The reason for the low specificity and sensitivity of monitoring these cytokine serum proteins may be due to variable removal from the blood by proteolytic breakdown in the liver and excretion in urine by the kidney.

1.5.3 Immunomonitoring by Urine Analysis

A number of studies have investigated the use of urine analysis in immunomonitoring of renal transplant patients. Urine cytology, examining the white cell count with culture is regularly carried out to exclude infection as a cause of renal transplant dysfunction. Several groups have looked at urinary lymphocytes and renal tubular cells and found some correlation with acute rejection [Eggensperger et al, 1988; Sandoz et al, 1986; Simpson et al, 1987]. Reliable diagnosis is difficult, however, due to cell disintegration in the urine from proteolytic enzymes, hypertonic urine and cellular contamination from other parts of the urinary system and genital tract.

Cytokines, adhesion molecules, complement cleavage products and nitrite and nitrate levels amongst others in urine have been analysed with varying success in correlation to acute rejection episodes [Albrecht et al, 2000; Budde et al, 1994; Simpson et al, 1989; Bechtel et al, 1994]. A major drawback is differentiating between infection and rejection and also the problem of urine collection in oliguric and anuric patients.

1.5.4 Immunomonitoring by gene expression analysis

With the rapid and recent development in molecular biology and techniques for quantitative gene expression analysis there has been a lot of interest in its potential use in the field of transplantation. Most studies have looked at gene expression within an allograft. However, relatively few have investigated gene expression in peripheral circulating inflammatory cells which may be influenced by, or reflect events within, the allograft and therefore be potentially useful in immunomonitoring. Gorezynski et al investigated mRNA cytokine expression by semi-quantitative RT-PCR in peripheral blood, and simultaneously in the allograft in human liver transplantation. They found IL-2, IL-6 and IFN-γ transcription was significantly increased in peripheral blood lymphocytes in recipients with rejection compared to those without rejection. They

concluded that although some differences in the frequency of cytokine gene transcription were seen, a good correlation was found between the intragraft and peripheral blood lymphocyte cytokine profile, suggesting that similar lymphocyte subpopulations regulating graft rejection were predominant both locally in the graft and peripherally in the blood [Gorezynski et al, 1996].

Miura et al investigated cytokine and chemokine gene expression, by real-time RT-PCR, in peripheral blood mononuclear cells from patients following autologous stem cell transplantation with induced graft-versus-host disease (GVHD). They found IL-10, IFN-α, IL-2, CCL3 and CXCL10 mRNA levels elevated in autologous transplant patients with GVHD compared to healthy individuals as controls [Miura et al, 2002].

Previous work in this laboratory has examined cytokine gene expression in PBMCs in renal transplantation and demonstrated significant changes correlating with rejection episodes and treatment. Tan et al, using a semi-quantitative RT-PCR method, showed IL-4, IL-5 and IL-13 expression increased before and during acute rejection and decreased after successful anti rejection therapy. Also IL-10 expression fell during acute rejection with a subsequent rise with anti-rejection therapy [Tan et al, 2001]. Gibbs et al demonstrated significant increases IL-4 and TNF-α expression prior to rejection, with a return to baseline values with anti rejection therapy, using a real-time quantitative technique [Gibbs et al, 2001]. These studies demonstrate the potential for PBMC gene expression analysis in the immunomonitoring of allograft recipients and subsequent use in the diagnosis and monitoring of treatment of rejection.

Part of the aim of this work is to identify chemokine or chemokine receptor gene products in PBMC, which may be used in immunomonitoring, so as to build up a robust panel of gene expression markers.

1.6 METHODOLOGY

Molecular analysis of gene sequences and determination of gene expression at the mRNA level has been of interest to molecular biologists and geneticists for many years. Altered patterns of gene expression reflect changes in function and activity of cells and therefore the ability to quantitate mRNA transcription is a useful tool for research into gene function and cellular activity. This has developed with the use of RNA quantification methods in clinical diagnostics such as detection of viral pathogens [Holodniy et al,1994], molecular assessment of tumour stage [Bustin et al, 1998], monitoring the response to chemotherapy [Desjardin et al, 1999], and detection of circulating tumour cells in cancer patients [Ghossein et al, 1996].

Early methods were based on RNA molecular hybridization known as Northern blotting and in situ hybridization, and require relatively large amounts of target gene transcripts. In situ hybridization is the only method that allows localization of transcripts to specific cells within a tissue. The main disadvantages of these methods is that quantification is relative and that they are of low sensitivity. The advent of PCR amplification methods [Mullis et al, 1987] has revolutionised gene quantification providing much greater sensitivity, allowing very small gene transcript copy numbers to be quantified. It also led to more accurate quantification and the ability to determine gene transcript copy numbers.

Before PCR amplification, the target RNA is reverse transcribed into cDNA as RNA cannot serve as a template for PCR. This can be carried out in a combined one-tube reaction with a heat-stable DNA-dependent polymerase or in a separate reaction. In this work, this step was carried out separately generating a cDNA bank reservoir which can be stored and used to carry out additional gene expression studies at a later date.

Relative quantification of a small mRNA gene copy number can be achieved by using a fixed number of PCR cycles to amplify the reverse transcribed target gene transcript (target cDNA), followed by relative quantification of the PCR end-point products. End-

point product quantification can be achieved by isotope incorporation into the amplified product, blotting with a labeled probe, or the more sensitive ELISA technique.
This led on to more accurate quantitative reverse transcription PCR (RT-PCR) using a standard cDNA molecule, namely competitive and non-competitive RT-PCR.
Competitive RT-PCR involves the use of a standard cDNA molecule that competes with the target molecule for primers and enzyme in the same reaction tube. Using a dilutional series of standard cDNA the quantity of target cDNA can be deduced [Becker-Andre et al, 1989; Gilliland et al, 1990]. For this method to be accurate the standard and target amplification reaction efficiencies should be equal in all reactions. In non-competitive RT-PCR a dilution series of standard cDNA is co-amplified with the target. The standard signal is plotted against the target signal, and where the lines intersect (equivalence point) their quantities are equal (with competitive RT-PCR log standard/ target signal is plotted against log standard cDNA, and target cDNA quantity determined at the equivalence point). These are known as end point quantitative methods, as quantification is based on the amount of amplified material obtained at the last amplification cycle. After the PCR reaction a further process is required to determine the quantity and confirm the identity of the amplified target gene transcript.

Another step in the advance of more fully quantitative PCR technology was taken by Higuchi et al, when they described the simultaneous amplification and detection of specific DNA sequences [Higuchi et al, 1992]. This is called kinetic or real-time PCR, and avoids the use of standard cDNA curves with the problems of developing, storing, and accurately quantifying the standard itself. Also with real-time PCR, quantification can be performed in the early exponential cycles of the PCR, at which point the reaction is less likely to be influenced by amplicon accumulation and changes in rate limiting reagents, thereby making quantification more precise compared to end-point PCR. After amplification, further processing to quantify amplicon accumulation is not required, greatly increasing the speed of the quantification process. Another advantage with real-time PCR is that the linear range of the assay (5-6 logs) is greater when compared to end-point quantification (2-3 logs), in other words real-time PCR

provides accurate measurement over a very large range of relative starting target quantities.

Further advances through the description by Holland et al of the 5' nucleolytic activity of Taq polymerase [Holland et al, 1991], and the development of fluorescent energy transfer hybridization probes [Bassler et al, 1995; Lee et al, 1993; Livak et al, 1995] led to the development of the ABI Prism ® 7900 HT Sequence Detector used in this work. The ABI Prism ® 7900 HT Sequence Detector can run a 384 well reaction plate allowing rapid analysis of large numbers of samples, including necessary replicates, controls and standards. For a description of the molecular mechanism of the PCR reaction and amplicon detection by the ABI Prism® 7900 HT Sequence Detector (TaqMan® system) see section 2.1.4.1. The Sequence Detector generates a numerical value for each PCR reaction on completion, namely the CT value, which is the number of PCR cycles to the point at which the reaction becomes exponential. The CT value is inversely correlated to the number of target sequence copies in the PCR reaction mix, see section 2.2.4.iii and Figure 2.2.

Absolute quantification of transcription levels by using a standard curve can be achieved and allows the precise determination of target copy number per cell, per total RNA concentration or per unit mass of tissue. A standard curve is constructed by making serial dilutions of a known quantity of the target gene of interest (or section of the target recognized by the primers and probe). A plot is made of the CT value (y-axis) versus log of the quantity of target (x-axis), and the quantity of target in a sample can then be determined by extrapolation to the x-axis of the standard curve from the sample CT value.

Whelan et al developed a novel method for absolute quantification by using the transcript copy number per microgram of cDNA allowing possible comparison of results between laboratories [Whelan et al, 2003].

In this work standard curves were not constructed as relative quantification was determined, showing changes in transcription levels of a target gene with time within a patient, measured as CT values, and making no comparisons between patients.

There is, however, variability within RT-PCR reactions and non-reproducibility particularily with the need for two sequential enzymatic steps. The coefficient of variation for C_T data has been shown to be very low at less than 2% for the TaqMan [Heid et al, 1996] compared to 14% reported for conventional RT-PCR [Zhang et al, 1997].

A method for minimizing this variability is to simultaneously amplify, with the target, a cellular RNA that serves as an internal reference against which other RNA values can be standardized. Part of this work was to investigate possible candidate gene RNA transcripts which could be used as endogenous contols within the gene expression analysis model of this study. Variability within the RT-PCR method and use of endogenous control genes are discussed in chapter 4.

1.7 HYPOTHESIS BEHIND THIS WORK

The basis of this work was to demonstrate if changes in mRNA and therefore gene expression levels within mononuclear cells in peripheral blood of particular chemokines and their receptors can be detected over the early post-transplant period. In addition, correlation of any detected changes with clinical events was also investigated.

There is evidence that chemokines and their receptors are up-regulated and down-regulated as they circulate around the body and come into contact with other cells such as endothelial and dendritic cells. When a naïve T lymphocyte enters a lymph node from blood and comes into contact with its specific antigen, presented by a dendritic cell, it is activated and undergoes a change in its chemokine expression pattern. Activation leads onto clonal proliferation and transformation into T effector and memory cells which drain back into the blood to migrate to the inflamed area that was the origin of the initial activating antigen, guided by their expression of inflammatory chemokine receptors. It is theoretically at this time point that it may be possible to detect the increased inflammatory chemokine receptor gene expression in circulating T lymphocytes.

Chemokine expression can be induced in T lymphocytes infiltrating an inflamed area. Whether their gene expression and synthesis occurs at the site of inflammation or maybe at the same time as the inflammatory chemokine receptors are up-regulated is unclear. It is possible that chemokine gene expression could be up-regulated prior to entry into the target tissue and therefore changes detected in circulating T lymphocytes.

1.8 AIMS OF PROJECT

1. To determine whether changes in chemokine ligand/receptor gene expression can be detected by sequential monitoring in peripheral blood mononuclear cells, in the early post-transplant period of human renal allografts.

2. To determine whether any changes detected correlate with clinical events, including acute rejection, and/or response to therapy. The ultimate aim of this work is to determine whether monitoring of expression levels of one or more chemokine ligand/receptors can be used in immunomonitoring, or as a predictive factor of impending rejection prior to clinical manifestation. Such markers can then be incorporated into an expanded panel of markers for more robust immunomonitoring.

3. To test a selection of possible candidate genes that could be used as endogenous controls for gene expression in peripheral blood mononuclear cells. Many studies use popular housekeeping genes as endogenous controls but these often vary between tissues and clinical settings. A reliable endogenous control gene would help to validate results, but must be thoroughly tested within the system used.

Chapter 2

Patients, Materials and Methods

In this chapter patient recruitment and the clinical steps taken to establish a diagnosis of rejection are detailed, followed by the materials and methods used in this study.

2.1 PATIENTS

In this section the procedure for patient recruitment is described followed by the clinical steps taken to make the diagnosis of rejection.

2.1.1 Patient Recruitment

Ethical approval for this work was obtained from the South and West Local Research Ethics Committee.

Patients admitted to the Wessex Renal and Transplant Unit for a renal transplant or living donor nephrectomy were asked to participate in this study. Live donors were included in the study to observe any possible effect on chemokine gene expression of surgery alone without the presence of an allograft or immunosuppressive therapy. The time period for recruitment by the author was between January 2002 and May 2003. Patients had also been recruited previously between June 1999 and August 2001, with cDNA samples created using the same method, which were available for use in this work. The purpose of the study was explained to each patient with plenty of opportunity to ask any questions. Once they had agreed to participate written consent was obtained (see Appendix I).

Data on each patient were collected at the time of transplantation or donor nephrectomy and transferred to a computer excell spread sheet. Any patient who developed an infection, i.e. chest, wound or urinary tract, within the study period was excluded.

2.1.2 Rejection Diagnosis

Rejection was diagnosed by renal allograft core biopsy and histological evaluation. In a functioning allograft rejection causes a rise in the serum creatinine of the transplant recipient. In the early post-transplant period daily blood samples were routinely taken to monitor the serum creatinine as well as other biochemical and haematological parameters. If a patient had successive rises in creatinine (usually >10%), the possibility of rejection was considered, however other causes were first excluded prior to biopsy. Firstly a urine sample for culture was sent to exclude infection. Secondly a duplex ultrasound scan of the allograft was performed to ensure adequate arterial perfusion, venous drainage and exclude urinary obstruction leading to hydronephrosis. Thirdly, and finally, blood was sent to monitor cyclosporin A levels, which if levels become too high (>1900ng/ml taken at two hours post-dose), or the patient has an abnormal absorption profile, can cause a rise in the serum creatinine. Once these were shown not to have caused the rise in creatinine, or their correction reverse the rise, an ultrasound guided core biopsy was performed. The biopsy was taken in the morning, histologically examined and reported, and anti-rejection therapy started, if necessary, the same day.

Patients who experienced delayed graft function, diagnosed by the failure of the serum creatinine to fall after 24 hours post-transplant or the need for dialysis, also had a biopsy every 5 to 7 days or until renal function recovered. Prior to biopsy they had a duplex ultrasound scan to ensure good allograft perfusion and no urinary obstruction.

2.2 MATERIALS AND METHODS

This section is divided into four subsections as listed below:

1. Blood collection, cell separation and RNA stabilisation.
2. Total RNA extraction, quantification, DNase Treatment and standardisation.
3. First-strand cDNA synthesis.
4. TaqMan gene expression analysis.

Consumables and equipment for each subsection are listed in the appendices II, III, IV and V.

2.2.1 Blood Collection, Cell Separation and RNA Stabilisation

These preliminary stages of the laboratory work were carried out in the Renal Laboratory at St Mary's Hospital, Portsmouth prior to 21st September 2002. Thereafter the Renal and Transplant Unit moved to the Queen Alexandra Hospital, Portsmouth, as did the Renal laboratory.

2.2.1.i Blood collection

Blood was collected in standard (4.5ml) sodium citrate Vacutainer® bottles. A total of 18ml of blood (i.e. four vacutainer bottles) was taken pre-operatively from patients undergoing transplantation or living donor nephrectomy. In the transplant patients this was usually done at the time samples were taken for cytotoxic cross-matching and in the the living donors on the morning of the operation.
Post-operatively blood samples (9ml) were taken daily by the phlebotomy service between 8 - 9.00am and immediately placed in a refrigerator at 4°C to minimise cellular RNA degradation prior to RNA stabilisation, which took place within 24 hours.

Samples were taken daily for fourteen days post-transplant or post-operatively, or until hospital discharge.

2.2.1.ii Cell Separation

Following blood collection and refrigeration the samples were taken to the Renal Laboratory the same day of collection, usually in the morning. The whole blood sample (9ml) was transferred into a 50 ml-skirted polypropylene tube and diluted up to a total volume of 22 ml with single strength phosphate buffered saline (PBS) and gently mixed by tipping the tube. The 18ml pre-operative transplant samples were split into 9 ml samples and processed in the same way. This diluted sampled was then split into two equal 11 ml aliquots and each layered carefully onto 4 ml of Lymphoprep® in a 15 ml conical polypropylene tube. Lymphoprep® has a density of 1.077 g/l which is higher than the density of most mononuclear cells and lower than that of granulocytes and erythrocytes. This allows separation of the mononuclear cells from the rest on centrifugation. The tubes were spun in a tipping bucket centrifuge at 1100 g for 20 minutes at 20 °C. No acceleration or braking rates were applied to the tubes.

After centrifugation the specimens could be seen to have separated into four distinct layers. The upper straw-coloured layer being plasma, with a narrow white 'buffy layer' separating the serum from the clear Lymphoprep® layer below. The 'buffy layer' contains the mononuclear white cells. The erythrocytes and granulocytes make up the bottom burgundy layer.
The 'buffy layer' was carefully removed using a sterile Pasteur pipette and placed in a 15 ml conical polypropylene tube. The volume of the mononuclear 'buffy layer' was around 1.5 – 2.5 mls for each Lymphoprep® tube. The total sample of between 3-5 ml was washed with 11ml of PBS with gentle shaking. The specimens were then centrifuged at 20°C and 400g for 10 minutes with no brake or acceleration rates applied. This forms a small white monocyte pellet at the bottom of the tube. The clear PBS and serum supernatant was then poured off leaving the pellet in about 200-300µl

of remaining fluid. The cells were re-suspended by vortexing the tube for a few seconds.

2.2.1.iii RNA Stabilisation

This step stabilises and prevents degradation of RNA and is the initial step in the RNA extraction process. Approximately 1 ml of RNAzol B ™ (Biogenesis, UK) was added to the mononuclear cell suspension. RNAzol B ™ contains guanidium thiocyanate, a denaturing agent, and βmercaptoethanol, a reducing agent, both of which are potent inactivators of ribonucleases. Addition of RNAzol B ™ causes lysis of the cells and promotes formation of RNA complexes with guanidium and water molecules as well as abolishing the hydrophilic interactions of DNA and proteins. This allows removal of the DNA and protein from the aqueous phase in which the RNA remains.

The cells were completely homogenised by passing the mixture up and down a sterile Pasteur pipette a few times, before being transferred to a sterile 1.5 ml eppendorf tube. The tubes were frozen at -20°C prior to the total RNA extraction.

2.2.2 Total RNA Extraction, Quantification, DNase Treatment and Standardisation

The specimens were transferred frozen to the Molecular Pathology Laboratory at Southampton General Hospital where the remainder of the laboratory work was performed.

It is essential to minimise the activity of RNases, which degrade RNA and can contaminate samples exogenously as well as being liberated on cell lysis. RNAzolB™ contains potent inactivators of RNases, used in the final step of the cell separation. To prevent exogenous contamination gloves were worn and changed regularly and the RNA extraction process performed in laminar flow cabinets. All disposable glassware

and plasticware was sterile and RNase free and RNase free water (Sigma®) was used for any dilutions. Between procedures samples were placed on ice.

2.2.2.i Total RNA Extraction

The total RNA extraction process used was a single step, acid guanidium thiocyanate–phenol-chloroform method [Chomczynski et al, 1987]. This method allows rapid RNA isolation providing both a high yield and good purity of undegraded RNA preparations.

On arrival at the laboratory in Southampton, the frozen samples were placed on ice. While the centrifuge was cooled to 4°C, 130 ml of chloroform was added to each sample. The then thawed samples were vigorously shaken for 15 seconds and allowed to stand on ice for a further 5 minutes before being centrifuged for 15 minutes at 12000 g and 4 °C. Following centrifugation three distinct layers were visible: the lower blue chloroform-phenol layer, the upper clear aqueous layer containing the total RNA in solution and a thin white interphase between them containing proteins and DNA.

The upper aqueous phase containing the RNA was carefully removed using a sterile glass Pasteur pipette and transferred into another sterile 1.5 ml eppendorf tube. The volume obtained was round 500-600 µl. Great care was taken to avoid disturbing the white protein/DNA interphase, if this did occur the sample was mixed and centrifuged again. A volume of 700 µl of isopropanol was then added to each RNA solution, mixed and left overnight in a refrigerator at 4°C. This step precipitates the RNA out of solution.

The following day the samples were centrifuged for 15 minutes at 12000 g and 4°C, which formed a small visible white pellet of RNA at the bottom of the eppendorf tube. The clear supernatant was removed and discarded using a sterile glass Pasteur pipette making sure not to disturb the RNA pellet.

The pellet was then washed with 500 µl of 75% ethanol, which had been stored at -20°C. The tubes were vortexed for 15 seconds to aid the washing, which helps remove any residual isopropanol, salts and protein from the RNA. This is important as these can inhibit the enzymatic reactions of reverse transcription and the polymerase chain reaction. The samples were then centrifuged for 8 minutes at 7500 g at 4°C to reform the purified RNA pellet. The ethanol was removed from each sample using a sterile glass Pasteur pipette and again taking great care to avoid disturbing the purified RNA pellet at the bottom of the eppendorf tube. The tubes were then placed in a 37°C warming cabinet to allow complete evaporation of the ethanol.

On drying, the RNA pellets become transparent as the ethanol evaporates, taking around 15 minutes. Once dried, 30 µl of RNase free water was added to the tube and placed in a 55°C water bath for 5 minutes. This improves the solubility of the RNA and on removal of the tubes from the water bath, with gentle flicking of the tube, the purified RNA pellet can be seen to dissolve. The RNA solution was then ready for quantification. If this did not occur on the same day, the samples were frozen overnight at -20°C.

2.2.2.ii RNA Quantification

A 6 µl sample of the purified stock RNA solution was used for quantification, and by adding 12 µls of RNase free water to it in a 0.75 ml eppendorf tube a 1:3 dilution was prepared.

A spectrophotometric method was utilised for RNA quantification using the GeneQuant™ (Pharmacia, UK) spectrophotometer. This was set up for RNA quantification as follows:

Path length	=	5
Use 320 nm?	=	Yes
RNA factor	=	40
Base number	=	0
(A,C,G,T,U)		
Oligo length	=	1
MW Calc.	=	0.0
Ratio expected	=	2.000
Conc. Expected	=	0.000
Protein coeff 1	=	1.550
Protein coeff 2	=	0.760

Prior to use, the spectrophotometric cuvette was sequentially washed with 0.1M HCl, 0.1M NaOH, ultra high quality (UHQ) double distilled water and finally RNase free water. This removes any contaminating protein or nucleic acid that may interfere with quantification. The cuvette was aspirated to dryness using a 10 ml pipette before use.

Before quantification a reference was set using 6 µl of RNase free water. Between all measurements the cuvette was washed with UHQ water and RNase free water and then aspirated to dryness. A 6 µl aliquot of each 1/3 strength RNA solution was serially loaded into the cuvette and the absorbance at 260 nm and 280 nm recorded with the 260/280 ratio. A 260/280 ratio of 1.6 indicated good quality RNA.

Occasionally, the 260 nm absorbance exceeded the limit measurement of the spectrophotometer displaying as >3.000 reading. In these cases the remaining 12 ul of 1/3 strength RNA solution was diluted with 12 ul RNase free water to make a 1/6 strength solution. If this exceeded the 260 mm absorbance limit, a 1/12 solution was made. No specimens were found to need further dilution than 1/12.

2.2.2.iii DNase Treatment

The stock RNA solution was treated with RNase free Deoxyribonuclease (Sigma®) to remove any contaminating genomic DNA. This prevents any genomic DNA being amplified in the PCR reaction by primers and probes not designed across an exon-exon boundary (see section 2.2.4.ii). A volume of 1 ul containing 1.5 U of DNase was added to each sample and incubated for 15 minutes at room temperature. The mixture was then heated to 65°C in a water bath for 10 minutes to denature the enzyme.

2.2.2.iv RNA Standardisation

The concentration of RNA in each sample was calculated from the 260 absorbance measurement using the following formula:

RNA conc. (ng/ml) = 260 absorbance x 40 (RNA factor) x dilution factor x 2

A standard concentration of RNA (125 ng/µl) for each sample was created by appropriate dilution with RNase free water. This produces an aliquot of 1 µg of total RNA in an 8 ul volume used for cDNA synthesis.

2.2.3 First Strand cDNA Synthesis

Prior to first-strand cDNA synthesis the RNA was denatured, unravelling the RNA molecules and allowing optimal annealing of primers. This was achieved by transferring 32 ul of the RNA solution (125 ng/ul) into a sterile 0.75ml eppendorf tube. The capped tubes were then heated to 95°C for 10 minutes in a thermal reactor (Hybrid, UK) and then placed on ice.

The first stand cDNA synthesis was performed using a commercially available kit (Amersham, UK). The kit employs Moloney Murine Leukaemia Virus (M-MuLV)

reverse transcriptase and the *Not* I-d(T)$_{18}$ bifunctional primer. The conditions under which the reaction takes place have been pre-optimised by the manufacturer to allow full-length transcription of RNAs 7 kilobases or more in length, i.e. produces cDNA from mRNA fraction of total RNA only.

Four 0.75ml sterile eppendorf reaction tubes were set up for each RNA sample. the eppendorf tubes were UV irradiated for 10 minutes to cross link any contaminating DNA and prevent its subsequent amplification. To each tube was added 5µl bulk first-strand reaction mix, 1µl of 200mM DTT, 1µl of *Not* I-d(T)$_{18}$ primer and 8µl (1µg) of denatured RNA which was then incubated at 37°C for one hour in an organ bath. After incubation, the four cDNA samples of each patient were combined and frozen at -70°C to await subsequent molecular analysis.

2.2.4 TaqMan® PCR for Gene Expression Analysis

Applied Biosystems developed the TaqMan® system as a research tool for real time, in vitro quantitative evaluation of polymerase chain reaction (PCR) products. In this work, the ABI Prism® 7900 HT sequence detection system was used.

2.2.4.i Theory of Operation

The TaqMan® real time PCR system utilises the 5' nuclease activity of Ampli Taq Gold® DNA Polymerase which is able to cleave a fluorescent labelled probe during the PCR reaction. The TaqMan probe has a reporter dye at the 5' end of the probe and a quencher dye at the 3' end of the probe.

In this study the 5' reporter dye used was 6-carboxy fluorescein (FAM) and 3' quencher dye, 6-carboxy-tetramethylrhodamine (TAMRA). During the PCR reaction the probe is cleaved which separates the reporter dye from the quencher dye resulting

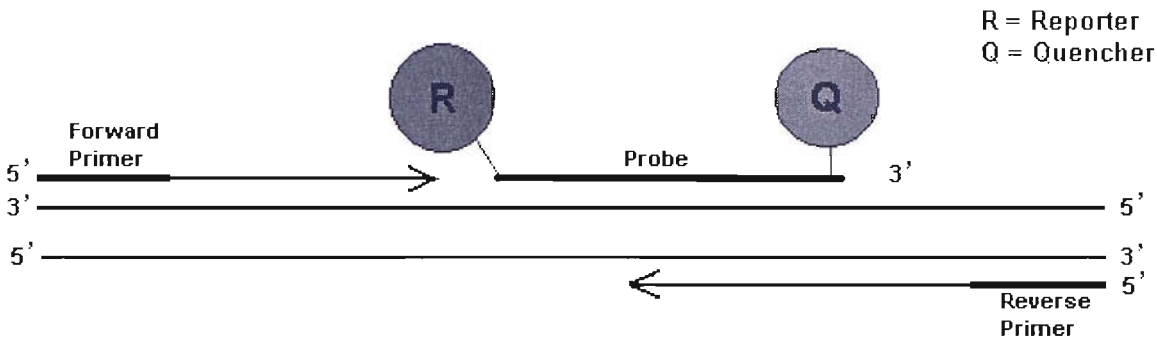
in increased fluorescence of the reporter dye (Figure 2.1.c.) When the probe is intact, the proximity of the reporter dye to the quencher dye leads to suppression of the fluorescence of the reporter dye by Förster-type energy transfer.

During the PCR, if the sequence of interest is present, the probe specifically binds between the forward and reverse primer sites. The 5'-3' nucleolyte activity of the Ampli Taq Gold ® DNA Polymerase only cleaves the probe releasing the reporter dye if the probe is hybridised to the target sequence. Probe fragments are displaced from the target sequence and polymerisation continues (Figure 2.1.d.). The 3' end of the probe is blocked to prevent probe extension during PCR.

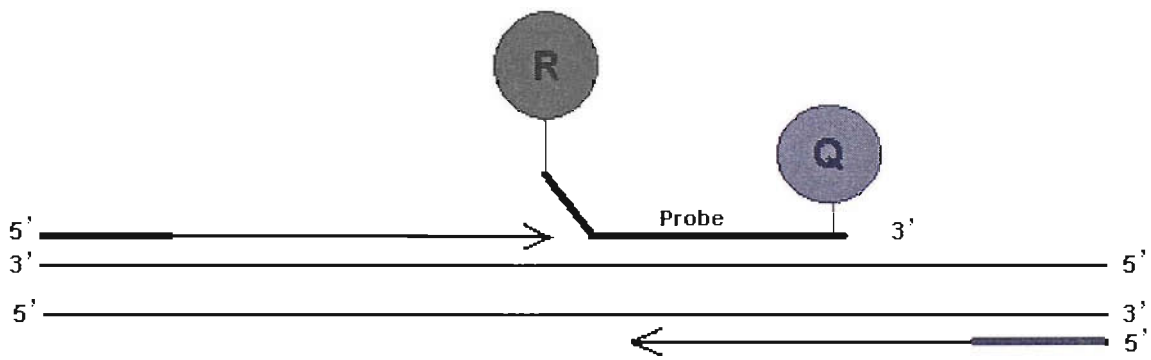
Accumulation of PCR products is directly related to the rising levels of reporter dye fluorescence. During PCR an argon ion laser is sequentially directed to each well of the microplate, which excites the fluorescent dyes present. The resulting fluorescent emission is collected from each well, with a complete collection of data from all wells approximately once every 7-10 seconds, by a charged coupled device (CCD) camera. The sequence detector software collects the fluorescent signals from the CCD camera and applies data analysis algorithms (see section 2.2.4.iii regarding generation of CT data).

Figure. 2.1 Diagramatic Representation of the Taqman PCR Assay

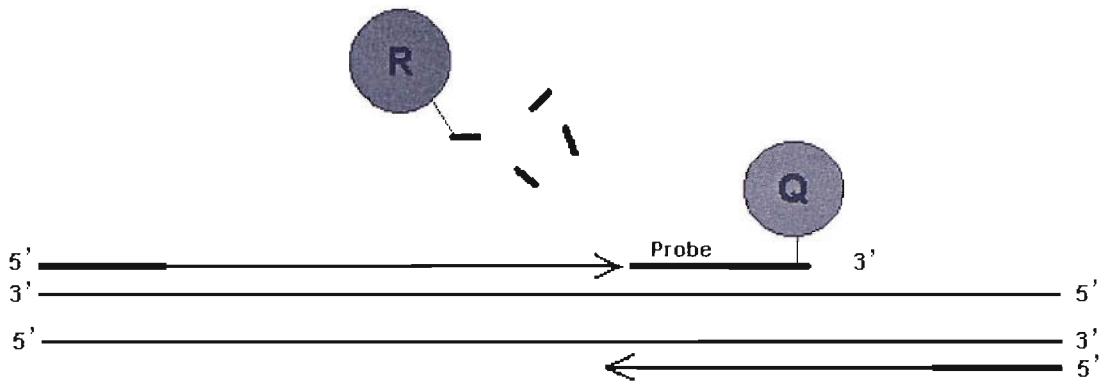
a. Polymerisation



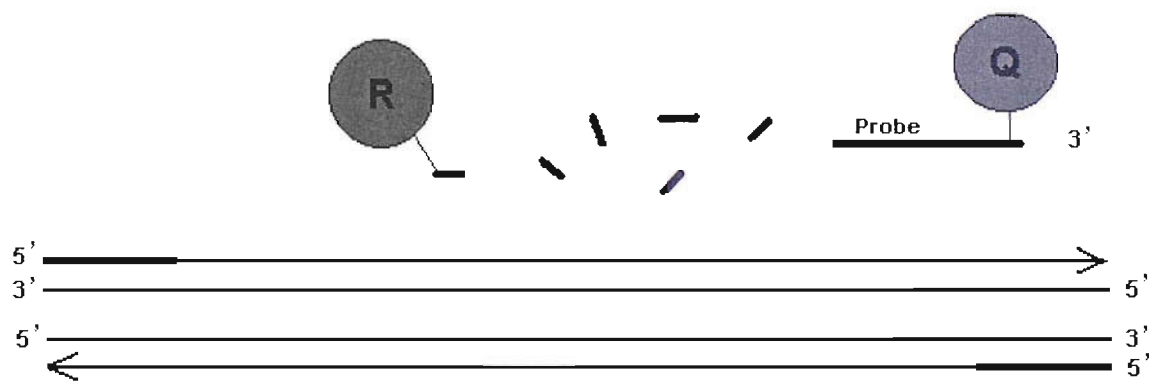
b. Strand Displacement



c Cleavage



d Polymerisation Complete



2.2.4.ii TaqMan Primer and Probe Design

All primers and probes used in this study were designed using Primer Express Version ® 2.0 software (Applied Biosystems, UK). This software allows primers and probes to be designed, which will perform optimally under standard PCR conditions using the 7900 HT Sequence Detector.

The cDNA sequence of the target gene was imported into Primer Express ®, the exon/exon boundaries marked and the software automatically searches for appropriate primers and probes. The primers and probes need to fulfil certain criteria to work optimally (Table.2.1). The probe preferentially lies across an exon/exon boundary so as to minimise the risk of amplifying any genomic DNA within the specimen. For some gene target sequences the software did not come up with any suitable primers and probes spanning an exon/exon boundary. In these incidences the primers and probes were designed individually and found that they could be designed within the criteria of Table 2.1 and across an exon/exon boundary, and were found to work well. If the primers and probes could not have been designed across exon/exon boundaries they could still have been used with the RNA samples prepared by the author but not with the historic samples, as the author's samples were treated with DNAse to remove contaminating genomic DNA, .

The oligonucleotide sequences for the primers and 5' FAM, 3' TAMRA dye labelled probes were ordered from Eurogentec DNA services (UK).

Primer	Tm 58-60°C (Melting Temp) 20-80% GC content Length 9-40 bases <2°C difference in Tm between the two primers Maximum of 2/5 G or C's at 3' end
Probe	Tm 10°C higher than Primer Tm 20-80% GC content Length 9-40 bases No G on the 5' end < 4 contiguous G's Must not have more G's than C's
Amplicon	50-150 bp in length 3' end of primer as close to the probe as possible without overlapping

Table.2.1. Criteria for Optimal TaqMan Primer and Probe Design

Primers and probes used in this study are detailed below, two bases either side of the exon/exon boundaries are highlighted. All probes were 5' FAM and 3' TAMRA labelled.

Primer and Probe Sequences

CCL3

Forward Primer	ACAGAATTTCATAGCTGACTACTTTGAGA
Reverse Primer	CGGCTTCGCTTGGTTAGGA
Probe	CAGTGCTCCAAGCCCGGTGTC**AT**

CCL4

Forward Primer	CTGCTCTCCAGCGCTCTCA
Reverse Primer	TTCCTCGCGGTGTAAGAAAAG
Probe	CACC**AATG**GGCTCAGACCCTCCCT

CCL5

Forward Primer	TCTGCGCTCCTGCATCTG
Reverse Primer	GCGGGCAATGTAGGCAAA
Probe	**ATAT**TCCTCGGACACCACACCCTGCTGT

CXCL10

Forward Primer	CGATTCTGATTTGCTGCCTTATC
Reverse Primer	GCAGGTACAGCGTACGGTTCT
Probe	TTCTGACTCTAAGTGGCATTC**AAGG**AGTACCTCTC

CCRI

Forward Primer	CACGGACAAAGTCCCTTGGA
Reverse Primer	TGTGGTCGTGTCATAGTCCTCTGT
Probe	A**GAGA**GAAGCCGGGATGGAAACTCCAAAT

CCR5

Forward Primer	CCAGAAGAGCTGAGACATCCGT
Reverse Primer	GTCATAGATTGGACTTGACACTTGATAAT
Probe	AACTCTCCC**CGGG**TGGAACAAGATG

CXCR3

Forward Primer	CCAGCAGCCAGAGCACCA
Reverse Primer	CCTCGGCGTCATTTAGCACTT
Probe	CCATGGTCCTTG**AGGT**GAGTGACCACC

GAPDH

Forward Primer	CCACATCGCTCAGACACCAT
Reverse Primer	CCAGGCGCCCAATACG
Probe	AAGGTGAAGGTCGGAGTCAAC**GGAT**TTG

MLM5I

Forward Primer	CCACAGGGCATGCTTGTG
Reverse Primer	GGGATAGAGGCCTGGATTGG
Probe	CCCCACCC**AGGT**TTACATCCCCA

YWHAZ

Forward Primer	TGAAAATGAAAGGAGATTACTACCGTTA
Reverse Primer	ACTGATCGACAATC**CCTT**TCTTG
Probe	TGGCTGAGGTTGCCGCTGGTG

EF-1α

Forward Primer	TGACCCACCAATGGAAGCA
Reverse Primer	GCGCTTATTTGGCCTGGAT
Probe	CTGGCTTCACTGCTC**AGGT**GATTATCCTGA

UBch5B

Forward Primer	TGAAGAGAATCCACA**AGGA**ATTGA
Reverse Primer	CCAACAGGACCTGCTGAACA
Probe	TGATCTGGCACGGGACCCTCCA

2.2.4.iii TaqMan Gene Expression Analysis

The 7900HT Sequence Detection System has a large capacity for specimen throughput with a potential of 384 wells in each plate that could be prepared for gene expression analysis.

Multiple patients were analysed on each plate and sometimes more than one gene target, usually no more than two for ease and efficiency of laboratory working and to minimise the risk of error. Each sample with each target gene primer and probe was run in duplicate to reduce error from sub-optimal PCR reactions. If there was a discrepancy of more than one cycle between the duplicate samples the patient series was repeated (the basis of the cycle readout is explained later on in this section). The mean of the duplicate samples was used for all subsequent statistical analysis.

Prior to setting up a run, a PCR reaction mix was made up for each gene to be analysed. The volumes of the individual constituents for each duplicate patient sample and their concentrations are shown in Table 2.2.

Components and Stock Concentration	Working Concentration	Volume used (per duplicate)
TaqMan Universal PCR Master Mix (x2)	1x	25µl
Rnase/Dnase Free Water	-	20µl
Forward Primer (15 µM)	300nM	1µl
Reverse Primer (15 µM)	300nM	1µl
Probe (5µM)	100nM	1µl
Total Volume per duplicate sample		**48µl**

Table. 2.2. Components of the PCR Reaction Mix for each Patient Duplicate Sample

The concentrations of the primers and probes used were previously established by colleagues within the laboratory and were found to work well.

Following preparation of the PCR reaction mix, a 384 well plate was set up using a paper template of the plate with patient sample numbers and the target gene analysed for each well. A 19µl aliquot of PCR reaction mix was added to each appropriate well with an electronic multi-dispensing pipette and then 1µl of cDNA from the patient samples added to the wells in accordance to the template. Four duplicate wells were loaded with 1µl of water instead of cDNA, each duplicate placed separately on the plate, which were used as negative controls. This was to identify any cross contamination between the wells during pipetting. Two positive control samples, i.e standards, were also analysed in duplicate on each plate. This allowed variations between each plate to be detected, with minimal variations adjusted by adjustment of the threshold baseline, or for the whole plate to be re-run for larger variations (more than one cycle difference between plates).
Following plate set up, an adhesive cover (Applied Biosystems UK) was applied, which sealed the wells. The plate was centrifuged at 1000g for 15 seconds to remove any air

bubbles from the bottom of the wells, which could interfere with the thermal profile of the PCR mix.

The plate was transferred to the 7900HT Sequence Detector. For each run the thermal cycling conditions (Table 2.3),reaction volume and dye detection system were checked or selected as appropriate. The plate was then run for 45 PCR cycles.

STAGE 1 (Hold)	STAGE 2 (Hold)	STAGE 3 (45 Cycles)	
2 Minutes at 50°C	10 Minutes at 95°C	15 Seconds at 95°C	60 Seconds at 60°C

Table. 2.3. Standard TaqMan PCR Thermal Cycling Conditions

On completion of the run, the computer analyses the data produced by the sequence detector, on request. This generates a numerical value expressed as the number of cycles of PCR at which point the reaction in each well becomes exponential. This value is the C_T (Threshold Cycle) value which is inversely correlated to the number of target sequence copies in the cDNA added to each PCR reaction mix. The data output is also shown in a graphical form (Amplification plot Figure 2.2) allowing adjustments to the CT values, relative to the positive controls, by moving the threshold bar up or down on the graph.

The data generated was transferred to an excell spreadsheet for statistical analysis.

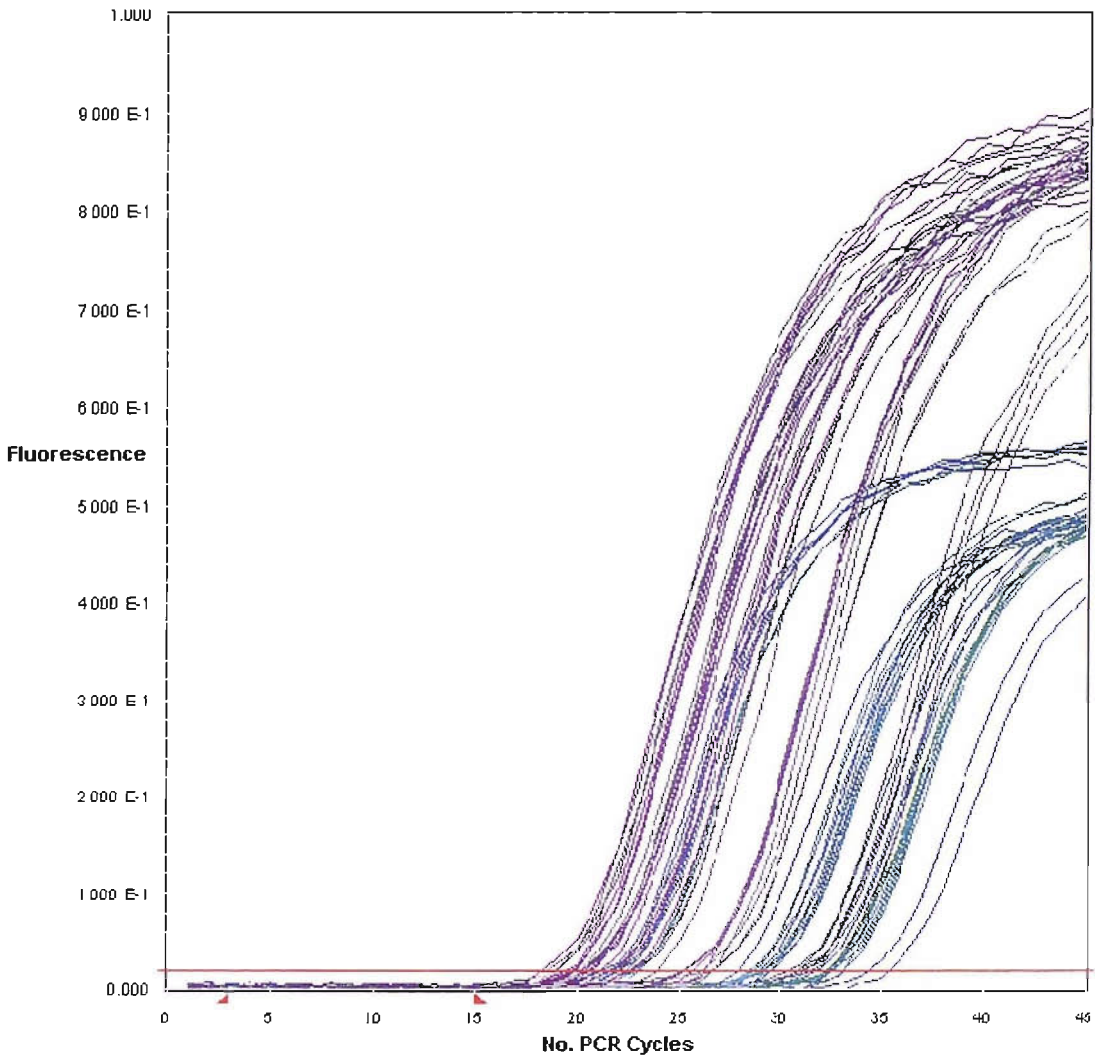


Figure. 2.2. Amplification plot showing real-time PCR amplicon production for two gene target sequences. Purple/brown lines - gene target sequence 1: Blue/green lines - gene target sequence 2: Horizontal red line - adjustable threshold bar.

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2.2.5 Statistical Analysis

The statistical software SPSS10 (SPSS Inc, USA) was used to analyse the data produced in this work. The software generated the box and whisker and scatter diagram plots as well as applying statistical tests and producing p values. The statistical tests used in the analyses of the data were originally chosen following consultation with a medical statistician of the University of Southampton (Dr R Pickering) .

A p value of <0.05 was deemed statistically significant and a value <0.01 was deemed highly statistically significant (i.e. less than one in 100 probability that the observation was a chance association).

The tests used were the correlation coefficient and Wilcoxon signed rank sum test as described below.

2.2.5.i Correlation Coefficient

The Pearson correlation coefficient, r, was used to analyse the relationship between two quantitative variables, for example, gene expression as a CT value and the A260/A280 ratio, represented graphically by a scatter diagram (Figures 4.1-4.5). It is based on the sum of products about the mean of two variables as shown in the equation below.

$$r = \frac{\sum (x_i - \bar{x})(y_i - \hat{y})}{\sqrt{(\sum (x_i - \bar{x})^2)(\sum (y_i - \hat{y})^2)}}$$

x_i, y_i – n pairs of variables

$\bar{x}, \hat{y}$ – mean x, y values

The p value can then be read off from a table equating it with r, and the number of observations, n.

2.2.5.ii Wilcoxon signed rank sum test

The Wilcoxon signed rank sum test is a non parametric test (population variable assumed not to have a Normal distribution) for paired data. The paired data compared in this work, being the target gene expression levels (as CT values), of a patient, between the day pre-transplant and subsequent days post-transplant, or between successive days post-transplant. Firstly the differences in the paired observations are ranked, ignoring the sign. The ranks of the positive and negative differences are then summated, of which the lesser of the sums is the test statistic and designated T. This T value is then put in the equation below to produce a *z* value. From the *z* value a p value can be read off from a Normal distribution table.

$$Z = \frac{T - n(n+1) / 4}{\sqrt{n(n+1)(2n+1) / 24}}$$

Chapter 3

Patient Demographics

This chapter presents the demographics of the 135 patients who were recruited between June 1999 and April 2003 and whose samples were used in this study. The non-rejector group who received a renal transplant and did not have any proven rejection episodes within the 14 day post-transplant study period comprised 84 patients. The rejector group who experienced an episode of proven rejection within the study period comprised 22 patients. The live donor group comprised 29 patients.

The causes of renal failure in both the rejector and non-rejector groups combined are shown in table 3.1. The demographics and donor to recipient CMV status of the patients and their immunosuppressive regime, comparing the rejector and non-rejector groups are shown in tables 3.2, 3.3 and 3.4 respectively.
The demographics of the living donor group are shown in table 3.5.

Cause of renal failure	No. of patients
Diabetic nephropathy	13
Polycystic kidney disease	27
Henoch-Scholein Purpura	1
Reflux nephropathy	8
Congenital obstructive uropathy (posterior urethral valves)	5
Obstructive uropathy	2
Membrano-proliferative GN	1
Focal segmental glomerulosclerosis	4
Crescentic GN	2
Wegener's granulomatosis	1
GN	10
Mesangio-proliferative GN	3
Neurogenic bladder	2
Proliferative GN	1
Membranous GN	2
Analgesic nephropathy	1
Cyclosporin nephropathy	2
Unknown	9
Pyelonephritis/interstitial nephritis	1
IgA nephropathy	4
Alports syndrome	1
Single/dysgenic kidney	1
Reno-vascular disease	2
Malignant hypertension	1
Vasculitis	2

Table 3.1 Causes of end-stage renal failure in all renal transplant recipients.

Demographic	Non-rejectors	Rejectors
Number in group	84	22
Female	29 (34.5%)	9 (40.9%)
Male	55 (65.4%)	13 (59.1%)
Mean donor age (years) Range	46.6 4-73	39.7 18-63
Live donors	16 (19%)	9 (41%)
Mean mismatch (A : B : DR)	0.9 : 1.0 : 0.6	0.9 : 1.1 : 0.7
Mean C.I.T (live donors) (hours) Range	3.0 2.0-5.0	2.1 0.2-4.0
Mean C.I.T (cadaveric) (hours) Range	18.7 10-38	16.5 5-36
Delayed graft function	12 (14.3%)	6 (27.3%)

Table 3.2 Demographics of the non-rejector and rejector groups of renal transplant recipients. C.I.T = Cold Ischaemic Time

Delayed graft function is defined as the need for dialysis post-transplant or a drop of less than 10% in the serum creatinine in the first 24 hours following transplantation.

Donor to Recipient CMV status	Non-rejectors	Rejectors
Positive to Positive	20 (24%)	7 (32%)
Positive to Negative	20 (24%)	9 (41%)
Negative to Positive	20 (24%)	1 (4%)
Negative to Negative	24 (28%)	5 (23%)

Table 3.3 CMV status of donor-recipient in the non-rejector and rejector groups of renal transplant recipients.

Immunosuppressive regime	Non-rejectors	Rejectors
Cyclosporin Azathioprine Prednisolone	32 (38%)	5 (23%)
Cyclosporin Azathioprine Prednisolone+Simulect	6 (7%)	1 (4%)
Cyclosporin MMF Prednisolone	20 (24%)	9 (41%)
Cyclosporin MMF Prednisolone+Simulect	12 (15%)	1 (4%)
Rapamycin Azathioprine Prednisolone	11 (13%)	3 (14%)
Rapamycin MMF Prednisolone	2 (2%)	3 (14%)
Rapamycin MMF Prednisolone+Simulect	1 (1%)	-

Table 3.4 Distribution of immunosuppressive regimes used in the non-rejector and rejector groups of renal transplant recipients. MMF=Mycophenolate mofetil

Donor type	Number	Mean age (years)	Male	Female
Living related	19	47	4 (21%)	15 (79%)
Living unrelated	10	50	3 (30%)	7 (70%)

Table 3.5 Demographics of living donor group.

All rejection episodes were biopsy proven and initially treated with three pulses of 500mg methyl prednisolone over three days. No patients were treated with OKT3 or ATG (anti-thymocyte globulin) within the study period for steroid resistant rejection. Not all biopsies were reported by the same pathologist and the Banff grades of rejection were unavailable as they were not routinely reported. Table 3.6 shows the biopsy results for the rejector group with eight patients having two biopsies within the study period.

Biopsy Report	**Number of Patients**
Acute Cellular Rejection	6
Acute Vascular Rejection	2
Acute Vascular and Cellular Rejection	1
Borderline Rejection	5
1st Biopsy – Borderline Rejection 2nd Biopsy – Acute Cellular Rejection	3
1st Biopsy – ATN / CyA toxicity 2nd Biopsy – Acute Cellular Rejection	2
1st Biopsy – ATN / CyA toxicity 2nd Biopsy – Acute Cellular and Vascular Rejection	2
1st Biopsy – Normal 2nd Biopsy – Borderline Rejection	1

Table 3.6 Histological reports of biopsies taken within the 14 day post-transplant study period in the rejector group ; ATN – Acute tubular necrosis, CyA – Cyclosporin A .

In the non-rejector group 36 patients had biopsies and the results of these are shown below in table 3.7. Two patients had two biopsies within the study period and all four were reported as normal.

Eight patients from the non-rejector group subsequently went on to have a biopsy proven rejection episode outside of the study period. One of these had had a biopsy during the study period that was reported as borderline rejection and not given methyl prednisolone. The time post-transplant at which rejection was diagnosed in this group ranged from 18 to 44 days with an mean of 28 days.

Biopsy Report	**Number of Patients**
Cyclosporin toxicity	4
Cyclosporin toxicity / ATN	2
Donor Disease	3
Normal	12
Non-specific changes	4
Query ? Cyclosporin toxicity	4
Borderline Rejection	5
ATN	2

Table 3.7 Histological reports of biopsies taken within the 14 day post-transplant study period in the non-rejector group ; ATN-Acute tubular necrosis.

Chapter 4

Critical Assessment of Method of Gene Expression Analysis and the Use of Endogenous Control Genes for Standardisation.

The method used in this work is based on determining the number of cycles of PCR required to achieve a given level of PCR product accumulation, set in the early exponential phase of the PCR reaction, and expressed as a C_T value (threshold cycle). The C_T value is inversely correlated to the sample target copy number of cDNA of the gene to which the primers and probes have been designed. The greater the C_T value, the smaller the quantity of gene cDNA in the PCR reaction, as more PCR cycles are required to reach the exponential phase of the reaction.
This chapter outlines the variables within the methodology used in this work, followed by a discussion on the use of endogenous control genes for standardisation of gene expression analysis. The results of five potential endogenous control genes investigated in this study are presented and then discussed.

4.1 RNA Extraction / Quantification

In this laboratory, the method of standardisation to date was to reverse transcribe the mRNA in a fixed quantity of total RNA to cDNA (i.e. 1μg of total RNA in an 8μl volume). This requires quantification of the RNA following extraction, and appropriate dilution. This should normalize variables such as the number of mononuclear cells extracted from the 9ml of blood, as the white cell count between patients and sample

days can vary. Another approach to this would be to standardise to the number of mononuclear cells, but further cell manipulation, stressing the cells, may cause changes in cellular transcriptional activity and therefore differences in mRNA levels in vitro. Also, although the mononuclear cells were separated and digested in RNAzol as quickly as possible after blood collection, there was a variation in the time that this was carried out. Blood specimens were placed in a 4°C refrigerator immediately after collection where they remained for between zero and 24 hours before cell separation and RNAzol digestion. This helps minimize any changes in gene expression levels due to storage. Tanner et al. (2002) looked at the effect of time and temperature on gene expression of stored human whole blood using TaqMan® quantitative real-time PCR. They demonstrated that blood stored at ambient temperature (23°C) generated an inflammatory response with general up-regulation of inflammatory cytokines and down-regulation of anti-inflammatory cytokines. These changes were minimal after 1 hour of storage but increased after 4 hours up to 24 hours, with 100 fold changes in some gene expression levels. With storage at 4°C for 24 hours these changes were not observed, however a two to threefold change was seen in some genes, for example CXCL8, but greatly reduced compared to the > 100-fold up-regulation of CXCL8 at ambient temperature for 24 hours. At ambient temperature for 24 hours they observed a 10-100 fold up-regulation in CCL3 expression, a 2-10 fold down-regulation in CXCL10 expression and no change in CCL4 and CCL5 expression levels. Although no values were quoted for storage at 4°C, on visual inspection of a graph of chemokine and cytokine gene expression plotted against time at 4°C, no changes in expression levels were obvious for up to 24 hours, in contrast to the graph at ambient temperature. This group of Tanner et al. looked at only two chemokine receptors, CCR3 which down-regulated 10-100 fold and CCR4 with no change in expression levels at ambient temperature at 24 hours. As with the chemokines, no changes were noted graphically for receptors at 4°C up to 24 hours [Tanner et al, 2002]. This study demonstrates that blood storage at 4°C minimizes changes in gene expression levels up to 24 hours of storage. Also RNA is relatively unstable and storage at 4°C helps prevent its degradation.

The RNA quantification method used was to measure the OD value at 260nm using a spectrophotometer. The OD value measures total RNA, which consists predominantly of ribosomal RNA (rRNA), and therefore may not always be representative of the mRNA fraction [Solanos et al, 2001]. The purity of the extracted RNA is indicated by the A260/A280 absorption ratio. A ratio of 1.7 or higher is considered sufficient for good reverse transcription [Sambrook et al, 1989]. Ratios below 1.7 were often observed in this work, despite careful avoidance of the aqueous/organic interface during pipetting of the aqueous RNA solution after chloroform extraction to reduce protein/DNA contamination. Through their work with RT-PCR reactions, Yamaguchi et al (1992) showed an increase in GAPDH signal strength for samples with higher A260/A280 absorbance ratios using detection by Southern hybridization of amplified PCR products [Yamaguchi et al, 1992]. In an attempt to investigate any possible correlation between the A260/A280 ratio and C_T value of samples used in this work, these two measures have been plotted, in the form of a scattergram, against one another for each of the endogenous contol genes investigated (Figures 4.1-4.4). The pre-operative and pre-transplant samples were analysed to avoid the influences that may occur post-operatively and post-transplant. Each scattergram has a linear best-fit line and the statistical analysis showing the Pearsons correlation coefficient for each gene is shown in Table 4.1.

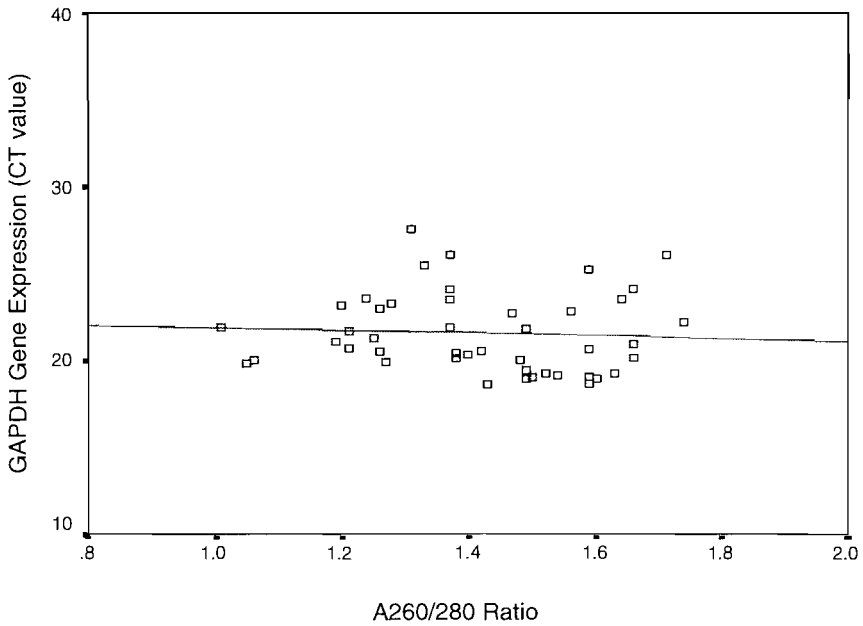

Figure 4.1 Scatter diagram showing correlation between RNA A260/A280 ratio (x-axis) and GAPDH gene expression levels (y-axis).

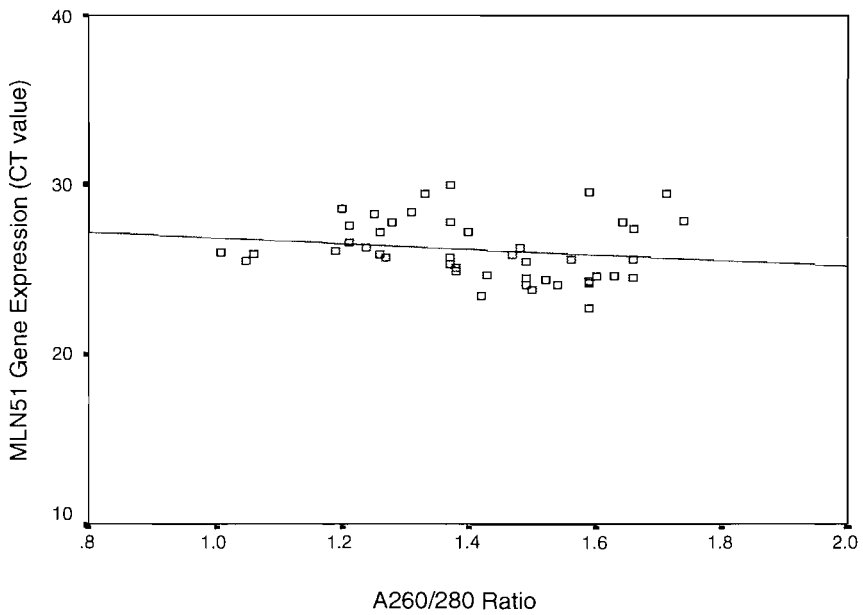

Figure 4.2 Scatter diagram showing correlation between RNA A260/A280 ratio (x-axis) and MLN51 gene expression levels (y-axis).

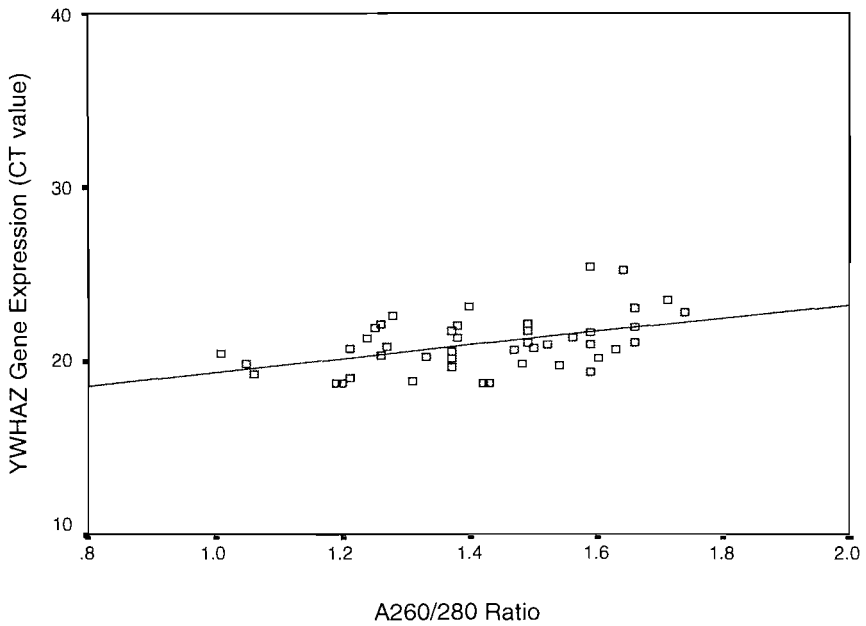

Figure 4.3 Scatter diagram showing correlation between RNA A260/A280 ratio (x-axis) and YWHAZ gene expression levels (y-axis).

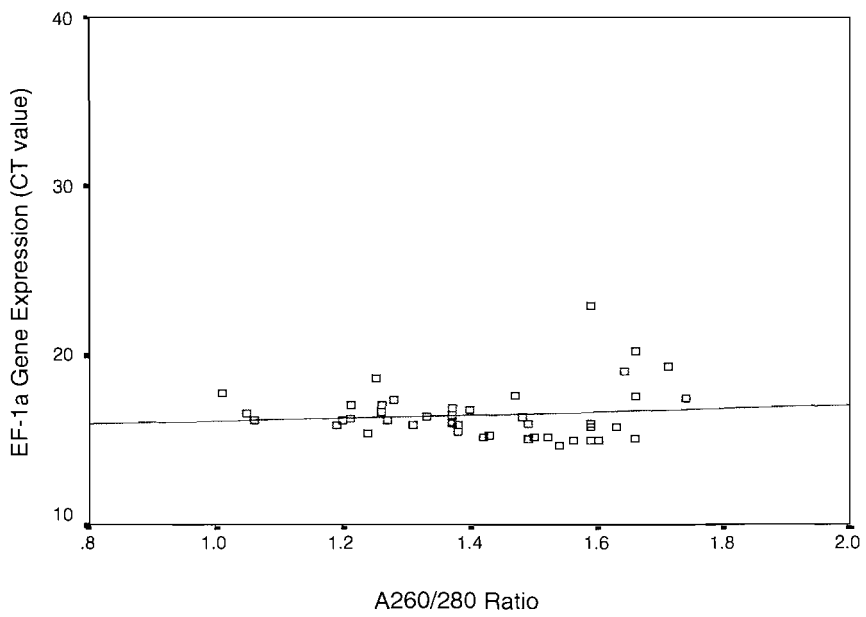

Figure 4.4 Scatter diagram showing correlation between RNA A260/A280 ratio (x-axis) and EF-1α gene expression levels (y-axis).

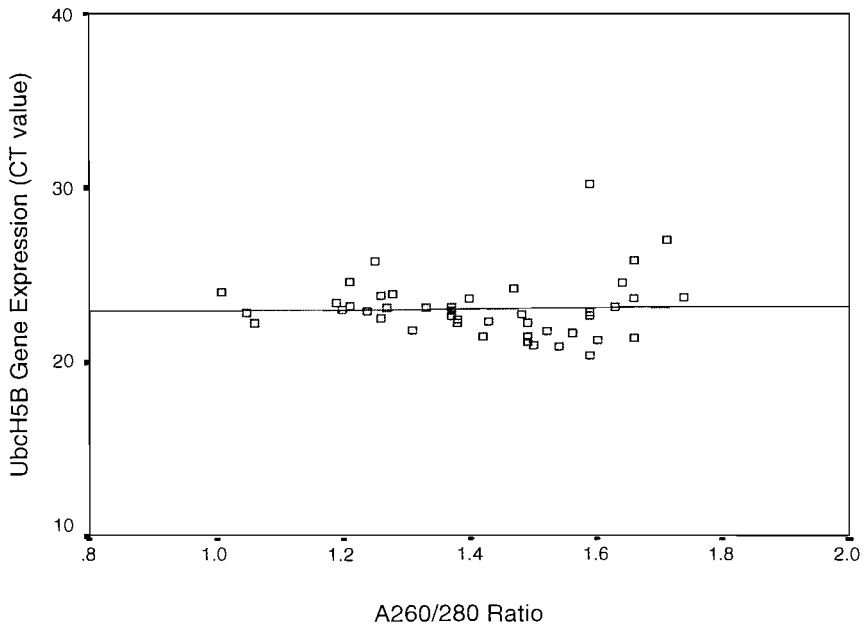

Figure 4.5 Scatter diagram showing correlation between RNA A260/A280 ratio (x-axis) and UbcH5B gene expression levels (y-axis).

Gene	Number of samples	Pearsons Correlation coefficient (r)	P value
GAPDH	45	-0.062	0.685
MLN51	45	-0.169	0.269
YWHAZ	45	0.454	0.002
EF-1α	45	0.105	0.491
UbcH5B	45	0.032	0.835

Table 4.1 Correlation coefficients and significance of gene expression levels and RNA A260/A280 ratios for the pre-operative and pre-transplant samples.

The scattergrams for GAPDH and MLN51 (Figures 4.1 and 4.2) show a very weak negative correlation (r= -0.062 and -0.169 respectively) between the CT value and

A260/280 ratio. In other words the higher the A260/280 ratio the lower the CT value and therefore the greater the quantity of target gene detected. Between the extreme A260/280 ratio values (1.0 and 1.8) the average difference in CT value was 1.0 for MLN51 and 0.75 for GAPDH (equivalent to a 2-fold and 1.75-fold increase in target gene detected respectively). This suggests increased efficiency of the reverse transcription and/or PCR reaction with higher A260/280 ratios. Yamaguchi et al, using the same RNA extraction method as this work, demonstrated the same relationship with GAPDH expression in human saphenous vein endothelial cells and the A260/280 ratio, but with a stronger correlation (r=0.714). However they analysed only six samples and found no statistical significance (p=0.111), compared to the 45 samples in this work, where also no statistical significance was demonstrated (p=0.685). They also used Southern blot to quantify the PCR end products which is less sensitive than real-time PCR quantitation [Yamaguchi et al, 1992]. In contrast the scattergrams for YWHAZ, EF-1α and UbcH5B (Figures 4.3 to 4.5) showed a positive correlation (r=0.454, 0.105 and 0.032 respectively) which was very weak for EF-1α and UbcH5B but greater for YWHAZ. Between the extreme A260/280 ratio values (1.0 and 1.8) the average difference in CT value was 1.0 for EF-1α, 0.25 for UbcH5B and 2.5 for YWHAZ (equivalent to a 2-fold, 1.25-fold and 6-fold decrease in target gene detected respectively). This was not statistically significant for EF-1α and UbcH5B (p=0.491 and 0.835 respectively), but was for YWHAZ (p=0.002). These results show a variable and mostly weak affect of the A260/280 ratio on the CT gene expression levels. The only significant correlation was with YWHAZ, where the higher the A260/280 ratio the less target gene was detectable suggesting that ratios lower than 1.7 can be sufficient for good reverse transcription. This variability in correlation coefficients may reflect differences in the binding properties of the primers and probes of the genes, and their influence by any impurities in the reaction mix.

By analyzing all the samples to increase the numbers, although values may be affected by post-operative/transplant events, 4 out of 5 genes showed a very weak negative correlation (max. r=-0.280) but were statistically significant ($p<0.05$) and one showed no correlation (see Table 4.2 below).

Gene	Number of samples	Pearsons Correlation coefficient (r)	P value
GAPDH	473	-0.166	<0.001
MLN51	463	-0.280	<0.001
YWHAZ	472	-0.099	0.031
EF-1α	469	0	0.992
UbcH5B	470	-0.099	0.031

Table 4.2 Correlation coefficients and significance of gene expression levels and RNA A260/A280 ratios for all samples.

Overall, however, it can be concluded that although the A260/280 ratios were often below 1.7 in this work, they had a variable and limited influence on the gene expression levels.

Another aspect of RNA quantification is that differential gene expression may, theoretically, lead to artefactual changes in the expression of the gene under investigation. For example, an increase in expression of gene A in a fixed quantity of RNA could lead to the detection of a false decrease in expression of gene B in a sample. This would however be unlikely due to the vast numbers of mRNA species present within solution.

4.2 Reverse Transcription

Following quantification the next stage is reverse transcription of the mRNA to create a cDNA bank. Variability in this step can occur as the reverse transcriptase (in this case Moloney murine leukaemia virus (MMLV-RT)) enzyme is sensitive to salts, alcohols, or phenol remaining from the RNA extraction process [Ferre et al, 1994]. The levels of these contaminants are likely to be variable, especially because of the level of RNA dilution required in the RNA standardization step (section 2.2.2.iv). Thus, the reverse

transcription efficiency is likely to have some variation between different reactions. Studies have reported variability of efficiency of the reverse transcription step ranging from 5% to 90% [Noonan et al, 1990; Henrard et al, 1992; Simmonds et al, 1990], and Gilliland et al reported the yield of amplification of a cDNA fragment can vary as much as 6-fold among duplicates [Gilliland et al, 1990]

The variation and reproducibility of the RT-PCR method in this work was tested, which would give an indication of the variability in the reverse transcriptase (RT) step by analysing the PCR step separately for the same set of samples. Twenty pre-operative/transplant blood samples were duplicated and their target gene expression determined (MLN51 was chosen as its CT expression levels lay midway between the CT levels of the 13 target genes analysed in this work (average CT value for these samples being 26.4 with a range of 23.3 to 29.6)). Each cDNA sample of the blood duplicates was run in duplicate in the PCR step. The results with the means and standard deviations are shown below in Table 4.3.

	Blood duplicates (N=20)	cDNA duplicates (N=40)
Mean of differences in CT value	1.08 ± 0.80	0.43 ± 0.36
Mean of difference as % of CT value	4.1 ± 3.0	1.61 ± 1.34

Table 4.3 Mean of CT differences, with standard deviation, between blood duplicate and cDNA duplicate samples.

These results show a mean variation in the RT-PCR method of 1.08 ± 0.80 in the CT value which is equivalent to a 2-fold change in target gene detection. The PCR step itself showed a mean variation of 0.43 ± 0.36 which is equivalent to a 1.4-fold change in target gene detection. This demonstrates that a greater proportion of the variability in the method of this work occurs in the pre-PCR steps (RNA quantitation and reverse transcription) compared to the PCR step (approx. 60% versus 40% respectively).

4.3 TaqMan PCR Reaction and avoidance of signal from contaminating DNA

The RT-PCR amplification reaction has been shown to be affected by salt [Freeman et al, 1999], therefore any salt contamination from the RNA precipitation may influence its efficiency. This was minimised by washing of the isolated RNA with 75% etnanol (section 2.2.2.i).

In the patient group recruited by myself samples were treated with a DNAase after RNA quantification. This process digested any contaminating genomic DNA extracted and thereby allowed the use of primers and probes that could not be designed across exon/exon boundaries. However all gene primers and probes used in this work were successfully designed across exon/exon boundaries so as to avoid amplification of any contaminating DNA. Each set of designed primers and probes was run with 1μg of genomic DNA (in a 1μl volume) instead of 1μl of sample cDNA as described in section 2.1.4.3. This was performed to check that the designed primers and probes were not able in practice to pick up genomic DNA that may be present within the samples. After 45 PCR cycles no fluorescence was detected showing no significant genomic DNA amplification. This was a robust double check of the system, to determine that results from the historic samples, untreated with DNAse, should not differ from the later samples.

Each PCR plate had several control wells where the cDNA was replaced by water to detect any possible cross-contamination between the wells. Also, to check for reproducibility, each plate had a standard cDNA solution run with the primers and probes in that plate. This ensured the detection of any variations in primer/probe solution composition which may have affected Taqman function between runs and any systematic shift in quantitation with each new batch of reagents would also be detected.

4.4.1 Standardisation using Endogenous control gene

Standardisation by RNA quantification does not control for the variability within the reverse transcription reaction, and such control is necessary if the gene expression results of RT-PCR are to be accurate, precise and reproducible. It was therefore decided to investigate the use of a gene whose expression could be used as a standard against which a target gene expression could be measured. This could be in the form of an endogenous internal or synthetic (heterologous or homologous) standard. To develop a synthetic standard involves many different stages, including plasmid formation and DNA quantification for each target gene investigated, and as such is time consuming. An endogenous standard, or endogenous contol gene, requires just primer and probe design and may possibly be used for multiple target genes. It also has the advantage of providing a control on the yield of amplifiable targets from the pool of successfully isolated total RNA.
A suitable endogenous control gene should show uniform expression in the tissue or cells under investigation, or in response to experimental treatment and five candidate genes were investigated in this work. A lot of studies that use endogenous control genes do not investigate whether their expression varies within the gene expression model used in the study. Many studies have shown that genes frequently used as endogenous controls (often referred to as housekeeping genes) actually vary in expression, indicating that ideal and universal endogenous control genes do not exist [Suzuki et al, 2000; Thellin et al, 1999; Warrington et al, 2000; Bustin et al, 2000]. It is therefore imperative to validate their stability of expression in the model in which they are to be used, which in this case was human peripheral blood mononuclear cells in solid organ transplantation.

In this laboratory Gibbs et al, using the same TaqMan method of gene expression analysis of human peripheral blood mononuclear cells from renal transplant patients, investigated three housekeeping genes as endogenous controls, namely β Actin, β2 microglobulin and transferrin receptor. No significant changes were found in the mean expression of these genes in the donor nephrectomy group, suggesting that the

methodology used for RNA standardization by quantification and cDNA transcription did give constant cDNA concentrations. However, levels of expression of all three genes were influenced by transplantation, acute rejection, and anti-rejection therapy, making them unsuitable for use in this experimental protocol [Gibbs et al, 2003].

In this study five further genes were investigated for their suitability as good endogenous control genes in our transplant model. Reasons for selecting these genes are outlined below. They were glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor 1α (EF-1α), MLN51, UbcH5B, and YWHAZ.

There is variation in gene expression between different tissues of individual genes. Therefore the potential endogenous control genes tested here were selected due to evidence that their gene expression levels were stable in leukocytes as described below, however they were not tested for stability over time. Vandesompele et al tested ten commonly used housekeeping genes in thirteen different human tissues, and found GAPDH, $\beta_2$microglobulin, and YWHAZ were the most stably expressed genes in leukocytes between 13 different normal individuals. They also outlined a procedure for calculating a normalization factor based on multiple housekeeping genes for a more accurate normalization of gene expression data. β_2Microglobulin was found to be the least stable control gene across the thirteen different tissues, but was, however a good choice for leukocyte expression levels [Vandesompele et al, 2002]. $\beta_2$microglobulin has already been tested in our transplant model and found to be unsuitable, therefore GAPDH and YWHAZ were chosen for testing.
In 2001 Hamalainen et al tested ten novel housekeeping genes for use in the human leukocyte differentiation process. The selection of these was based on the results from a microarray screen of approximately seven thousand human genes in adult and fetal tissues identifying five hundred and thirty five housekeeping genes [Warrington et al, 2000]. Using real-time PCR, Hamalainens' group identified the expression of MLN51, EF-1α and UbcH5B genes to be most stably expressed during the T-cell differentiation process. The expression of all three were found to be relatively similar in naïve T cells and T cells differentiated to Th1 or Th2 cells in vitro with time. Also

these genes were expressed at similar levels independent of the donor both in Th1 and Th2 cells. The T-cell differentiation process carried out in vitro involved exposure to the cytokines IL-2, IL-12, and IL-4 [Hamalainen et al, 2001]. Therefore as our model involves exposure to immunosuppressive agents and allograft rejection, both of which modulate cytokine levels, it seemed appropriate to test these three.

4.4.1.i GAPDH

Glyceraldehyde-3-phospate dehydrogenase is an enzyme that catalyzes an energy-yielding step in carbohydrate metabolism, oxidative phosphorylation of glyceraldehyde-3-phospate. The GAPDH gene is located on chromosome 12p13 and pseudogenes are known to exist, one of which is located to Xp21-p11.
GAPDH has been used as a housekeeping gene in numerous gene expression studies [Lipman et al, 1998; Gorzelniak et al, 2001]. There have been reports of variations in GAPDH expression in response to various factors, which suggests that it is unsuitable as a housekeeping gene [Sabek et al,2002; Suzuki et al, 2000; Thellin et al, 1999; Weisinger et al, 1999; Zhong et al, 1999; Hamalainen et al, 2001]. However this has not been shown in human leukocytes.

4.4.1.ii MLN51

The MLN51 gene was characterized due to over expression in breast carcinoma [Tomasetto et al, 1995]. It is located at chromosome 17q11-q21.3. The function of the expressed protein is unknown. To date no group has used it as an endogenous control gene in quantitative gene expression studies.

4.4.1.iii YWHAZ

The YWHAZ gene is located at chromosome 2p25.2-p25.1. Its gene product is tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta isoform. It has phospholipase A2 activity whose function is signal transduction by binding to phosphorylated serine residues on a variety of signaling molecules and thought to have a role in diverse biochemical activities. YWHAZ has also not been used as an endogenous control gene in quantitative gene expression studies.

4.4.1.iv EF-1α

The Elongation Factor-1α (EF-1α) gene is located at chromosome 6q23q24. Its protein product is a GTP-binding protein. EF-1α has been used as an endogenous housekeeping gene for quantitative PCR in non-human studies.

4.4.1.v UbcH5B

The UbcH5B gene encodes the protein ubiquitin-conjugating enzyme EZDZ, and is localized to chromosome 5q31-3. It is thought to catalyse ubiquitination of cellular proteins prior to degradation. UbcH5B has also not been used as an endogenous housekeeping gene in any quantitative PCR expression studies.

4.4.2 METHOD

Primers and probes for these five genes were designed and run with patient samples using the TaqMan as previously described in chapter 2. The primer and probe sequences are detailed in section 2.2.4.ii and were all designed across exon/exon boundaries as highlighted.

4.4.3 Results of endogenous control gene expression analysis

In this section, the results of the endogenous control gene expression analysis, using real time TaqMan quantitative PCR are set out below.
For each endogenous control gene receptor gene there are four graphs. The first are the results from the non-rejector group, the second from the rejector group, the third the rejector group adjusted to the time of rejection diagnosis and finally, the fourth, the live donor group. Each graph is in a box and whisker format with the CT value along the y-axis which is the number of PCR cycles to the point at which the PCR reaction becomes exponential. The box of a graph (coloured red) is the interquartile range, with the median represented as a black bar. The outliers (designated ≅) are values between 1.5 and 3.0 box lengths from the upper or lower edge of the box. Extreme cases (designated M) are values >3 box lengths from the upper or lower edge of the box. All outliers and extreme cases are included in the statistical analysis. Along the x-axis, in the first, second and fourth graphs, is the sample day post-transplant (non-rejector group and non-adjusted rejector group) or post–operative (live donor group), up to day 14 with PT being the pre-transplant and PO the pre-operative baseline samples. The third graph shows the results of the rejector group adjusted to the time at which rejection was diagnosed and treatment started (in all cases treatment was started on same day as rejection diagnosed), with the x-axis being the sample day either before (B) or post (P) rejection diagnosis, and PT the pre-transplant baseline sample.

On the x-axis of all four grafts are the N numbers, which are the number of patient samples for that day. This number varies as some patient blood samples were missed, occasionally had insufficient RNA extracted, or had failure of reverse transcription. The number also may vary between genes analysed for a particular group and reflects a limited cDNA supply in the historical samples and occasionally in the more recent samples.

Statistical analysis of changes in gene expression between time points are shown in Tables 4.1 – 4.5. The analysis for each graph is shown in tabloid format with the graph figure number and analysis table number being identical for each set of results (i.e. table 4.6.a. / figure 4.6.a.). Analysis was performed using the Wilcoxon rank signed test (see section 2.2.5.ii). The tables show two forms of analysis, firstly comparing each time point to the pre-transplant or pre-operative baseline, which will show any significant gene expression changes post-transplant or post-operatively compared to the pre-transplant or pre-operative levels. Secondly comparing in a stepwise fashion to the adjacent time point which will show any significant changes between adjacent time points, for example due to the start of anti-rejection therapy . The p value is shown for each comparison and the direction of any significant ($p<0.05$) gene expression change shown.

4.4.3.i GAPDH

The results of GAPDH gene expression and statistical analysis are shown in figures / tables 4.6.a-d.

In the non-rejector group there were significant increases in expression (i.e lower CT values) on the first five days ($p=0.001$, 0.032, 0.013, 0.003, 0.003 respectively), and 9th day ($p=0.005$) post-transplant compared to the pre-transplant baseline value. There was a significant decrease in expression between days 5 and 6 ($p=0.002$) post-transplant.

In the rejector group few significant changes were seen sequentially post-transplant, however with adjustment to rejection diagnosis significant increases in GAPDH expression occurred on days 1 ($p=0.011$), 2 ($p=0.018$), 3 ($p=0.025$) and 5 ($p=0.046$) before rejection diagnosis and anti-rejection therapy started. A significant decrease in expression occurred between day 1 before and day 1 post start of anti-rejection therapy.

In the live donor group no significant changes in GAPDH gene expression were demonstrated.

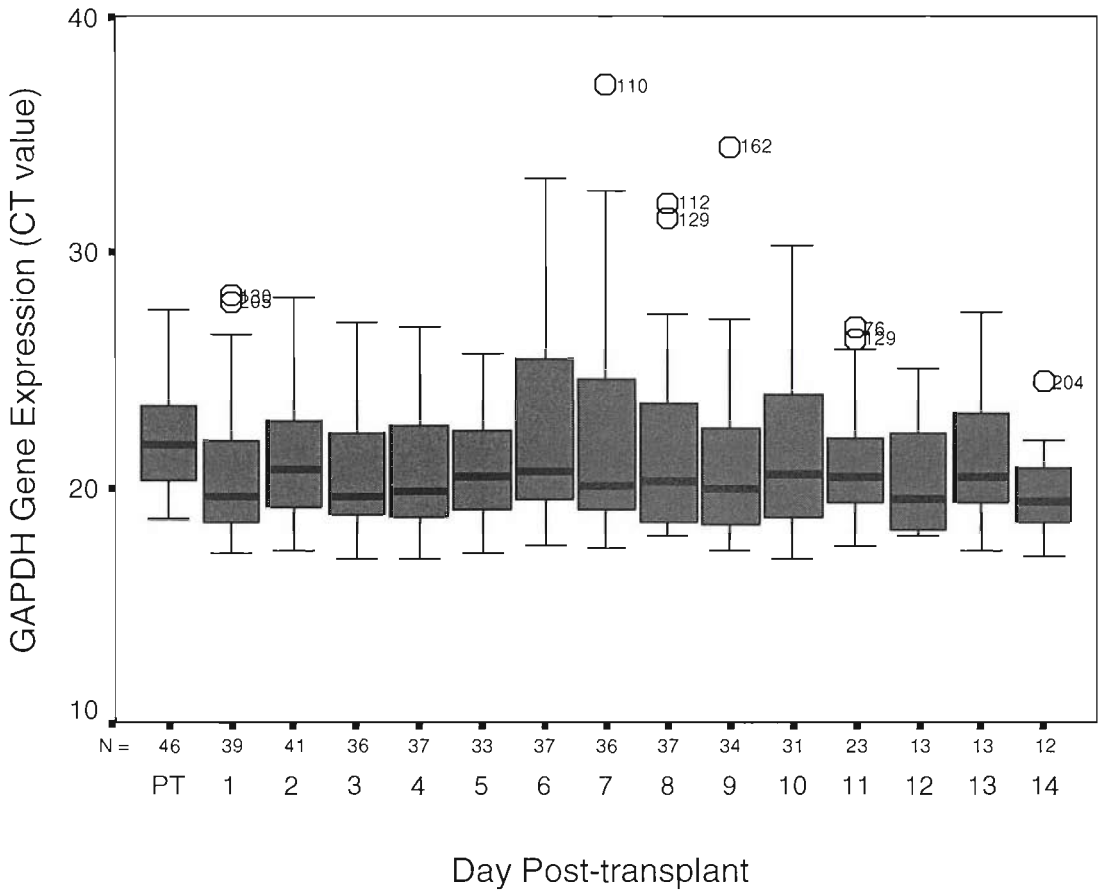

Fig. 4.6.a. Box and whisker plot showing **GAPDH** gene expression levels in the **non-rejector group**. The y-axis is GAPDH gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

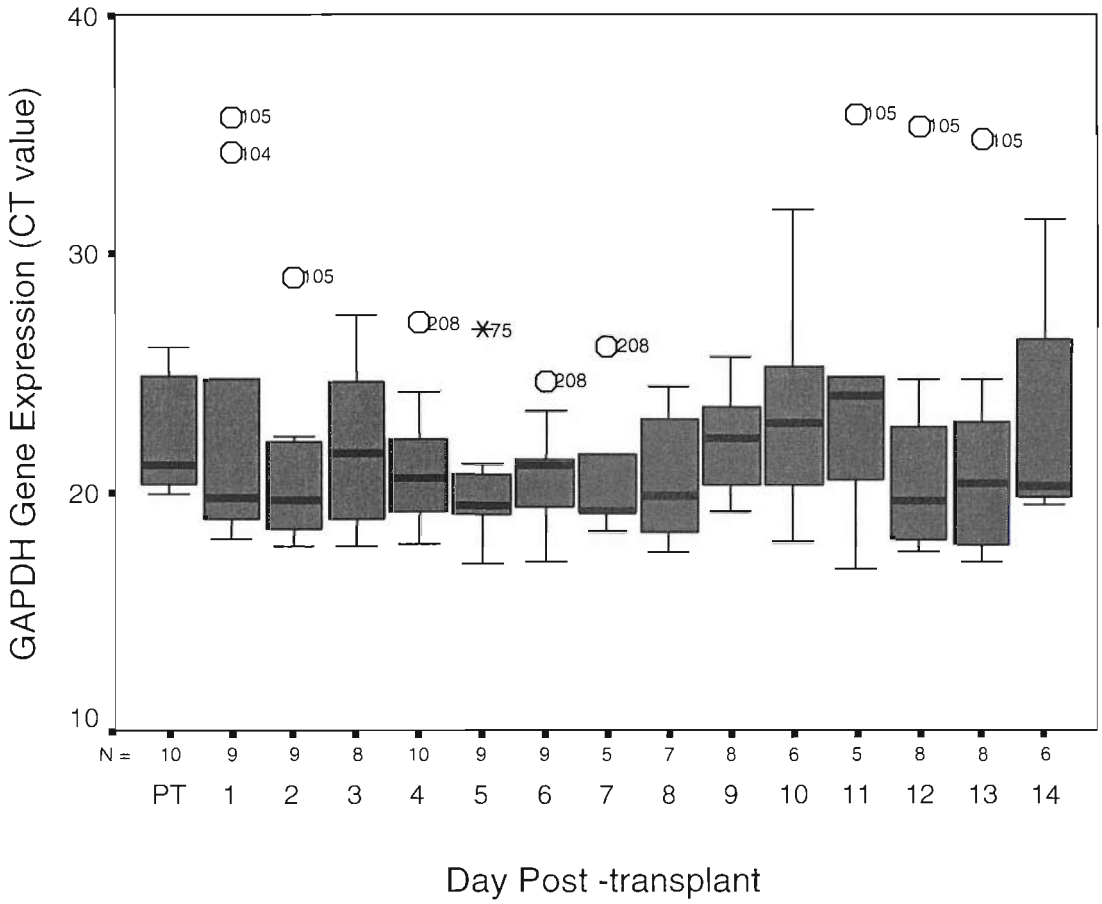

Fig. 4.6.b. Box and whisker plot showing **GAPDH** gene expression levels in the **rejector group**. The y-axis is GAPDH gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	39	41	36	37	33	37	36	37	34	31	23	13	13	12
Direction of change	↑	↑	↑	↑	↑				↑					
p-value	**0.001**	**0.032**	**0.013**	**0.003**	**0.003**	0.868	0.470	0.319	**0.005**	0.232	0.386	0.055	0.780	0.071
Stepwise	PT-	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	39	37	33	32	27	27	29	32	31	27	18	9	10	9
Direction of change	↑					↓								
p-value	0.001	0.394	0.695	0.465	0.981	**0.002**	0.577	0.859	0.112	0.990	0.500	0.953	0.139	0.192

Table. 4.6.a. Statistical significance of **GAPDH** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector** group using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	9	9	8	10	9	9	5	7	8	6	5	8	8	6
Direction of change					↑									
p-value	0.594	0.123	0.310	0.203	**0.038**	0.214	0.273	0.271	1.000	0.753	0.686	0.575	0.327	0.917
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	9	8	8	8	9	8	5	4	6	6	5	4	7	4
Direction of change		↑												
p-value	0.594	**0.036**	0.233	0.833	0.086	0.176	0.686	0.715	0.345	0.753	0.500	0.465	0.735	1.000

Table. 4.6.b. Statistical significance of **GAPDH** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

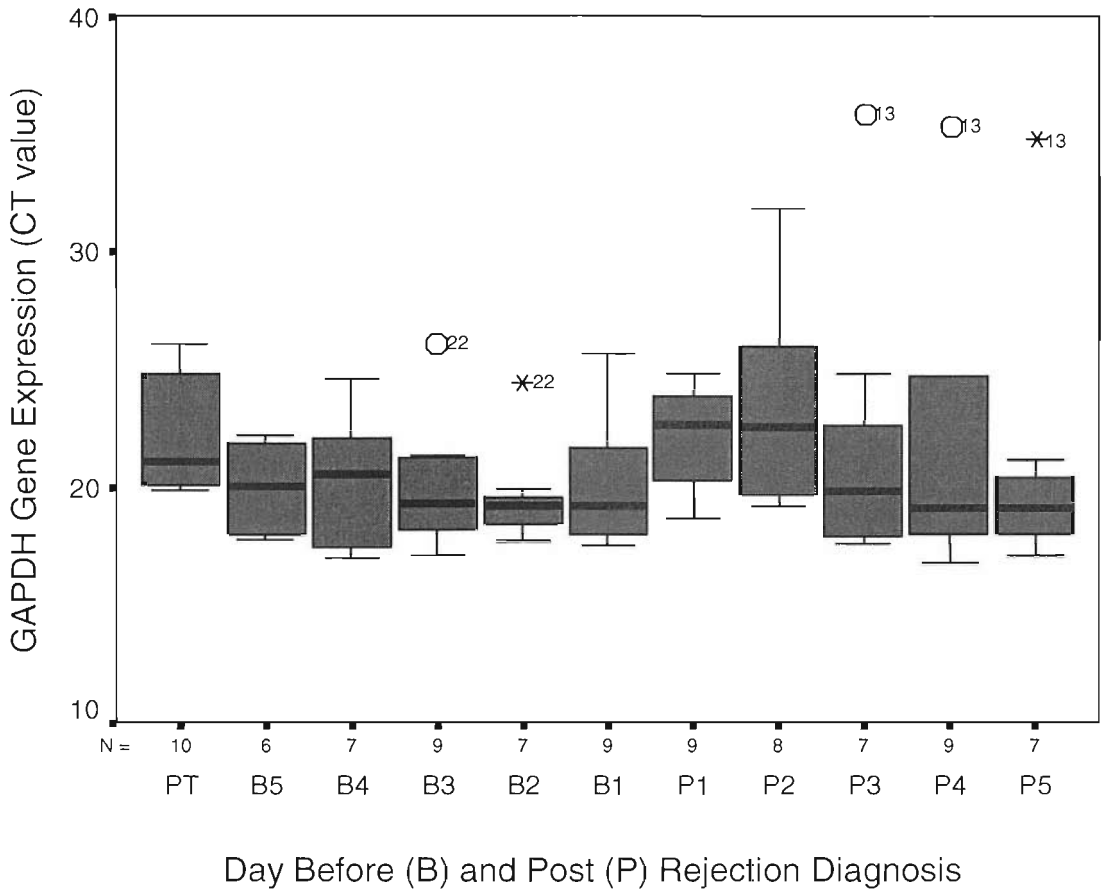

Fig. 4.6.c. Box and whisker plot showing **GAPDH** gene expression levels in the **rejector group adjusted** to time of rejection diagnosis and treatment. The y-axis is GAPDH gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day before (B) and post (P) rejection diagnosis and start of anti-rejection therapy with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

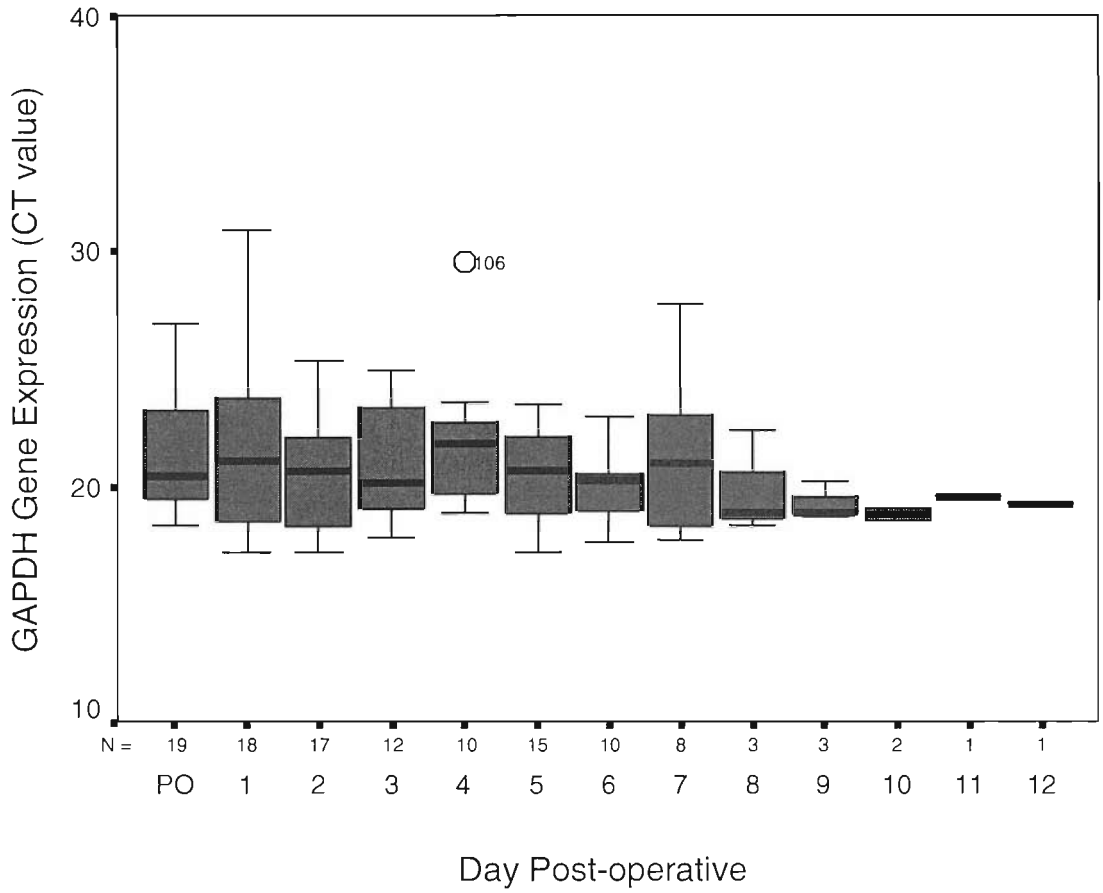

Fig. 4.6.d. Box and whisker plot showing **GAPDH** gene expression levels in the **live donor group**. The y-axis is GAPDH gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-operative with PO being the pre-operative baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-B5	PT-B4	PT-B3	PT-B2	PT-B1	PT-P1	PT-P2	PT-P3	PT-P4	PT-P5
n	6	7	9	7	9	9	8	7	9	7
Direction of change	↑		↑	↑	↑					
p-value	**0.046**	0.176	**0.025**	**0.018**	**0.011**	0.859	0.779	0.866	0.441	0.237
Stepwise	PT-B5	B5-B4	B4-B3	B3-B2	B2-B1	B1-P1	P1-P2	P2-P3	P3-P4	P4-P5
n	6	5	6	6	7	8	8	6	7	7
Direction of change	↑									
p-value	**0.046**	0.686	0.345	0.225	1.00	0.050	0.778	0.463	0.446	0.866

Table. 4.6.c. Statistical significance of **GAPDH** gene expression changes for each day before (B) and post (P) rejection diagnosis and treatment commencement compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PO-1	PO-2	PO-3	PO-4	PO-5	PO-6	PO-7	PO-8	PO-9	PO-10	PO-11
n	18	17	12	10	15	10	8	3	3	2	1
Direction of change											
p-value	0.223	0.149	0.695	0.838	0.222	0.093	0.575	1.000	0.109	0.655	-
Stepwise	PO-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11
n	18	16	12	6	7	9	5	3	2	2	1
Direction of change											
p-value	0.223	0.623	0.610	0.917	0.735	0.286	0.498	0.593	0.180	1.000	-

Table. 4.6.d. Statistical significance of **GAPDH** gene expression changes for each post-operative day compared to the pre-operative (PO) sample (baseline) and between successive days (stepwise) in the **live donor group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

4.4.3.ii MLN51

The results of MLN51 gene expression and statistical analysis are shown in figures / tables 4.7.a-d.

In the non-rejector group there was a significant increase in expression on day 1 ($p=0.007$) post-transplant compared to the pre-transplant level. There was also a stepwise decrease in MLN51 expression between days 12 and 13 ($p=0.004$) post-transplant.

In the rejector group a significant increase in MLN51 expression was seen on day 1 ($p=0.028$) post-transplant compared to the pre-transplant level and also a stepwise decrease occurred between days 5 and 6 ($p=0.043$). With adjustment to time of rejection a significant increase occurred on day 5 ($p=0.043$) post start of anti-rejection therapy.

In the live donor group no significant changes in MLN51 gene expression were demonstrated.

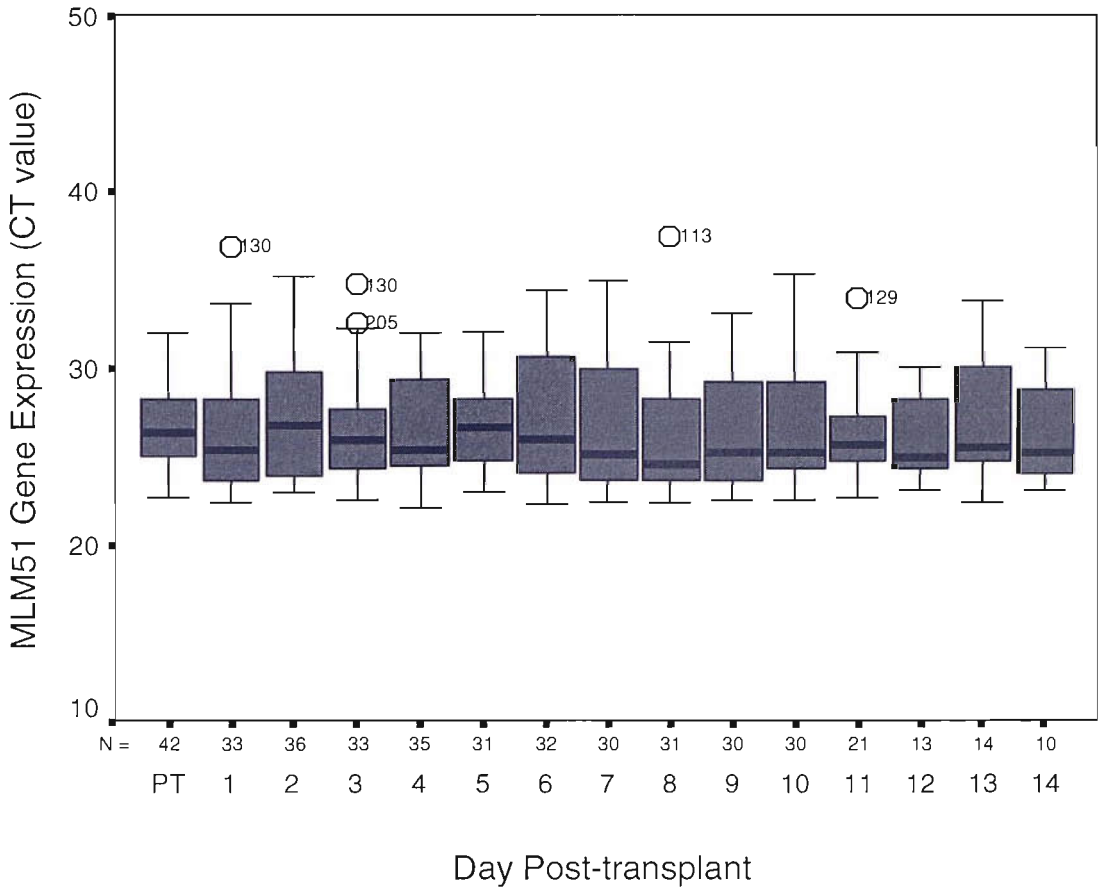

Fig. 4.7.a. Box and whisker plot showing **MLN51** gene expression levels in the **non-rejector group**. The y-axis is MLN51 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each.

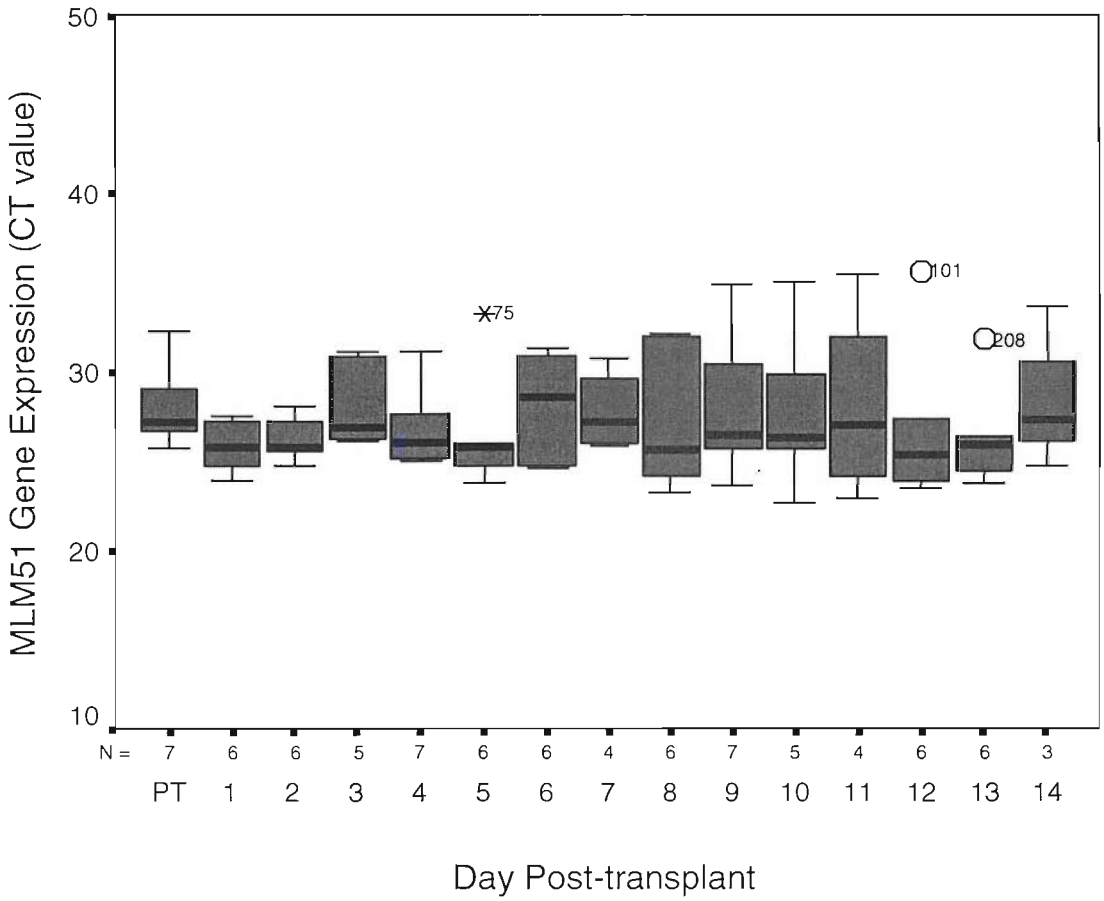

Fig. 4.7.b. Box and whisker plot showing **MLN51** gene expression levels in the **rejector group**. The y-axis is MLN51 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	33	36	33	35	31	32	30	31	30	30	21	13	14	10
Direction of change	↑													
p-value	**0.007**	0.712	0.475	0.343	0.428	0.274	0.600	0.367	0.271	0.497	0.702	0.456	0.530	0.508
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	33	30	29	30	26	23	23	26	25	25	16	9	11	8
Direction of change	↑												↓	
p-value	**0.007**	0.358	0.650	0.579	0.979	0.168	0.083	0.909	0.590	0.904	0.469	0.374	**0.004**	0.484

Table. 4.7.a. Statistical significance of **MLN51** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector** group using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	6	6	5	7	6	6	4	6	7	5	4	6	6	3
Direction of change	↑													
p-value	**0.028**	0.075	0.500	0.128	0.115	0.463	1.00	0.600	0.612	1.00	0.715	0.345	0.249	1.00
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	6	5	5	5	6	5	4	4	6	5	4	3	5	2
Direction of change	↑					↓								
p-value	**0.028**	0.500	0.080	0.080	0.600	**0.043**	1.000	0.715	0.600	0.225	1.000	1.000	0.893	0.655

Table. 4.7.b. Statistical significance of **MLN51** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

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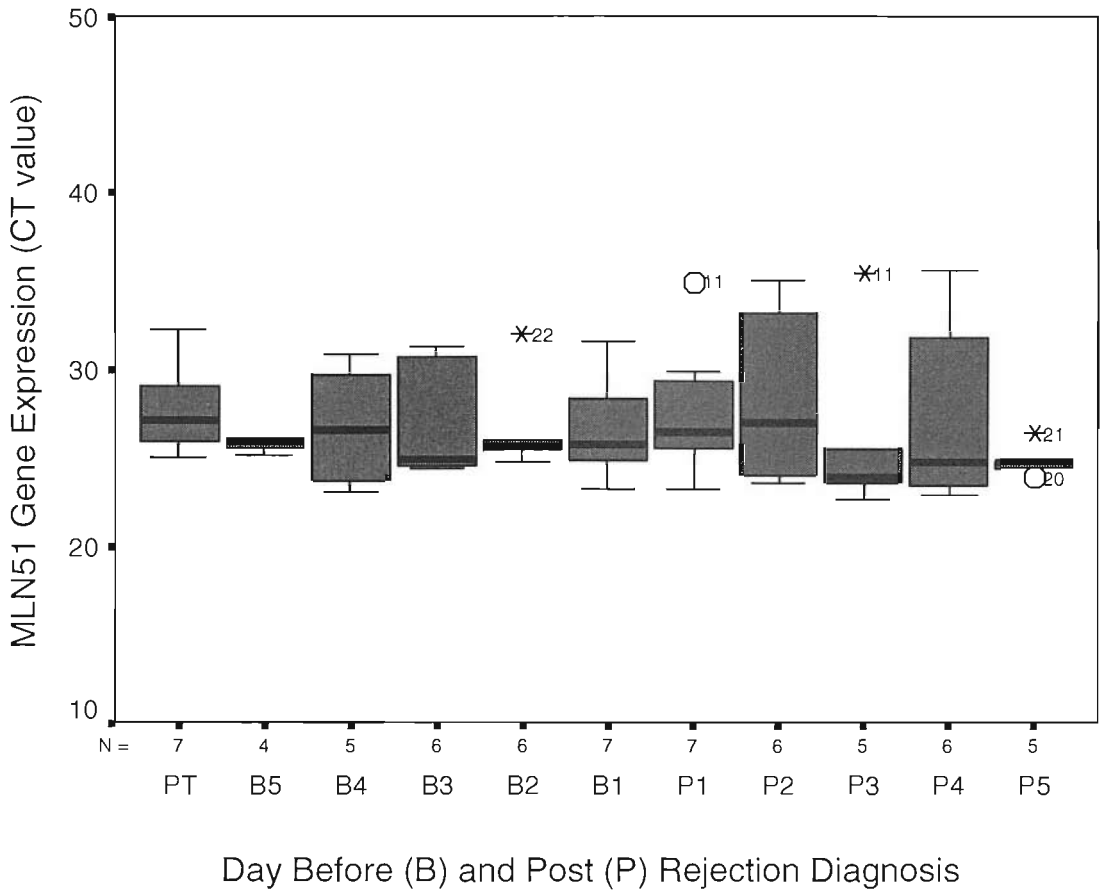

Fig. 4.7.c. Box and whisker plot showing **MLN51** gene expression levels in the **rejector group adjusted** to time of rejection diagnosis and treatment. The y-axis is MLN51 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day before (B) and post (P) rejection diagnosis and start of anti-rejection therapy with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

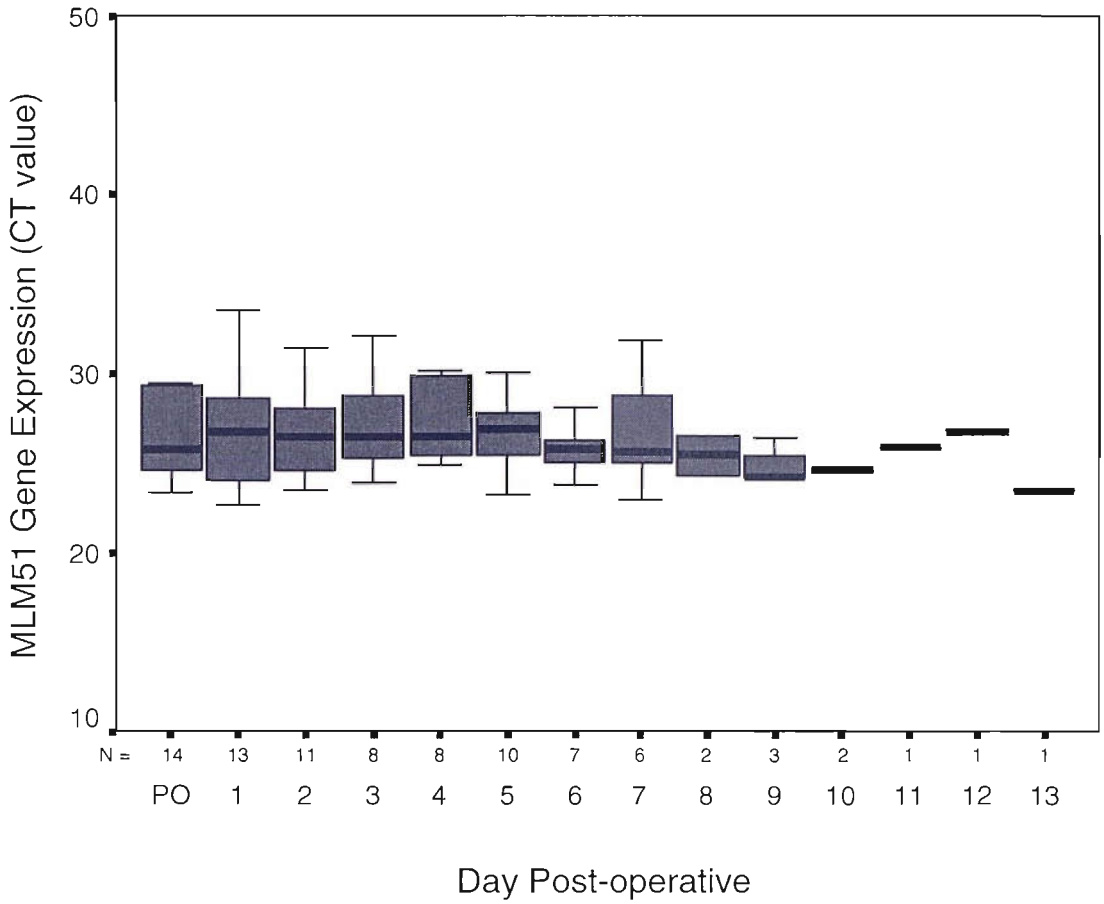

Fig. 4.7.d. Box and whisker plot showing **MLN51** gene expression levels in the **live donor group**. The y-axis is MLN51 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-operative with PO being the pre-operative baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-B5	PT-B4	PT-B3	PT-B2	PT-B1	PT-P1	PT-P2	PT-P3	PT-P4	PT-P5
n	4	5	6	6	7	7	6	5	6	5
Direction of change										↑
p-value	0.144	0.893	0.463	0.345	0.091	0.600	0.462	0.500	0.917	**0.043**
Stepwise	PT-B5	B5-B4	B4-B3	B3-B2	B2-B1	B1-P1	P1-P2	P2-P3	P3-P4	P4-P5
n	4	3	4	5	6	7	6	4	5	5
Direction of change										
p-value	0.144	0.285	0.715	0.500	0.917	0.735	0.753	0.273	0.416	0.686

Table. 4.7.c. Statistical significance of **MLN51** gene expression changes for each day before (B) and post (P) rejection diagnosis and treatment commencement compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PO-1	PO-2	PO-3	PO-4	PO-5	PO-6	PO-7	PO-8	PO-9	PO-10
n	13	11	8	8	10	7	6	2	3	2
Direction of change										
p-value	0.507	0.624	0.484	0.674	0.575	0.499	0.249	0.180	0.593	0.317
Stepwise	PO-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
n	13	11	8	4	5	6	4	2	2	2
Direction of change										
p-value	0.507	0.197	0.674	1.000	0.686	0.075	0.715	0.655	0.180	0.655

Table. 4.7.d. Statistical significance of **MLN51** gene expression changes for each post-operative day compared to the pre-operative (PO) sample (baseline) and between successive days (stepwise) in the **live donor group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

4.4.3.iii YWHAZ

The results of YWHAZ gene expression and statistical analysis are shown in figures / tables 4.8.a-d.

In the non-rejector group there was a significant increase in expression on day 9 (P=0.005) compared to the pre-transplant level and a stepwise decrease from day 12 to 13 (p=0.026) post-transplant.

In the rejector group a significant increase in YWHAZ expression occurred on day 2 (p=0.046) and 13 (p=0.028) post-transplant compared to the pre-transplant level, and a stepwise decrease in expression occurred between days 4 and 5 (p=0.043) post-transplant. With adjustment to the time of rejection diagnosis a significant increase in expression occurred on day 2 before (p=0.028) and day 5 post (p=0.043) rejection diagnosis, compared to the pre-transplant level.

In the live donor group no significant changes in YWHAZ gene expression were demonstrated.

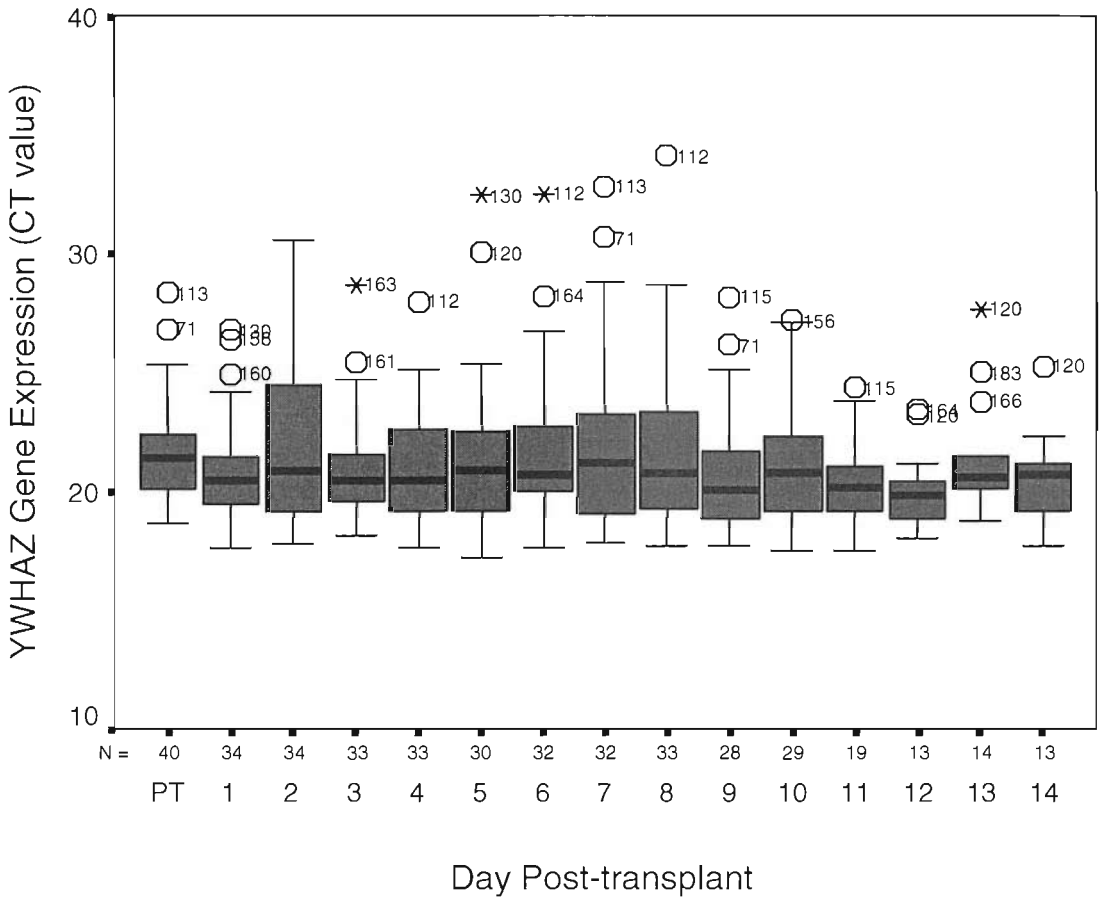

Fig. 4.8.a. Box and whisker plot showing **YWHAZ** gene expression levels in the **non-rejector group**. The y-axis is YWHAZ gene expression as the PCR threshold (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each.

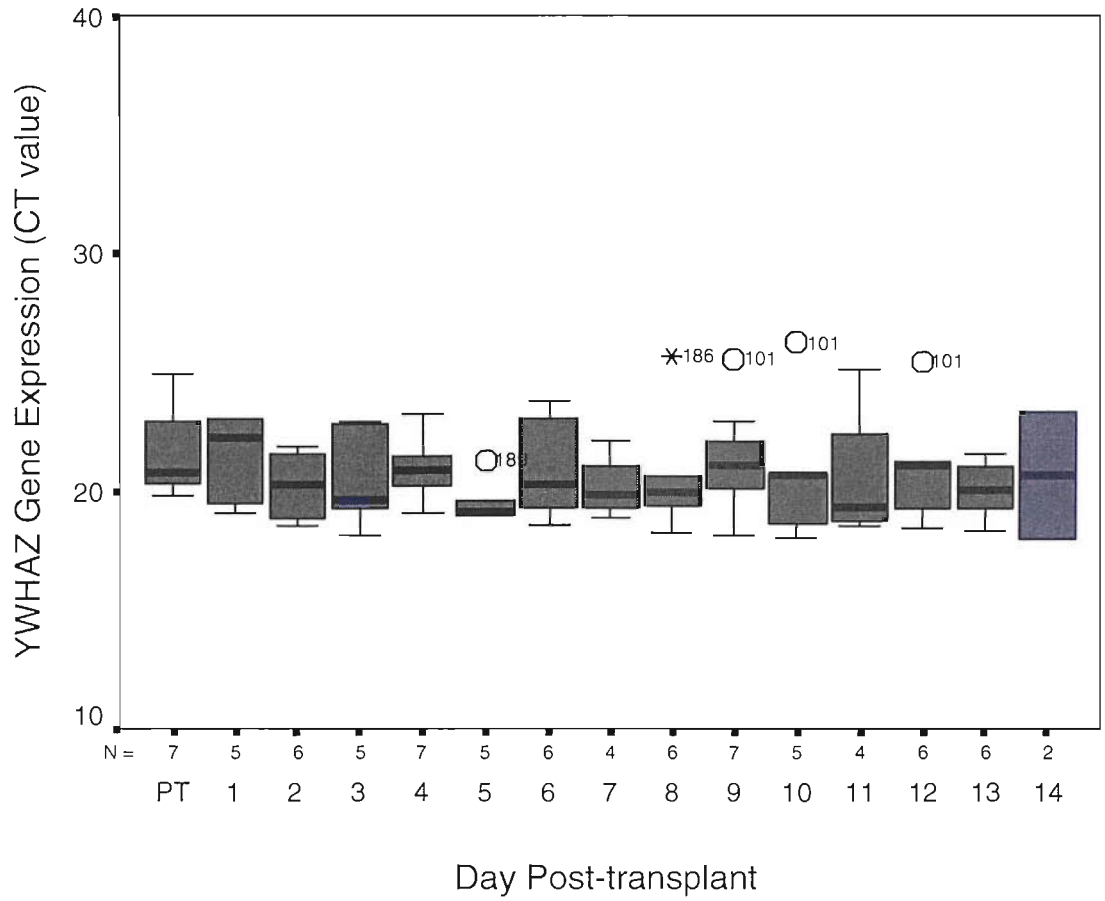

Fig. 4.8.b. Box and whisker plot showing **YWHAZ** gene expression levels in the **rejector group**. The y-axis is YWHAZ gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	34	34	33	33	30	32	32	33	28	29	19	13	14	13
Direction of change									↑					
p-value	0.089	0.986	0.186	0.210	0.434	0.969	0.647	0.348	**0.005**	0.552	0.061	0.173	0.730	0.442
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	34	31	30	29	26	24	25	30	26	23	16	9	12	11
Direction of change													↓	
p-value	0.089	0.229	0.411	0.641	0.485	0.424	0.294	0.162	0.741	0.761	0.609	0.477	**0.026**	0.328

Table. 4.8.a. Statistical significance of **YWHAZ** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector** group using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	5	6	5	7	5	6	4	6	7	5	4	6	6	2
Direction of change		↑											↑	
p-value	0.686	**0.046**	0.225	0.128	0.080	1.00	0.068	0.249	0.499	0.715	0.715	0.463	**0.028**	0.655
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	5	4	5	5	5	5	4	4	6	5	4	3	5	2
Direction of change					↑									
p-value	0.686	0.068	0.686	0.416	**0.043**	0.138	0.715	0.715	0.600	0.343	0.465	0.785	0.686	0.655

Table. 4.8.b. Statistical significance of **YWHAZ** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

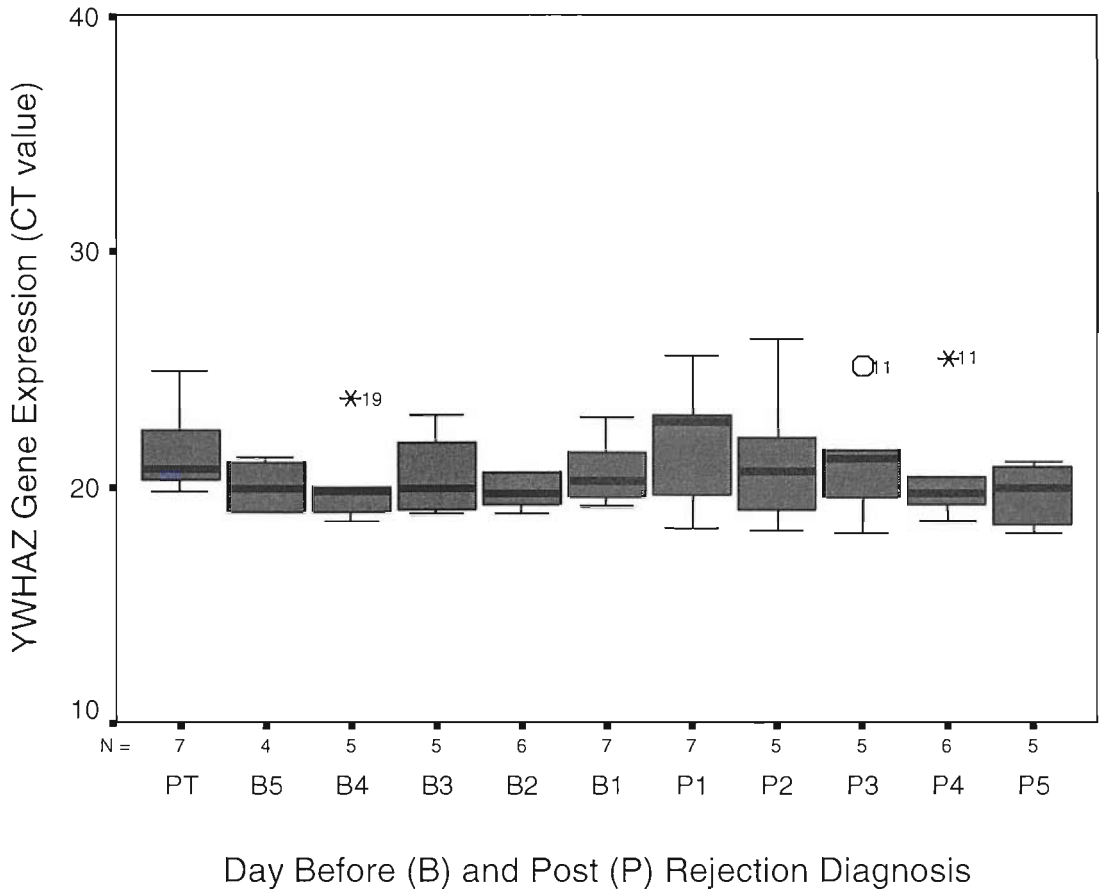

Fig. 4.8.c. Box and whisker plot showing **YWHAZ** gene expression levels in the **rejector group adjusted** to time of rejection diagnosis and treatment. The y-axis is YWHAZ gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day before (B) and post (P) rejection diagnosis and start of anti-rejection therapy with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

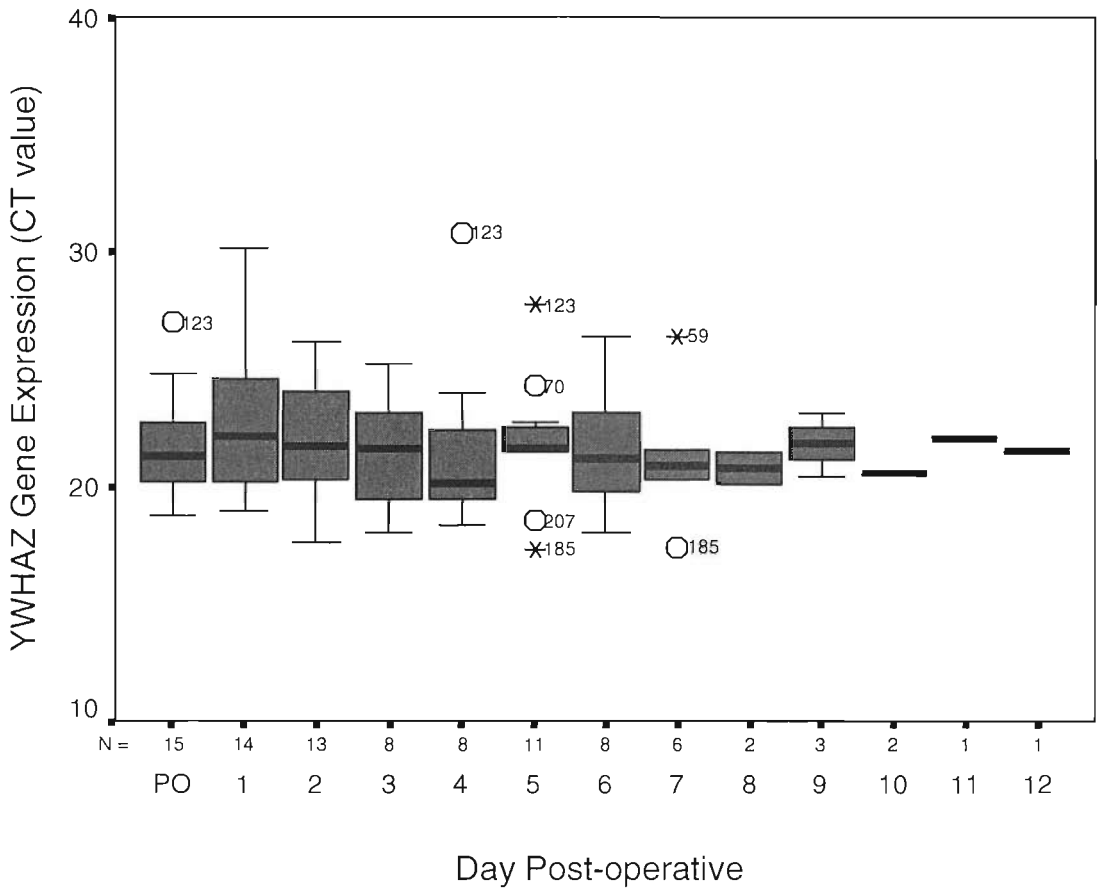

Fig. 4.8.d. Box and whisker plot showing **YWHAZ** gene expression levels in the **live donor group**. The y-axis is YWHAZ gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-operative with PO being the pre-operative baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-B5	PT-B4	PT-B3	PT-B2	PT-B1	PT-P1	PT-P2	PT-P3	PT-P4	PT-P5
n	4	5	5	6	7	7	5	5	6	5
Direction of change				↑						↑
p-value	0.144	0.686	0.416	**0.028**	0.091	0.753	0.686	0.893	0.345	**0.043**
Stepwise	PT-B5	B5-B4	B4-B3	B3-B2	B2-B1	B1-P1	P1-P2	P2-P3	P3-P4	P4-P5
n	4	3	4	4	6	7	5	4	5	5
Direction of change										
p-value	0.144	1.00	0.273	0.465	0.173	0.498	0.686	0.068	0.414	0.686

Table. 4.8.c. Statistical significance of **YWHAZ** gene expression changes for each day before (B) and post (P) rejection diagnosis and treatment commencement compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PO-1	PO-2	PO-3	PO-4	PO-5	PO-6	PO-7	PO-8	PO-9	PO-10
n	14	13	8	8	11	8	6	2	3	2
Direction of change										
p-value	0.379	0.972	0.575	0.889	0.593	0.674	0.753	0.655	0.285	0.180
Stepwise	PO-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
n	14	12	8	3	5	7	4	2	2	2
Direction of change										
p-value	0.379	0.583	0.779	1.000	0.686	0.735	0.144	0.655	0.180	0.655

Table. 4.8.d. Statistical significance of **YWHAZ** gene expression changes for each post-operative day compared to the pre-operative (PO) sample (baseline) and between successive days (stepwise) in the **live donor group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

4.4.3.iv EF-1α

The results of EF-1α gene expression and statistical analysis are shown in figures / tables 4.9.a-d.

In the non-rejector group there were no significant changes in EF-1α expression in the post-transplant period when comparing to the pre-transplant baseline value. There were however significant changes in expression at several time points on stepwise analysis, a decrease between days 5 and 6 ($p=0.045$) and 12 and 13 ($p=0.012$), and an increase between days 6 and 7 ($p=0.035$) and 13 and 14 ($p=0.008$).

In the rejector group a significant increase in EF-1α expression occurred on day 2 ($p=0.042$), with no other significant changes sequentially. With adjustment to time of rejection diagnosis a significant increase in EF-1α expression occurred on day 5 ($p=0.043$) post rejection diagnosis.

In the live donor group a significant decrease in EF-1α gene expression occurred on the first day post-operatively ($p=0.007$), with no other changes thereafter.

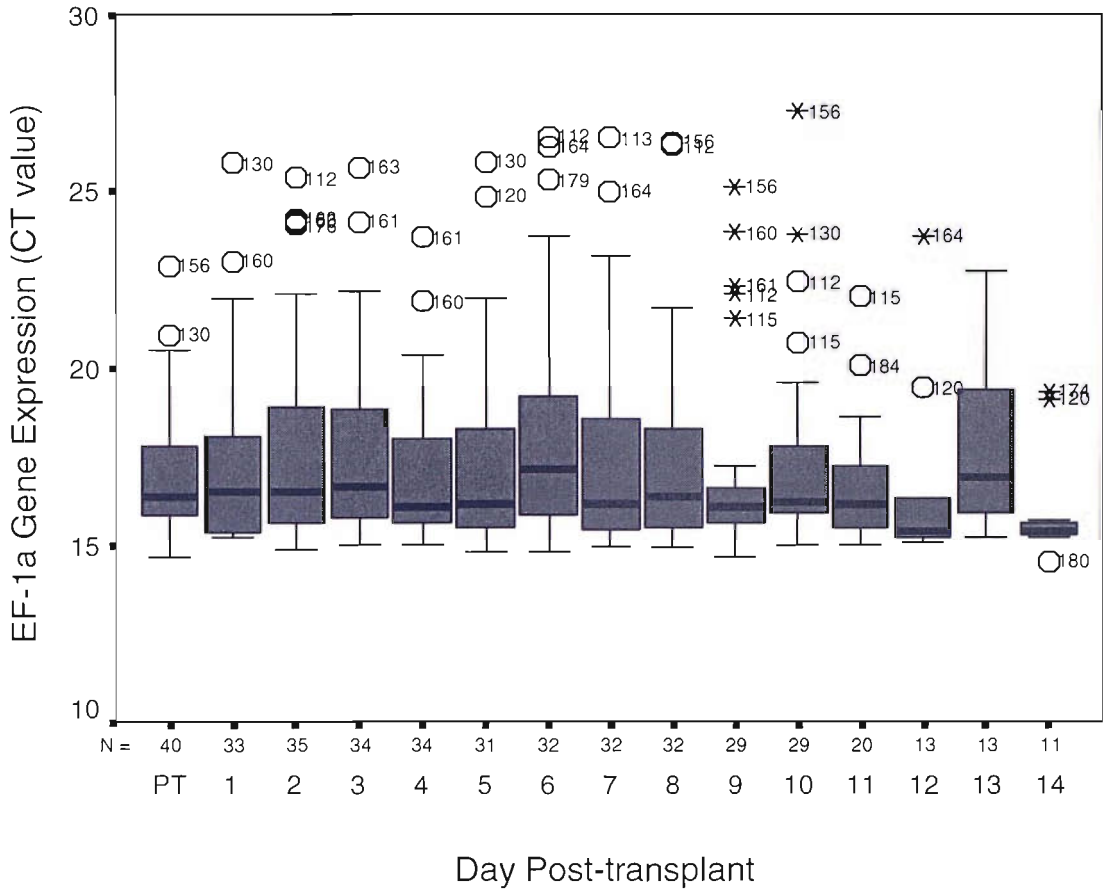

Fig. 4.9.a. Box and whisker plot showing **EF-1α** gene expression levels in the **non-rejector group**. The y-axis is EF-1α gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each.

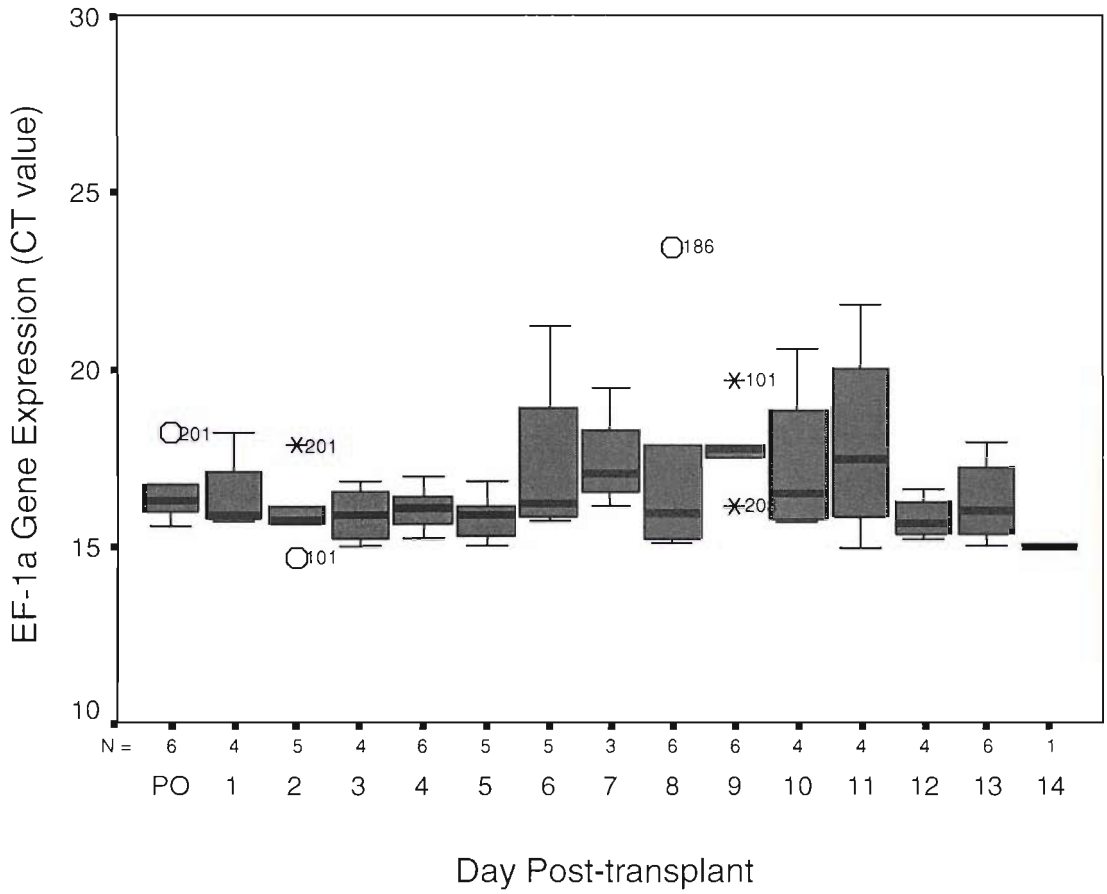

Fig. 4.9.b. Box and whisker plot showing **EF-1α** gene expression levels in the **rejector group**. The y-axis is EF-1α gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	33	35	34	34	31	32	32	32	29	29	20	13	13	11
Direction of change														
p-value	0.183	0.156	0.602	0.943	0.652	0.059	0.427	0.557	0.657	0.604	0.763	0.221	0.124	0.476
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	33	31	31	31	27	25	25	29	27	25	16	9	11	9
Direction of change						↓	↑						↓	↑
p-value	0.183	0.951	0.930	0.590	0.933	**0.045**	**0.035**	0.300	0.213	0.170	0.679	0.859	**0.012**	**0.008**

Table. 4.9.a. Statistical significance of **EF-1α** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector** group using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	4	5	4	6	5	5	3	6	6	4	4	4	6	1
Direction of change		↑												
p-value	1.00	**0.042**	0.715	0.225	0.080	0.893	0.109	0.917	0.075	0.715	0.465	0.068	0.500	-
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	4	3	4	4	5	4	3	3	6	4	3	2	4	1
Direction of change														
p-value	1.000	0.285	0.273	1.000	0.225	0.068	0.109	0.593	0.753	0.715	1.000	0.655	0.273	-

Table. 4.9.b. Statistical significance of **EF-1α** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

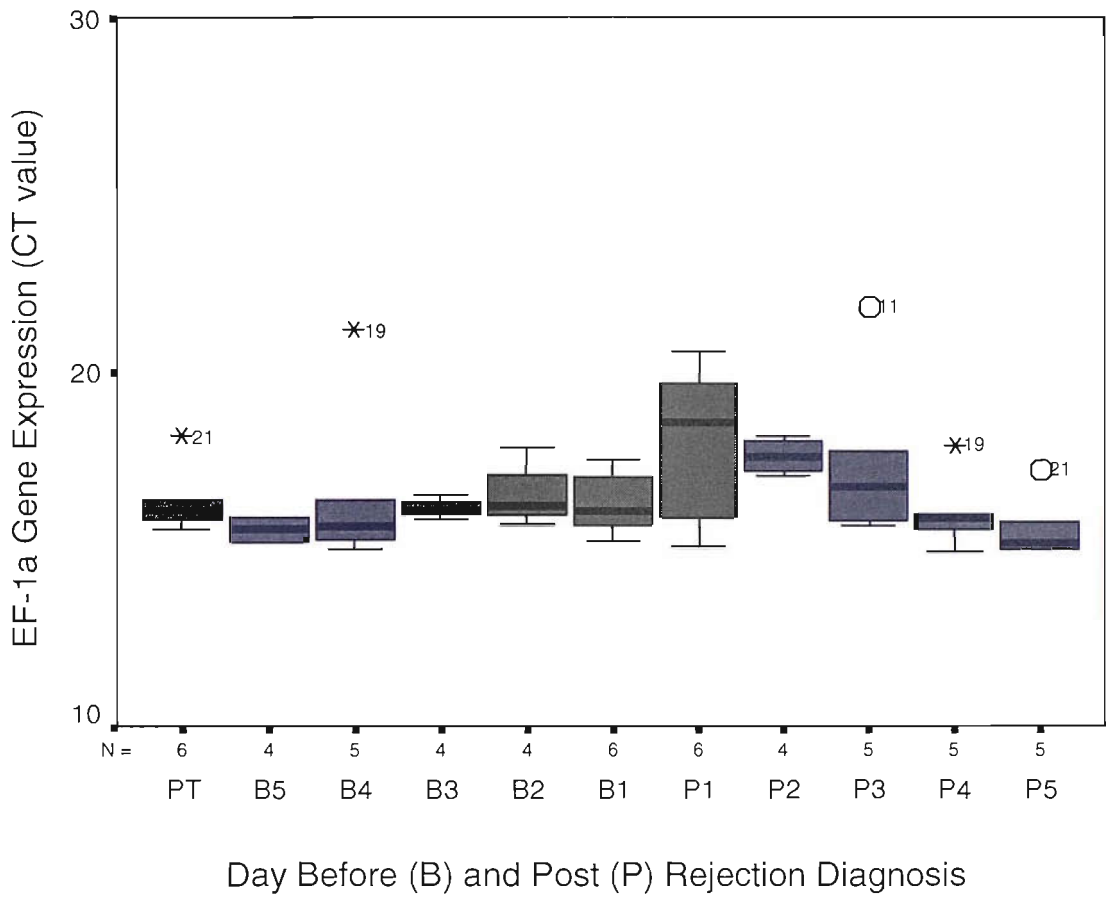

Fig. 4.9.c. Box and whisker plot showing **EF-1α** gene expression levels in the **rejector group adjusted** to time of rejection diagnosis and treatment. The y-axis is EF-1α gene expression as the PCR threshold cycle (C_T) value. The x-axis is the sample day before (B) and post (P) rejection diagnosis and start of anti-rejection therapy with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

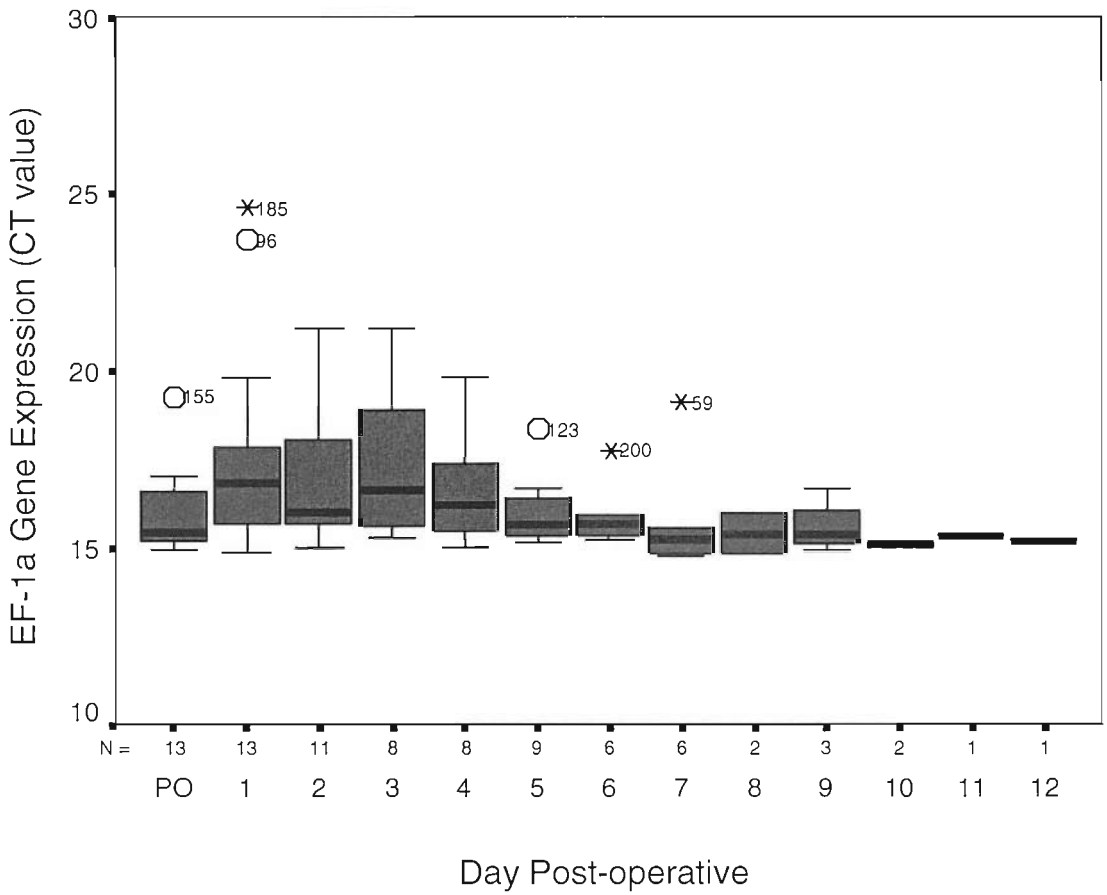

Fig. 4.9.d. Box and whisker plot showing **EF-1α** gene expression levels in the **live donor group**. The y-axis is EF-1α gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-operative with PO being the pre-operative baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-B5	PT-B4	PT-B3	PT-B2	PT-B1	PT-P1	PT-P2	PT-P3	PT-P4	PT-P5
n	4	5	4	4	6	6	4	5	5	5
Direction of change										↑
p-value	0.068	0.713	0.715	0.465	0.753	0.345	0.144	0.686	0.715	**0.043**
Stepwise	PT-B5	B5-B4	B4-B3	B3-B2	B2-B1	B1-P1	P1-P2	P2-P3	P3-P4	P4-P5
n	4	3	3	3	4	6	4	3	4	4
Direction of change										
p-value	0.068	0.285	0.285	1.00	0.465	0.116	0.465	0.593	0.715	0.715

Table. 4.9.c. Statistical significance of **EF-1α** gene expression changes for each day before (B) and post (P) rejection diagnosis and treatment commencement compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PO-1	PO-2	PO-3	PO-4	PO-5	PO-6	PO-7	PO-8	PO-9	PO-10
n	13	11	8	8	9	6	6	2	3	2
Direction of change	↓									
p-value	**0.007**	0.139	0.093	0.141	0.110	0.058	0.528	0.655	0.655	0.655
Stepwise	PO-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
n	13	11	8	4	5	5	4	2	2	2
Direction of change	↓									
p-value	**0.007**	0.534	0.401	1.000	0.225	0.715	0.141	0.655	0.655	0.655

Table. 4.9.d. Statistical significance of **EF-1α** gene expression changes for each post-operative day compared to the pre-operative (PO) sample (baseline) and between successive days (stepwise) in the **live donor group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

4.4.3.v UbcH5B

The results of UbcH5B gene expression and statistical analysis are shown in figures / tables 4.10.a-d.

In the non-rejector group there were no significant changes in UbcH5B expression in the post-transplant period when comparing to the pre-transplant baseline value. There was however a significant increase in expression between days 5 and 6 (p=0.030), and decrease between days 12 and 13 (p=0.041) post-transplant.

In the rejector group significant increases in UbcH5B expression occurred on days 1 (p=0.043), 2 (p=0.017), 4 (p=0.028) and 5 (0.046) post-transplant compared to the pre-transplant baseline value. Stepwise changes occurred with a decrease in expression from day 2 to 3 (p=0.018) and day 5 to 6 (p=0.043). With adjustment to time of rejection diagnosis significant increases were seen on days 1 (p=0.013), 3 (p=0.036) and 5 (p=0.043) before rejection diagnosis. A stepwise increase in expression occurred from day 2 to 3 (p=0.043) post rejection diagnosis.

In the live donor group a significant decrease in UbcH5B gene expression occurred on the third day post-operatively (p=0.022) compared to the pre-operative baseline value.

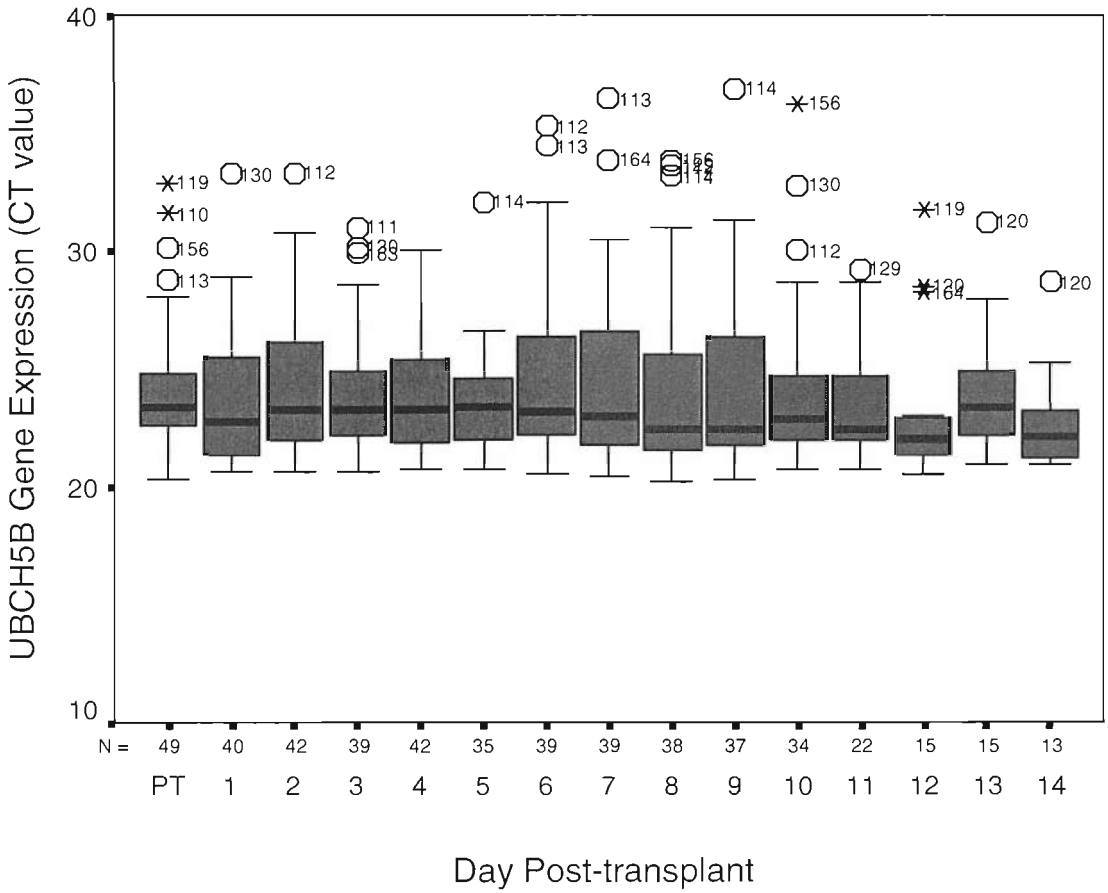

Fig. 4.10.a. Box and whisker plot showing **UbcH5B** gene expression levels in the **non-rejector group**. The y-axis is UbcH5B gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each.

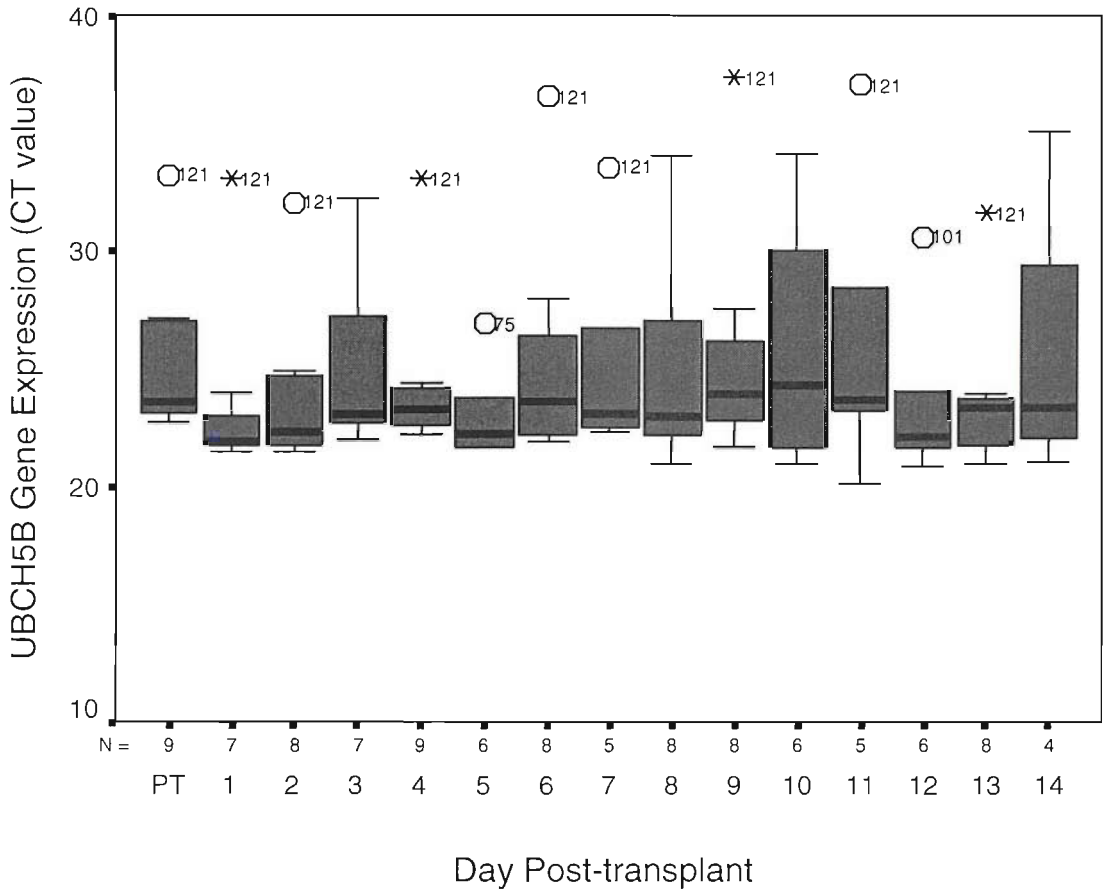

Fig. 4.10.b. Box and whisker plot showing **UbcH5B** gene expression levels in the **rejector group**. The y-axis is UbcH5B gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	40	42	39	42	35	39	39	38	37	34	22	15	15	13
Direction of change														
p-value	0.078	0.630	0.557	0.338	0.225	0.507	0.867	0.965	0.251	0.561	0.426	0.050	0.201	0.917
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	40	36	36	36	30	29	31	33	32	31	18	9	12	11
Direction of change						↑							↓	
p-value	0.078	0.232	0.875	0.712	0.559	**0.030**	0.967	0.660	0.501	0.339	1.000	0.767	**0.041**	0.083

Table. 4.10.a. Statistical significance of **UbcH5B** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	7	8	7	9	6	8	5	8	8	6	5	6	8	4
Direction of change	↑	↑		↑	↑									
p-value	**0.043**	**0.017**	0.236	**0.028**	**0.046**	0.889	0.893	0.575	0.528	0.600	0.416	0.463	0.141	0.465
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	7	6	7	7	6	5	5	5	7	6	5	3	5	3
Direction of change	↑		↓			↓								
p-value	**0.043**	0.225	**0.018**	0.735	0.753	**0.043**	0.500	0.686	0.499	0.753	0.686	0.593	0.893	0.593

Table. 4.10.b. Statistical significance of **UbcH5B** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

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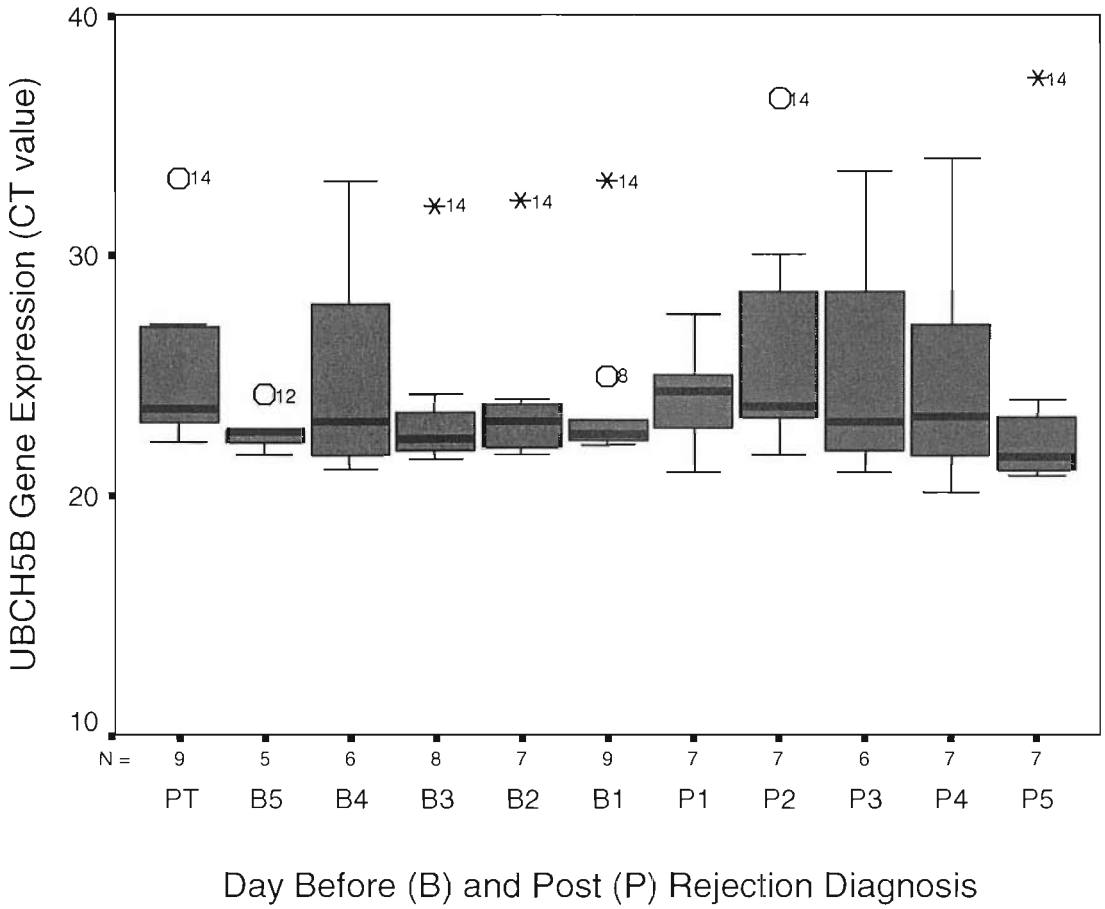

Fig. 4.10.c. Box and whisker plot showing **UbcH5B** gene expression levels in the **rejector group adjusted** to time of rejection diagnosis and treatment. The y-axis is UbcH5B gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day before (B) and post (P) rejection diagnosis and start of anti-rejection therapy with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

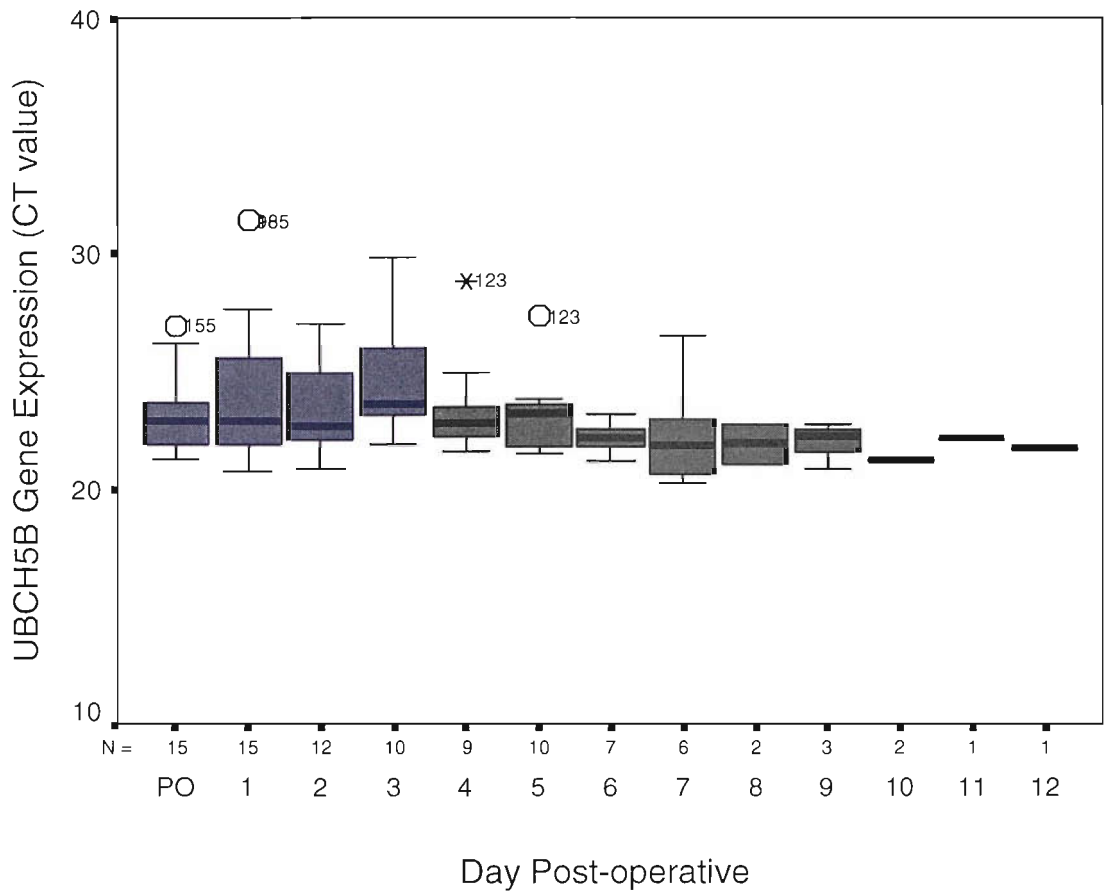

Fig. 4.10.d. Box and whisker plot showing **UbcH5B** gene expression levels in the **live donor group**. The y-axis is UbcH5B gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-operative with PO being the pre-operative baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-B5	PT-B4	PT-B3	PT-B2	PT-B1	PT-P1	PT-P2	PT-P3	PT-P4	PT-P5
n	5	6	8	7	9	7	7	6	7	7
Direction of change	↑		↑		↑					
p-value	**0.043**	0.753	**0.036**	0.176	**0.013**	0.612	0.310	0.833	1.00	0.237
Stepwise	PT-B5	B5-B4	B4-B3	B3-B2	B2-B1	B1-P1	P1-P2	P2-P3	P3-P4	P4-P5
n	5	3	5	6	7	7	6	5	6	6
Direction of change	↑							↑		
p-value	**0.043**	0.285	0.686	0.752	0.799	0.176	0.248	**0.043**	0.753	0.753

Table. 4.10.c. Statistical significance of **UbcH5B** gene expression changes for each day before (B) and post (P) rejection diagnosis and treatment commencement compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PO-1	PO-2	PO-3	PO-4	PO-5	PO-6	PO-7	PO-8	PO-9	PO-10
n	15	12	10	9	10	7	6	2	3	2
Direction of change			↓							
p-value	0.460	0.110	**0.022**	0.813	0.241	0.345	0.917	0.655	0.593	0.317
Stepwise	PO-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
n	15	12	9	5	5	6	4	2	2	2
Direction of change										
p-value	0.460	1.000	0.155	0.500	0.893	0.173	0.715	0.180	0.180	0.655

Table. 4.10.d. Statistical significance of **UbcH5B** gene expression changes for each post-operative day compared to the pre-operative (PO) sample (baseline) and between successive days (stepwise) in the **live donor group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

4.4.3.vi Correlation between expression of endogenous control genes

The results of the pair wise correlation between the expression of the five endogenous control genes investigated, and statistical significance, are shown below in Table 4.11.

Endogenous control gene pair	Pearson Correlation (r)	P value	Number
GAPDH / MLN51	0.865	< 0.001	584
GAPDH / YWHAZ	0.537	< 0.001	580
GAPDH / EF-1α	0.692	< 0.001	560
GAPDH / UbcH5B	0.752	<0.001	628
MLM51 / YWHAZ	0.605	< 0.001	557
MLM51 / EF-1α	0.655	< 0.001	555
MLM51 / UbcH5B	0.811	<0.001	589
YWHAZ / EF-1α	0.784	< 0.001	555
YWHAZ / UbcH5B	0.803	<0.001	571
EF-1α / UbcH5B	0.918	<0.001	565

Table. 4.11 Pearson correlations of expression between endogenous control genes

4.4.4 Discussion

A suitable endogenous contol gene should show constant expression in the tissue or cells under investigation, and in response to external influences. In this work a suitable endogenous control gene should have constant expression in PBMCs with time, in patients undergoing a renal transplant or donor nephrectomy. Its expression should therefore not be significantly altered by surgery, immunosuppressive drugs or the immunological processes of allograft rejection. Five candidate endogenous control genes were investigated in this work, selected as described in section 4.4.1.

The Pearson correlations between the expression of the five endogenous control genes investigated (Table 4.11) in all the groups (non-rejector, rejector and live donor) combined were positively correlated (r value range 0.537-0.865) and highly significant ($p<0.001$). This suggests that a change in expression of one of these endogenous control genes is also seen in the other four. However, it is possible that this may well reflect the differing efficiencies of the reverse transcription reaction between the samples (i.e differing amounts of cDNA in each actual TaqMan reaction), rather than simultaneous changes in expression levels of all five genes in the same direction. Variability of the efficiency of the reverse transcription reaction has been reported varying from 5% to 90% [Noonan et al, 1990; Henrard et al, 1992; Simmonds et al, 1990]. It is a purpose, therefore, of using a suitable endogenous control gene(s) to control for this variability, which would then make this method of gene expression analysis more robust.

In the live donor group no significant changes in gene expression occurred in the post-operative period for GAPDH, YWHAZ or MLN51 either on baseline or stepwise analysis. Their expression seems not to be affected by donor nephrectomy and with relatively constant expression levels demonstrated, could be used in this group of patients as endogenous control genes. With EF-1α and UbcH5B a significant change in expression occurred on day 1 and 3 post-operatively suggesting an influence of surgery on their expression levels.

In the non-rejector group there were significant changes in gene expression in all 5 endogenous control genes, particularly seen with GAPDH. A significant increase (equivalent to 2-to 6-fold change in target gene) in GAPDH expression was seen in the first 5 days following transplantation. Although gene expression levels of GAPDH, YWHAZ or MLN51 were not affected by donor nephrectomy, they were affected by renal transplantation, of which allograft reperfusion injury or immunosuppressive therapy are the likely causes. EF-1α and UbcH5B expression levels in the days following transplantation when compared to the pre-transplant level, showed no significant differences, however significant stepwise changes occurred at several time

points. These results suggest that, despite the selection criteria, all five genes are poor candidates as endogenous controls in the transplant setting due to significant changes in their gene expression.

In the rejector group significant changes in the expression levels of all five genes occurred at various time points post-transplantation. With adjustment to time of rejection diagnosis, significant changes also occurred for all five genes but particularly for GAPDH and UbcH5B. Increases in gene expression of GAPDH (2-3 fold) and UbcH5B (2 fold) were seen on several days before rejection diagnosis compared to pre-transplant baseline values. With commencement of anti-rejection therapy their expression levels returned back to baseline levels. Rather than GAPDH and UbcH5B being used as endogenous controls they may have a potential in immunomonitoring over the early transplant period. This correlates with a study by Sabath et al who showed an increase in GAPDH mRNA levels upon IL-2 stimulation in murine T lymphocytes [Sabath et al, 1990]. Also Radonic et al showed in the human T-cell line, CCRF-HSB-2, treated with TPA (12-O-tetradecanoylphorbol-13-acetate) and ionomycin which increased levels of IL-2 RNA transcription, that there was also increased GAPDH RNA transcription [Radonic et al, 2004].

These results suggest that none of the genes investigated here are suitable as endogenous control genes in this work, as they seem to be influenced either by renal transplantation, rejection or both and some by surgery itself.
The three genes, GAPDH, MLN51 and YWHAZ, which showed no significant changes in expression in the live donor group, however, does suggest that the method used for RNA standardization and reverse transcription was effective. With these results of 3 out of 5 possible endogenous control genes in this study and the 3 from the study by Gibbs et al [Gibbs et al, 2003], with no significant changes in gene expression in the live donor group, it gives added evidence for the robustness of this method and validity for use in post-transplant monitoring.
The number of samples in the group should be noted as this influences any statistical significance (in these cases there were 19, 14 and 15 live donor patients analysed with

GAPDH, MLN51 and YWHAZ respectively). The higher the number of patient samples with no significant changes in expression levels, the more likely it is that the method gives constant cDNA concentrations.

It may be that a suitable endogenous control gene does not exist for this gene expression model, and therefore another method of standardisation is required. The advantages of the use of an endogenous control over an exogenous (synthetic) control are outlined in section 4.4.1. Gene transcript number (or a reflective value of it) can be standardised to the number of cells (PBMCs in this study), however this does not contol for the variability in the reverse transcription reaction.

An alternative method of standardization is the use of structural RNA such as 18S or 28S ribosomal RNA, however this requires preparation of cDNA from total RNA, rather than only mRNA as with this study using an oligo(dT) primer (*Not* I-d$(T)_{18}$ bifunctional primer). This is because rRNA contains no poly(A) tail and therefore cannot be reverse transcribed in oligo(dT) primed cDNA synthesis. A drawback in the use of these ribosomal units is their expression levels are very high compared to a lot of target mRNA transcripts which makes it difficult to accurately subtract the baseline value in real-time RT-PCR data analysis. As mentioned in section 4.1, total RNA consists predominantly of rRNA, and therefore may not always be representative of the mRNA fraction [Solanos et al, 2001]. Also it has been reported that rRNA transcription is affected by biological factors and drugs [Spanakis et al,1993; Johnson et al,1995].

A method of standardization avoiding the use of endogenous control genes was described by Whelan JA et al. They expressed target gene expression as the copy number per microgram of cDNA by cloning real-time PCR products into plasmids and then used them to calibrate unknown samples [Whelan et al, 2003].

Standardisation can add robustness to gene transcript quantitation, however the use of controls can bring new variables into the system and therefore should be thoroughly investigated.

Chapter 5

Results of Chemokine and Chemokine Receptor Gene Expression

This chapter sets out the results of the gene expression analysis using real time TaqMan quantitative PCR for each chemokine and chemokine receptor gene studied. For each chemokine/chemokine receptor gene there are four graphs. The first being the results from the non-rejector group, the second from the rejector group, the third the rejector group adjusted to the time of rejection diagnosis and finally the live donor group. Each graph is in a box and whisker format with the C_T value along the y-axis which is the number of PCR cycles to the point at which the PCR reaction becomes exponential. An increase in the C_T value between samples indicates a reduction in gene expression and vice versa, and a one 'cycle' change equates to a 2-fold change in target gene transcript levels. The box of a graph (coloured red) is the interquartile range, with the median represented as a black bar. The outliers (designated ≅) are values between 1.5 and 3.0 box lengths from the upper or lower edge of the box. Extreme cases (designated M) are values >3 box lengths from the upper or lower edge of the box. All outliers and extreme cases are included in the statistical analysis. Along the x-axis is the sample day post-transplant (non-rejector group and non-adjusted rejector group) or post–operative (live donor group), up to day 14 with PT being the pre-transplant and PO the pre-operative baseline samples. The third graph shows the results of the rejector group but adjusted to the time at which rejection was diagnosed and treatment started (in all cases treatment was started on same day as rejection diagnosed). The x-axis time point codes are BS, which is the pre-transplant baseline sample, B1 to B5, which are the 5 days before the diagnosis of rejection, and P1 to P5, the 5 days post rejection diagnosis.

Rejection was treated with three pulses of methylprednisolone (500mg IV) on three consecutive days, therefore day P1, P2 and P3 are the samples after the 1st, 2nd and 3rd pulses respectively.

On the x-axis of all four grafts is also an N number, which is the number of patient samples for each day. This number varies as some patient blood samples were missed, occasionally had insufficient RNA extracted, or had failure of reverse transcription. The number also may vary between genes analysed for a particular group and reflects a limited cDNA supply in the historical samples and occasionally in the more recent samples.

Statistical analysis of changes in gene expression between time points are shown in Tables 5.1 – 5.28. The analysis for each graph is shown in tabloid format with the graph figure number and analysis table number being identical for each set of results (i.e. table 5.1.a. / figure 5.1.a.). Analysis was performed using the Wilcoxon rank signed test. The tables show two forms of analysis, firstly comparing each time point to the pre-transplant or pre-operative baseline, which will show any significant gene expression changes post-transplant or post-operatively compared to the pre-transplant or pre-operative levels. Secondly comparing in a stepwise fashion to the adjacent time point which will show any significant changes between adjacent time points, for example due to the start of anti-rejection therapy. The p value is shown for each comparison and the direction of any significant ($p<0.05$) gene expression change shown.
The results are presented for each chemokine and chemokine receptor gene below and discussed in the following chapter.

5.1 CCL3

The results of CCL3 gene expression and statistical analysis are shown in figures / tables 5.1.a - 5.1.d.
In the non-rejector group there were no significant changes in CCL3 gene expression levels on baseline analysis, however on stepwise analysis significant decreases between days 5 and 6 (equivalent to 4-fold change) (p=0.005), and, 7 and 8 (1.5-fold) (p=0.014) occurred.
Subdivision of the non-rejector group into those with primary graft function (N=57) versus delayed graft function (N=12) (Appendix VI, Tables A.1.a & b), and patients with late rejection episodes (N=7) versus those without rejection episodes (N=62) (Appendix VI, Tables A.1.c & d), revealed no significantly different patterns of CCL3 gene expression.

In the rejector group there were also no significant changes in CCL3 expression levels on baseline or stepwise analysis, however, on adjustment to rejection time there was a significant reduction (2-fold) on day 1 post rejection diagnosis compared to the baseline (p=0.041). This corresponds to the first anti rejection pulse. From day 2 to 1 before rejection (B2-B1) there was also a significant stepwise drop (32-fold) in CCL3 expression (p=0.013).
In the live donor group, there were no significant changes in CCL3 expression levels on baseline analysis and a single stepwise fall (8-fold) in expression between days 3 and 4 (p=0.041).

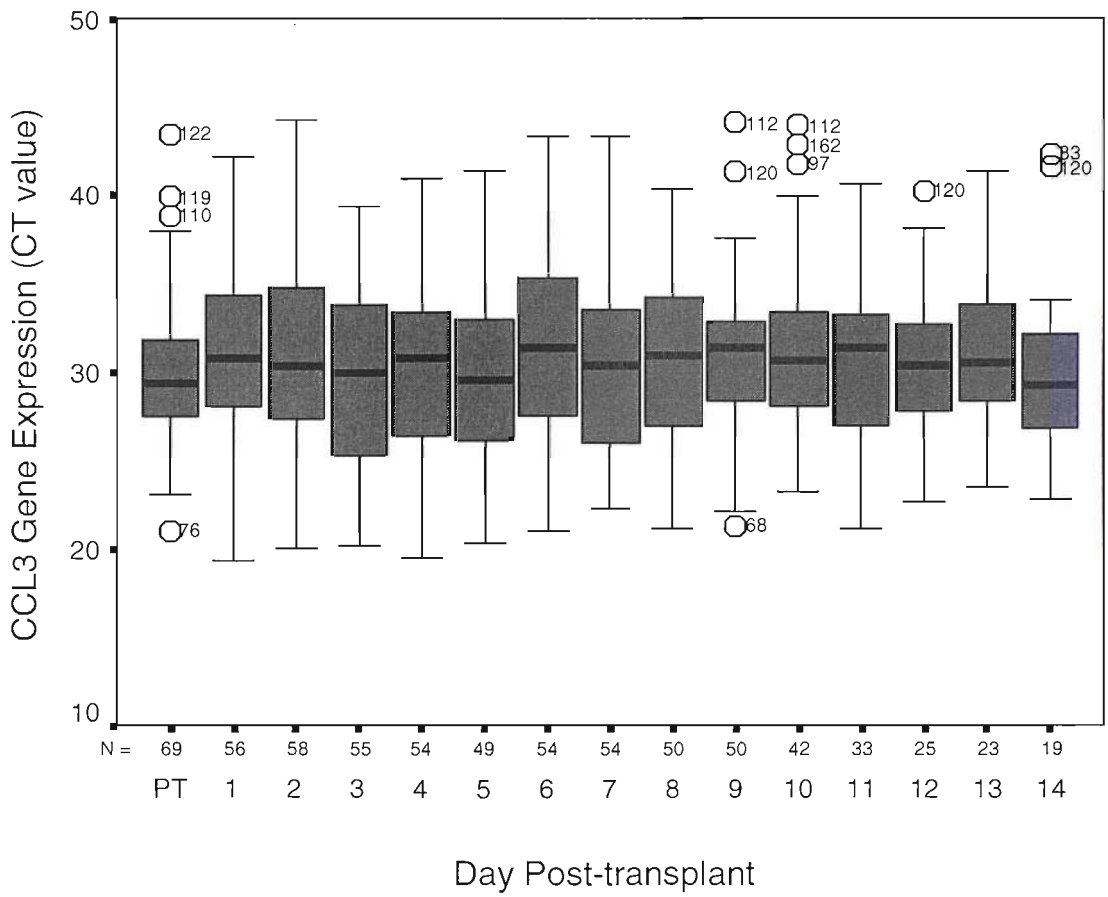

Fig. 5.1.a. Box and whisker plot showing chemokine **CCL3** gene expression levels in the **non-rejector group**. The y-axis is CCL3 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

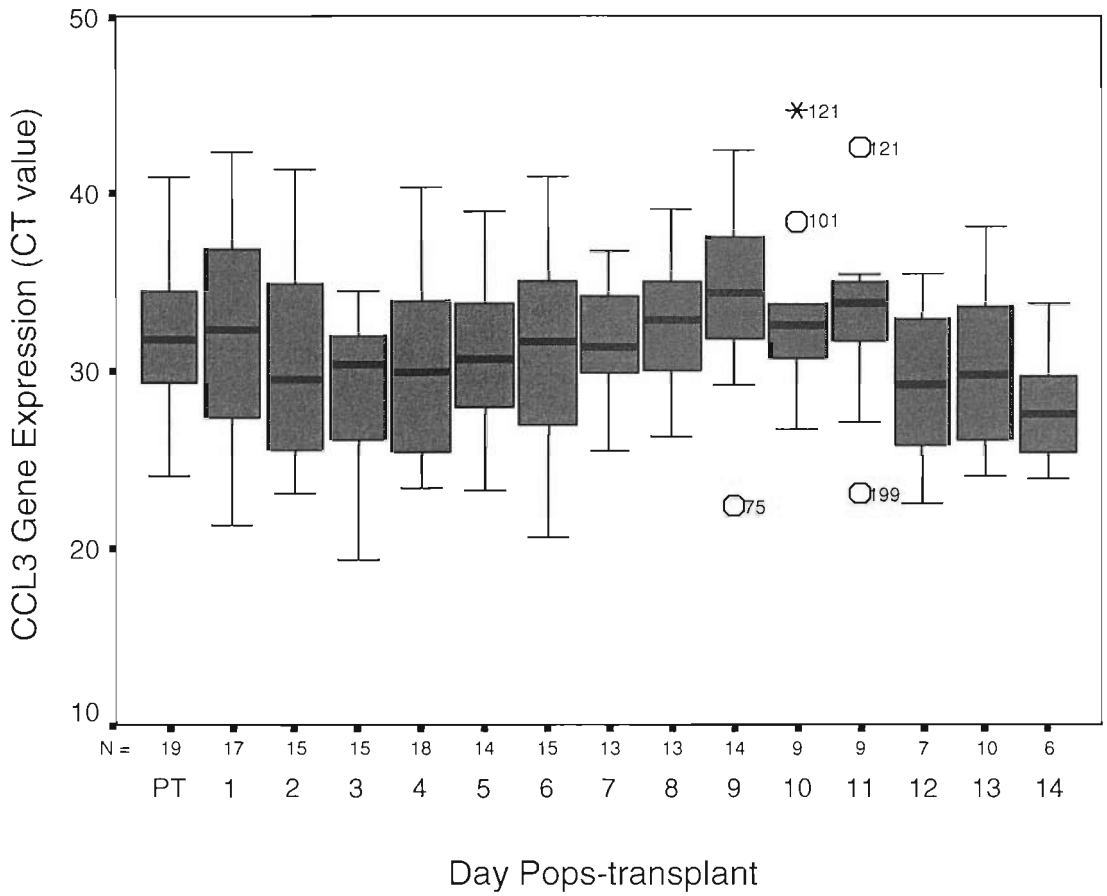

Fig. 5.1.b. Box and whisker plot showing chemokine **CCL3** gene expression levels in the **rejector group**. The y-axis is CCL3 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	56	58	55	54	49	54	54	50	50	42	33	25	23	19
Direction of change														
p-value	0.076	0.211	0.522	0.669	0.233	0.065	0.284	0.097	0.091	0.472	0.253	0.909	0.057	0.573
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	56	50	48	46	38	40	43	43	43	36	25	19	17	15
Direction of change						↓		↓						
p-value	0.076	0.985	0.213	0.227	0.627	**0.005**	0.340	**0.014**	0.346	0.140	0.211	0.872	0.227	0.244

Table. 5.1.a. Statistical significance of **CCL3** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	17	15	15	18	14	15	13	13	14	9	9	7	10	6
Direction of change														
p-value	0.554	0.211	0.112	0.557	0.778	0.875	0.701	0.382	0.109	0.477	0.859	0.310	0.241	0.075
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	17	14	13	14	14	12	12	11	11	8	7	4	6	6
Direction of change														
p-value	0.554	0.096	0.701	0.510	1.00	0.505	0.610	0.114	0.202	0.401	0.499	0.465	0.600	0.463

Table. 5.1.b. Statistical significance of **CCL3** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

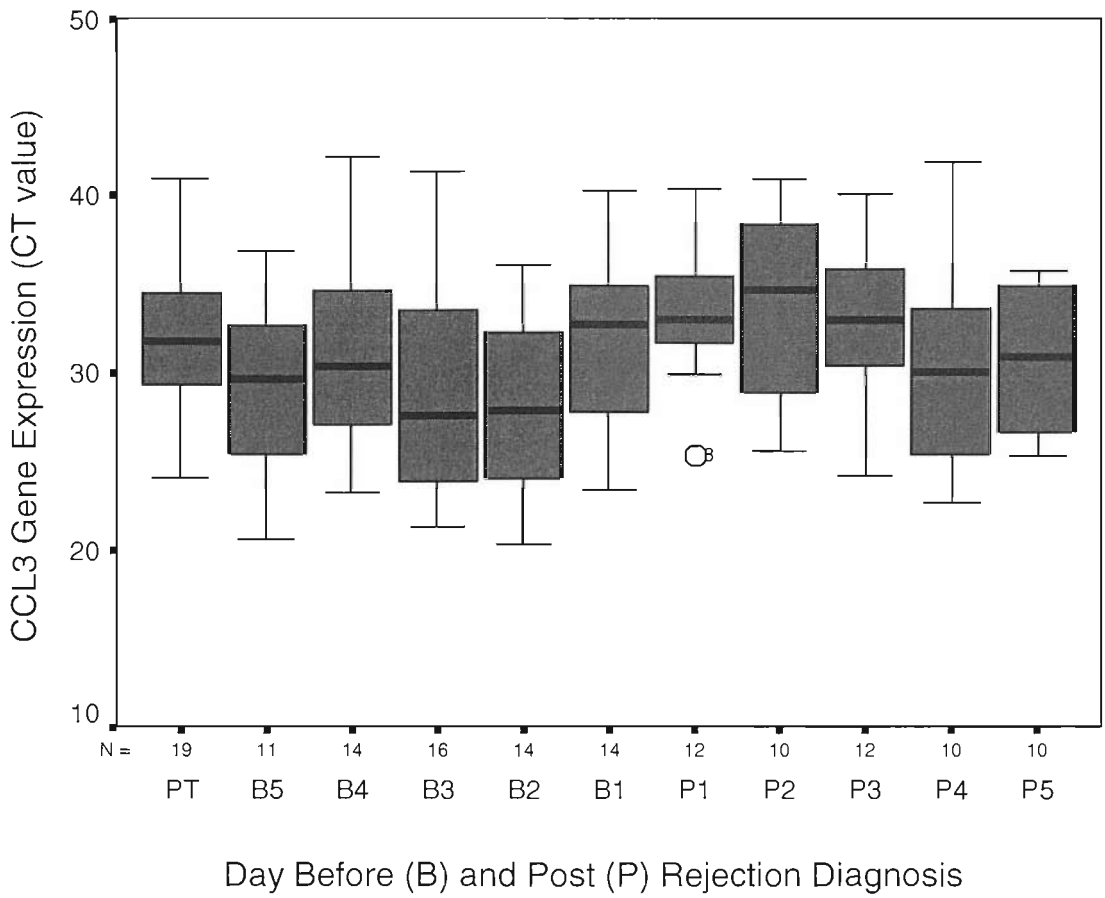

Fig. 5.1.c. Box and whisker plot showing chemokine **CCL3** gene expression levels in the **rejector group** adjusted to time of rejection diagnosis and treatment. The y-axis is CCL3 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day before (B) and post (P) rejection diagnosis and start of anti-rejection therapy with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

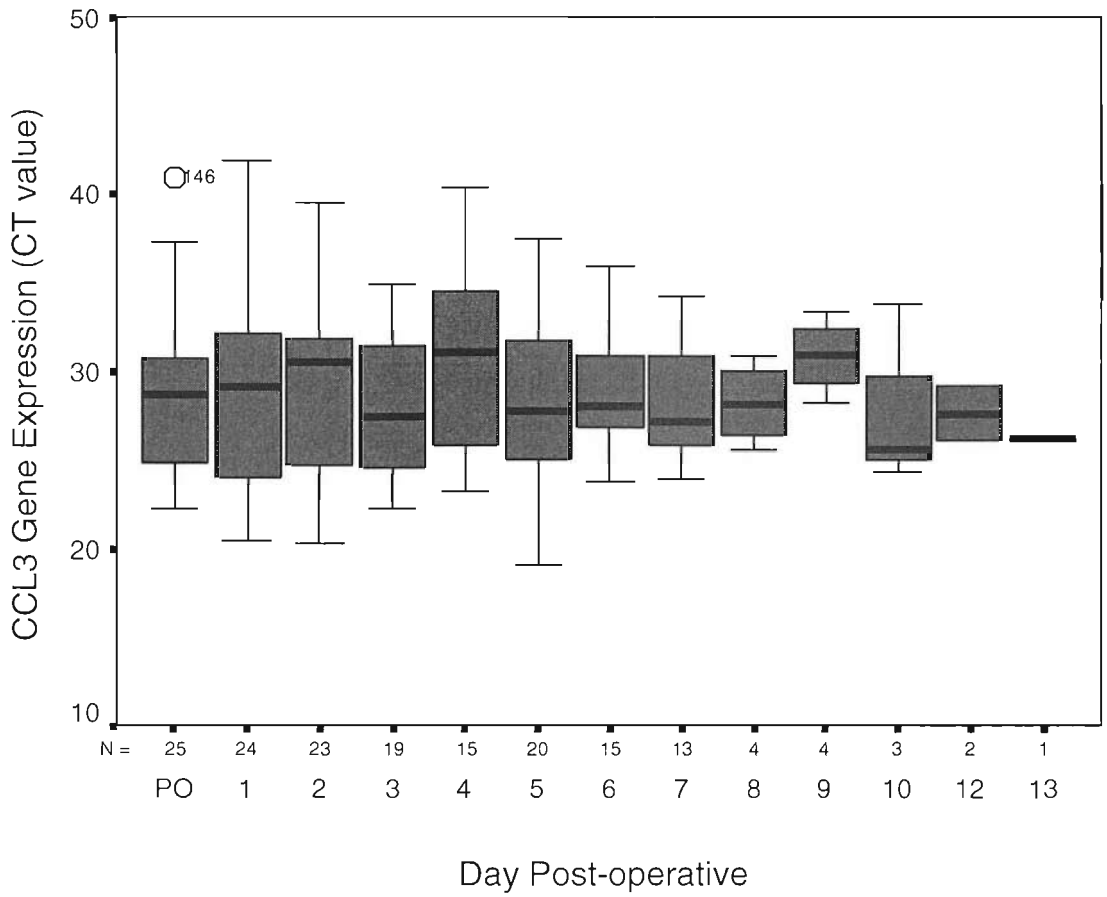

Fig. 5.1.d. Box and whisker plot showing chemokine **CCL3** gene expression levels in the **live donor group**. The y-axis is CCL3 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-operative with PO being the pre-operative baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-B5	PT-B4	PT-B3	PT-B2	PT-B1	PT-P1	PT-P2	PT-P3	PT-P4	PT-P5
n	11	14	16	14	14	12	10	12	10	10
Direction of change						↓				
p-value	0.374	0.451	0.234	0.096	0.826	**0.041**	0.327	0.695	0.445	0.221
Stepwise	PT-B5	B5-B4	B4-B3	B3-B2	B2-B1	B1-P1	P1-P2	P2-P3	P3-P4	P4-P5
n	11	9	12	12	12	11	9	8	9	9
Direction of change					↓					
p-value	0.374	0.314	0.110	0.814	**0.013**	0.965	0.813	0.401	0.093	0.678

Table. 5.1.c. Statistical significance of **CCL3** gene expression changes for each day before (B) and post (P) rejection diagnosis and treatment commencement compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PO-1	PO-2	PO-3	PO-4	PO-5	PO-6	PO-7	PO-8	PO-9	PO-10	PO-11
n	24	23	19	15	20	15	13	4	4	3	2
Direction of change											
p-value	0.753	0.465	0.445	0.320	0.654	0.955	0.311	0.715	0.465	0.109	0.655
Stepwise	PO-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11
n	24	23	18	11	11	14	11	4	3	3	2
Direction of change				↓							
p-value	0.753	0.484	0.163	**0.041**	0.722	0.683	0.398	0.465	0.109	0.285	0.655

Table. 5.1.d. Statistical significance of **CCL3** gene expression changes for each post-operative day compared to the pre-operative (PO) sample (baseline) and between successive days (stepwise) in the **live donor group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

5.2 CCL4

The results of CCL4 gene expression and statistical analysis are shown in figures / tables 5.2.a – 5.2.d.
In the non-rejector group there were significant decreases (equivalent to 2- to 4-fold change) in CCL4 gene expression levels on days 6 (p=0.018), 7 (p=0.030) and 8 (p=0.014) post transplant compared to the pre-transplant baseline level and a decrease (2-fold) between days 5 and 6 (p=0.013) on stepwise analysis.
With subdivision of the non-rejector group into those with primary graft function (N=57) versus delayed graft function (N=12) (Appendix VI, Tables A.2.a & b), the latter subgroup had significant decreases (2- to 4-fold) in CCL4 expression on days 8 (p=0.007), 9 (p=0.004) and 10 (p=0.009) post-transplant compared to baseline levels. This was not seen in the subgroup with primary graft function, which had a significant decrease in expression on day 13 (p=0.045) compared to baseline and also between days 5 and 6 (p=0.018). Subdivision of the non-rejector group into patients with late rejection episodes (N=7) versus those without rejection episodes (N=62) (Appendix VI, Tables A.2.c & d), showed that the latter subgroup had a similar CCL4 gene expression pattern to the non-rejector group, and the former had no significant changes on baseline or stepwise analysis.

In the rejector group there were no significant changes in CCL4 expression levels on baseline or stepwise analysis. On adjustment to rejection diagnosis there was a significant decrease (2-fold) in expression between days 2 and 1 before rejection (p=0.011). No significant changes were seen during the anti-rejection therapy.
In the live donor group, there were no significant changes in CCL4 expression levels on baseline or stepwise analysis.

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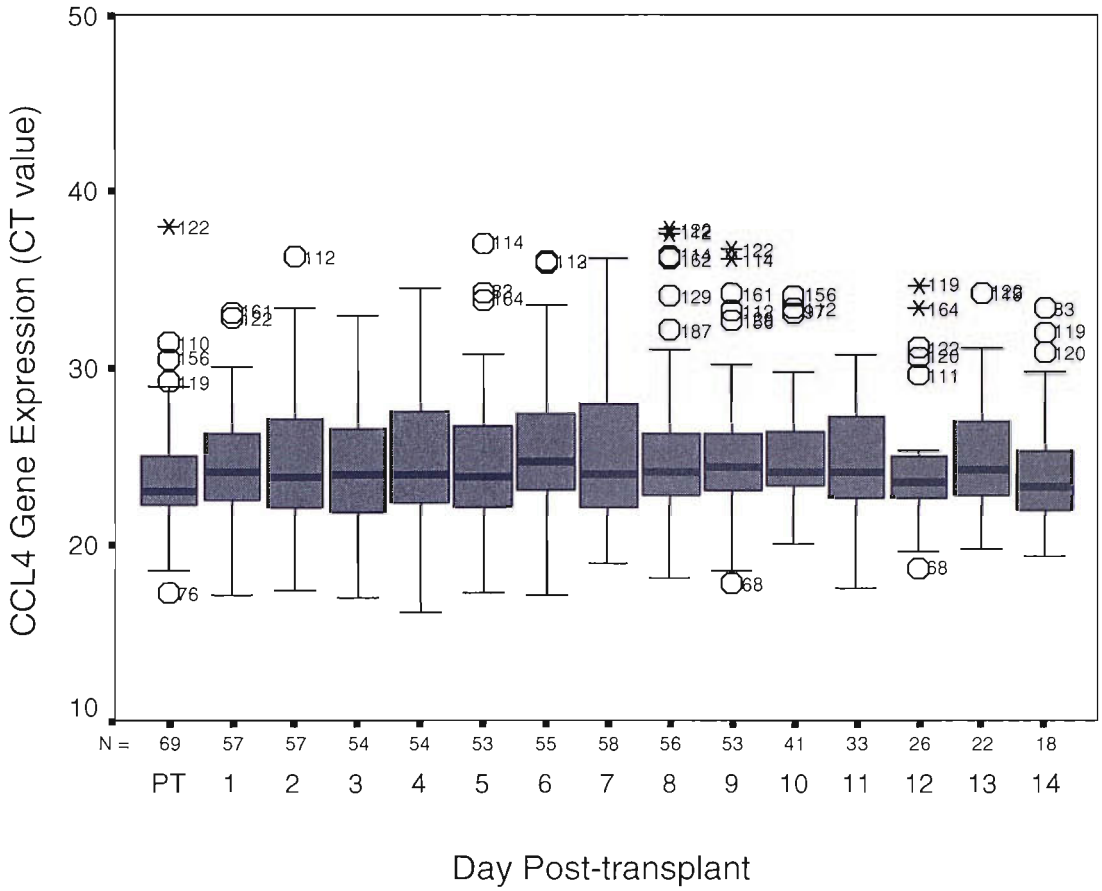

Fig. 5.2.a. Box and whisker plot showing chemokine **CCL4** gene expression levels in the **non-rejector group**. The y-axis is CCL4 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

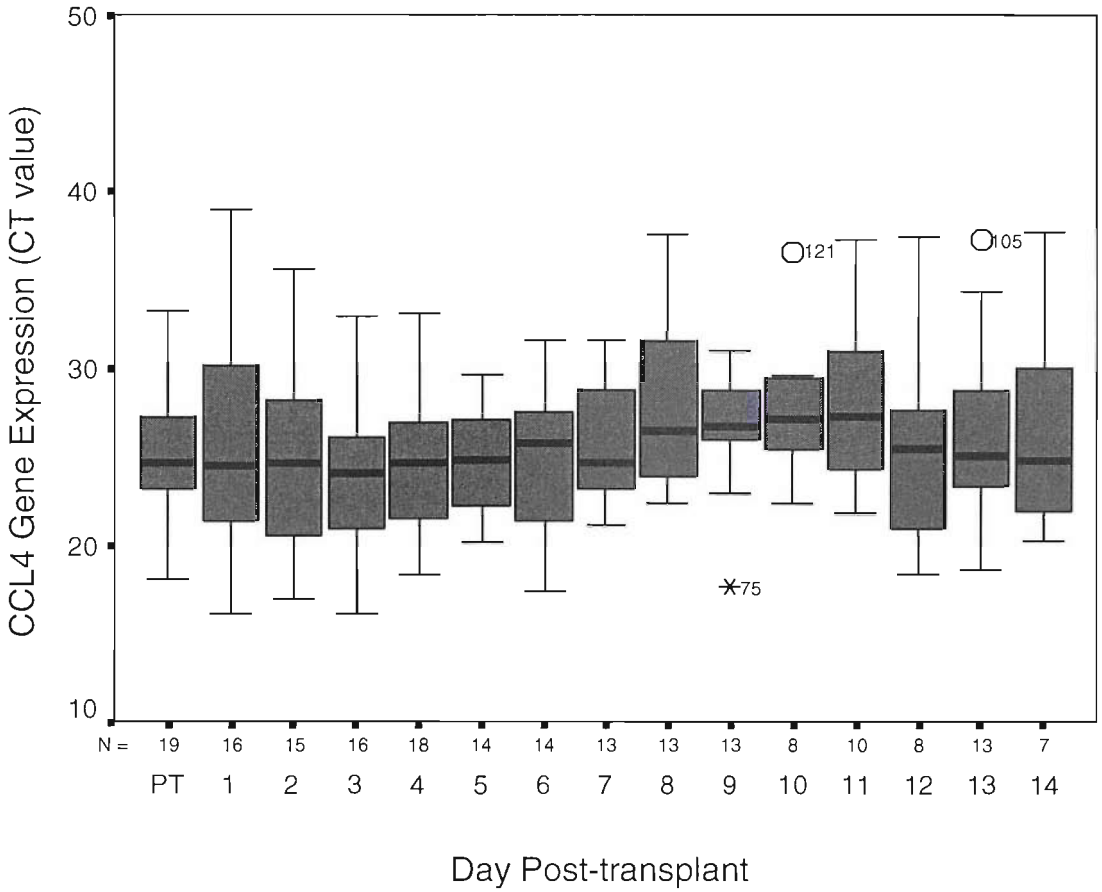

Fig. 5.2.b. Box and whisker plot showing chemokine **CCL4** gene expression levels in the **rejector group**. The y-axis is CCL4 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	57	57	54	54	53	55	58	56	53	41	33	26	22	18
Direction of change						↓	↓	↓						
p-value	0.196	0.150	0.692	0.136	0.797	**0.018**	**0.030**	**0.014**	0.058	0.091	0.421	0.648	0.021	0.122
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	57	51	48	45	42	43	46	51	48	39	23	19	17	14
Direction of change						↓								
p-value	0.196	0.579	0.862	0.252	0.488	**0.013**	0.676	0.232	0.472	0.238	0.637	0.214	0.093	0.084

Table. 5.2.a. Statistical significance of **CCL4** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	16	15	16	18	14	14	13	13	13	8	10	8	13	7
Direction of change														
p-value	0.408	0.691	0.501	0.896	0.397	0.875	0.650	0.055	0.050	0.326	0.114	1.00	0.649	1.00
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	16	13	14	15	14	12	11	11	9	6	6	5	8	7
Direction of change														
p-value	0.408	0.075	0.925	0.306	0.637	0.919	0.131	0.091	0.767	0.917	0.753	0.686	1.000	0.176

Table. 5.2.b. Statistical significance of **CCL4** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

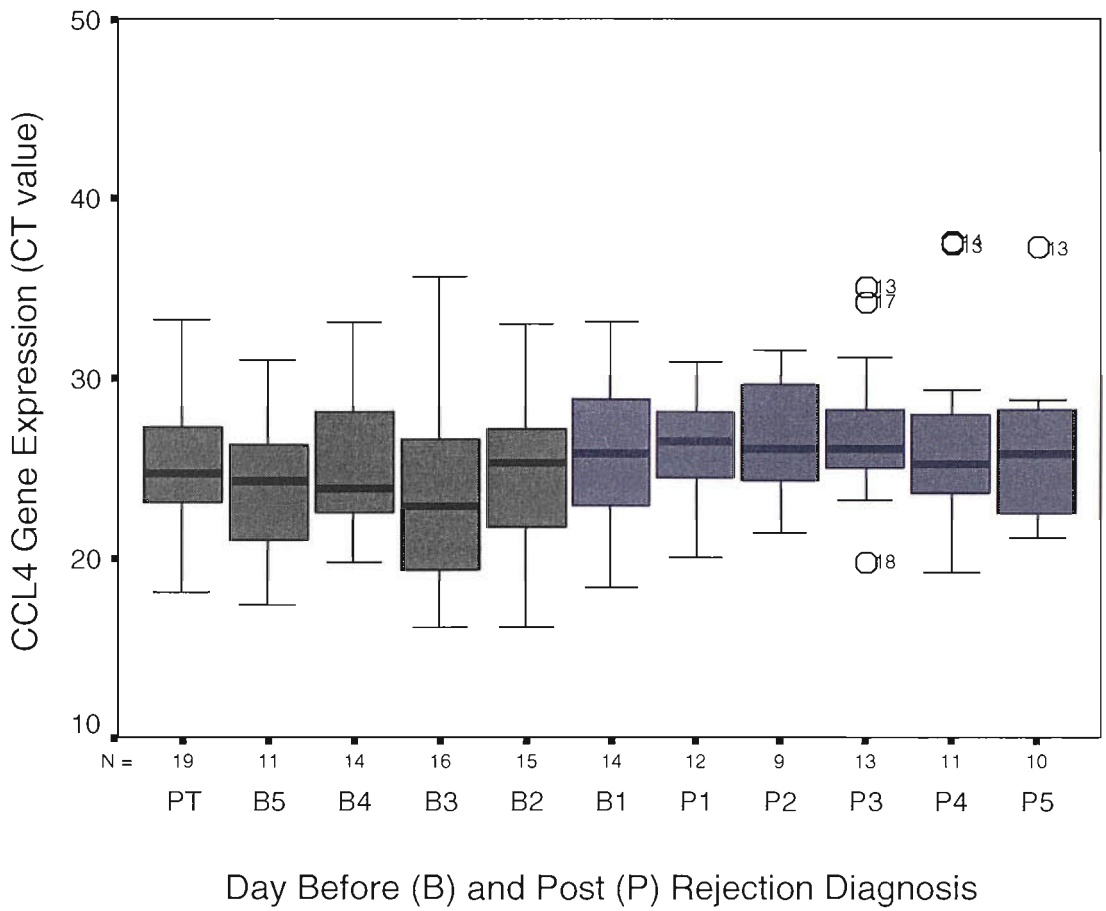

Fig. 5.2.c. Box and whisker plot showing chemokine **CCL4** gene expression levels in the **rejector group** adjusted to time of rejection diagnosis and treatment. The y-axis is CCL4 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day before (B) and post (P) rejection diagnosis and start of anti-rejection therapy with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

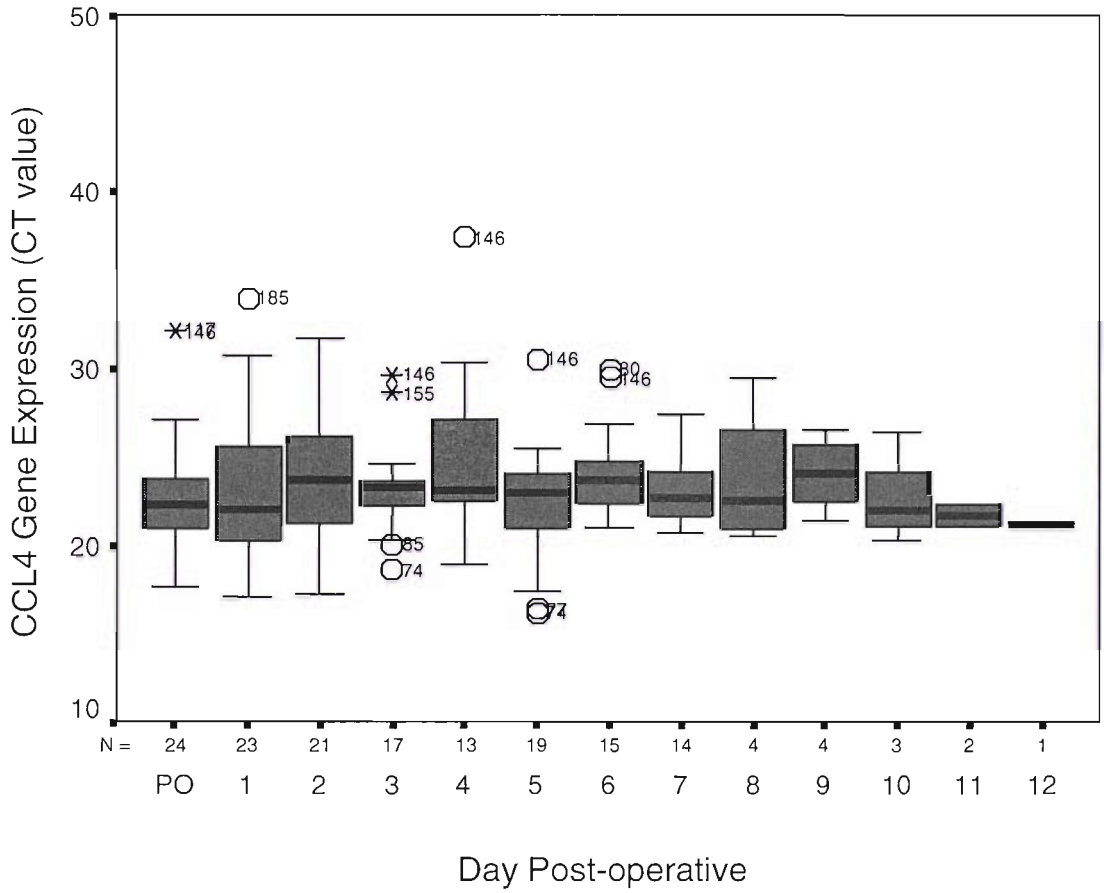

Fig. 5.2.d. Box and whisker plot showing chemokine **CCL4** gene expression levels in the **live donor group**. The y-axis is CCL4 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-operative with PO being the pre-operative baseline sample. The N number on the x-axis is the number of patient samples for each day

Baseline	PT-B5	PT-B4	PT-B3	PT-B2	PT-B1	PT-P1	PT-P2	PT-P3	PT-P4	PT-P5
n	11	14	16	15	14	12	9	13	11	10
Direction of change										
p-value	0.965	0.272	0.756	0.691	0.975	0.308	0.260	0.196	0.286	0.878
Stepwise	PT-B5	B5-B4	B4-B3	B3-B2	B2-B1	B1-P1	P1-P2	P2-P3	P3-P4	P4-P5
n	11	9	12	13	13	11	9	7	10	8
Direction of change					↓					
p-value	0.965	0.401	0.583	0.807	**0.011**	0.929	0.086	0.345	0.721	0.674

Table. 5.2.c. Statistical significance of **CCL4** gene expression changes for each day before (B) and post (P) rejection diagnosis and treatment commencement compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PO-1	PO-2	PO-3	PO-4	PO-5	PO-6	PO-7	PO-8	PO-9	PO-10	PO-11
n	23	21	17	13	19	15	14	4	4	3	2
Direction of change											
p-value	0.903	0.339	0.507	0.311	0.658	0.349	0.826	1.00	1.00	0.285	0.655
Stepwise	PO-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11
n	23	21	15	9	9	14	11	4	3	3	2
Direction of change											
p-value	0.903	0.263	0.551	0.139	0.767	0.158	0.068	0.465	0.285	0.109	0.655

Table. 5.2.d. Statistical significance of **CCL4** gene expression changes for each post-operative day compared to the pre-operative (PO) sample (baseline) and between successive days (stepwise) in the **live donor group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

5.3 CCL5

The results of CCL5 gene expression and statistical analysis are shown in Figures / Tables 5.3.a – 5.3d.

In the non-rejector group there were no significant changes in CCL5 gene expression levels comparing each post transplant day to the baseline pre-transplant sample or by stepwise analysis.

With subdivision of the non-rejector group into those with primary graft function (N=69) versus delayed graft function (N=11) (Appendix VI, Tables A.3.a & b), no significantly different patterns of CCL5 gene expression were revealed. Subdivision however, of the non-rejector group into patients with late rejection episodes (N=8) versus those without rejection episodes (N=72) (Appendix VI, Tables A.3.c & d), showed that the former subgroup had significant decreases (equivalent to 2- to 48-fold change) in CCL5 expression on days 2 ($p=0.043$), 3 ($p=0.043$), 4 ($p=0.028$), 5 ($p=0.028$), 6 ($p=0.043$), 8 ($p=0.025$) and 11 ($p=0.043$) compared to baseline levels. In the latter subgroup with no episodes of rejection, a similar expression pattern to the non-rejector group existed, except for an increase on day 5 ($p=0.005$) compared to baseline and stepwise increase between days 4 and 5 ($p=0.040$).

In the rejector group there were no significant changes in CCL5 gene expression levels by baseline analysis but a significant increase (2-fold) between days 4 and 5 ($p=0.049$) on stepwise analysis. With adjustment to time of rejection there were significant decreases in CCL5 expression (2- to 4-fold) on days 2 ($p=0.013$) and 3 ($p=0.015$) post rejection diagnosis compared to the baseline level. These changes correspond to the 2nd and 3rd pulses of methylprednisolone anti-rejection therapy. On stepwise analysis, there was a significant increase (1.5-fold) in CCL5 expression levels between days 3 and 2 before rejection diagnosis ($p=0.034$).

In the live donor group there were no significant changes on baseline or stepwise analysis.

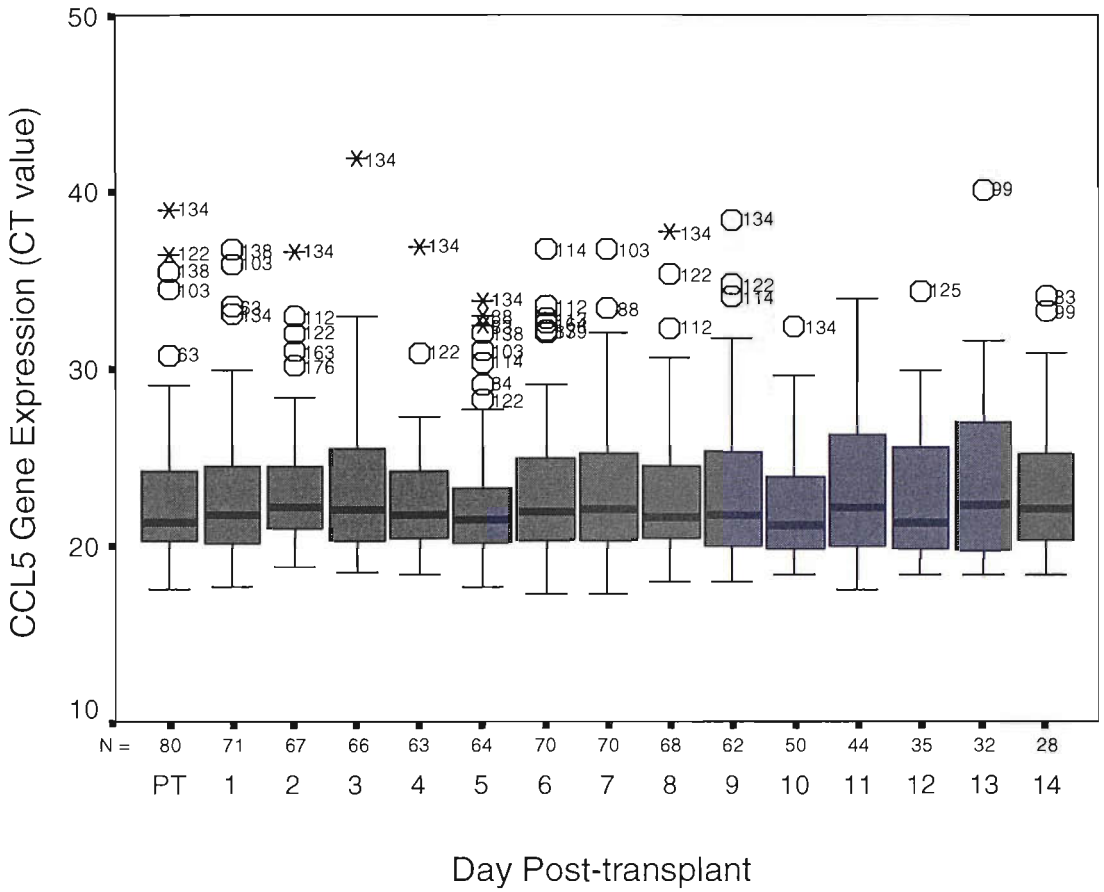

Fig. 5.3.a. Box and whisker plot showing chemokine **CCL5** gene expression levels in the **non-rejector group**. The y-axis is CCL5 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

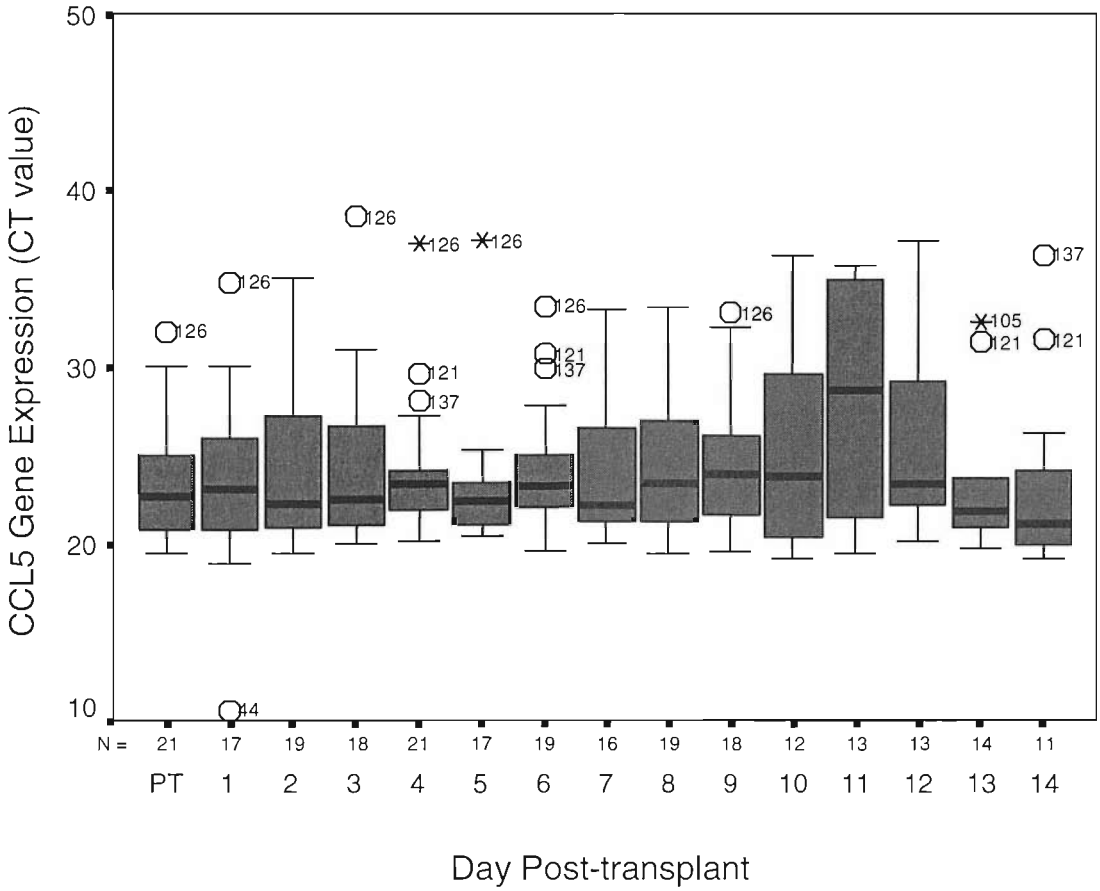

Fig. 5.3.b. Box and whisker plot showing chemokine **CCL5** gene expression levels in the **rejector group**. The y-axis is CCL5 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	71	67	66	63	64	70	70	68	62	50	44	35	32	28
Direction of change														
p-value	0.705	0.272	0.426	0.430	0.074	0.748	0.349	0.450	0.869	0.460	0.981	0.437	0.340	0.900
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	71	63	59	55	51	57	62	63	58	46	33	28	26	24
Direction of change														
p-value	0.705	0.366	0.310	0.136	0.247	0.143	0.453	0.347	0.954	0.092	0.085	0.624	0.228	0.331

Table. 5.3.a. Statistical significance of **CCL5** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	17	19	18	21	17	19	16	19	18	12	13	13	14	11
Direction of change														
p-value	0.136	0.084	0.349	0.566	0.740	0.573	0.605	0.717	0.170	0.060	0.101	0.345	0.875	0.790
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	17	16	17	18	17	15	16	16	17	12	9	9	11	9
Direction of change					↑									
p-value	0.136	0.605	0.836	0.983	**0.049**	0.334	0.717	0.255	0.653	0.695	0.161	0.678	0.050	0.767

Table. 5.3.b. Statistical significance of **CCL5** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

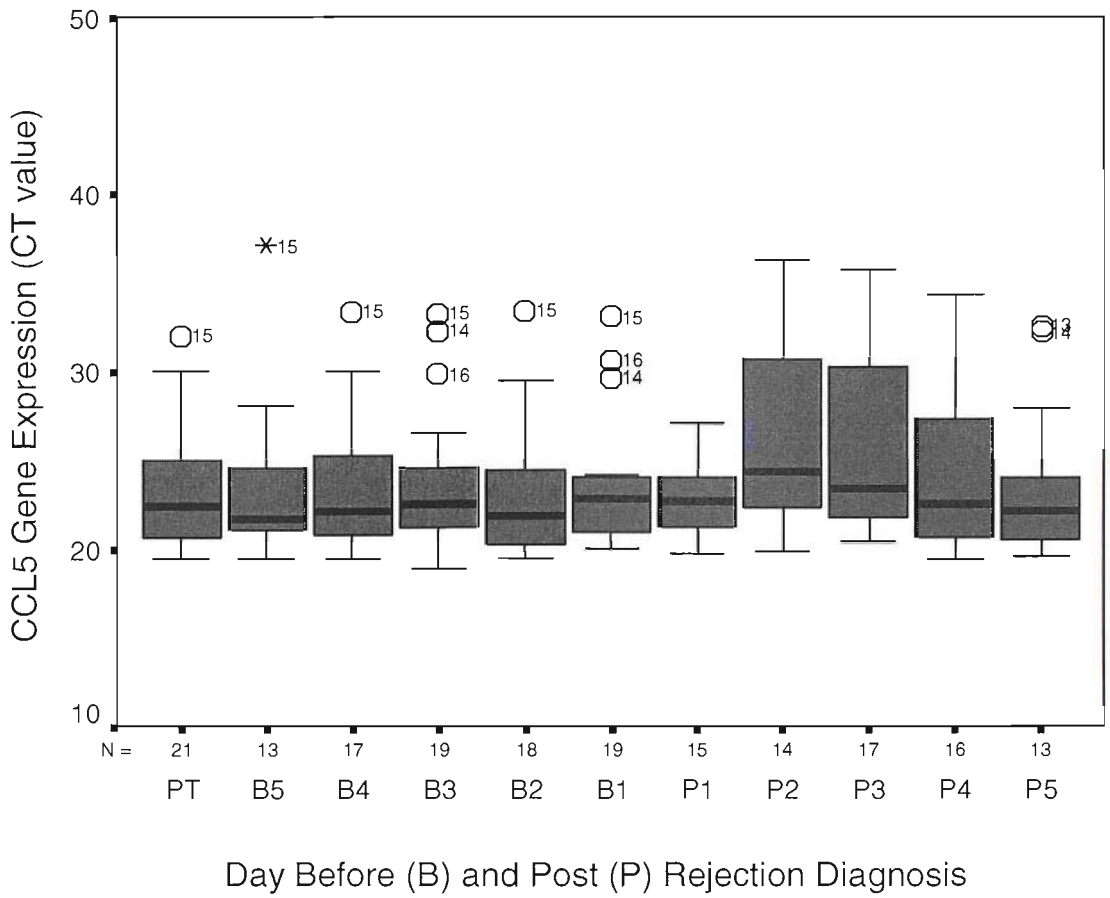

Fig. 5.3.c. Box and whisker plot showing chemokine **CCL5** gene expression levels in the **rejector group adjusted** to time of rejection diagnosis and treatment. The y-axis is CCL5 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day before (B) and post (P) rejection diagnosis and start of anti-rejection therapy with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

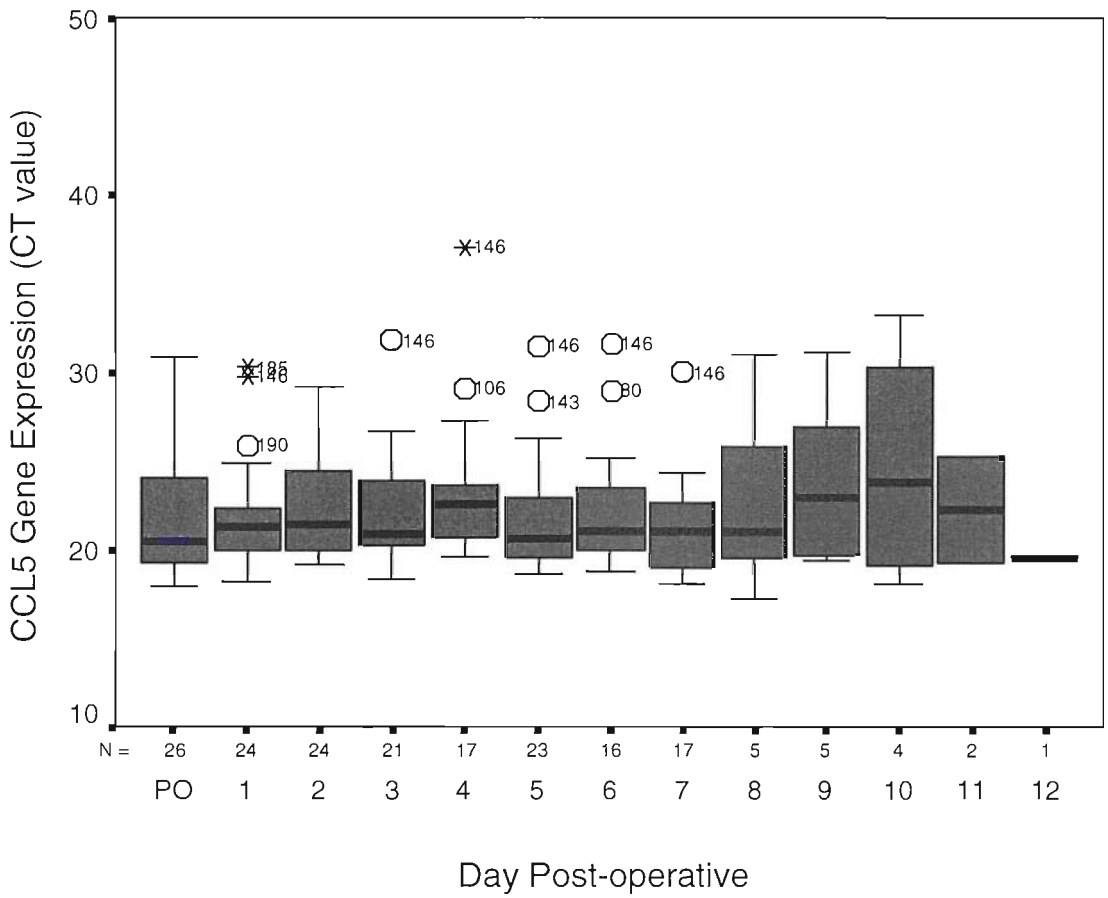

Fig. 5.3.d. Box and whisker plot showing chemokine **CCL5** gene expression levels in the **live donor group**. The y-axis is CCL5 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-operative with PO being the pre-operative baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-B5	PT-B4	PT-B3	PT-B2	PT-B1	PT-P1	PT-P2	PT-P3	PT-P4	PT-P5
n	13	17	19	18	19	15	14	17	16	13
Direction of change							↓	↓		
p-value	0.600	0.554	0.687	0.983	0.778	0.865	**0.013**	**0.015**	0.408	0.382
Stepwise	PT-B5	B5-B4	B4-B3	B3-B2	B2-B1	B1-P1	P1-P2	P2-P3	P3-P4	P4-P5
n	13	12	15	16	17	15	11	12	15	13
Direction of change				↑						
p-value	0.600	0.695	0.191	**0.034**	0.492	0.910	0.110	0.213	0.112	0.345

Table. 5.3.c. Statistical significance of **CCL5** gene expression changes for each day before (B) and post (P) rejection diagnosis and treatment commencement compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PO-1	PO-2	PO-3	PO-4	PO-5	PO-6	PO-7	PO-8	PO-9	PO-10	PO-11
n	24	24	21	17	23	16	17	5	5	4	2
Direction of change											
p-value	0.855	0.977	0.476	0.906	1.00	0.717	0.356	0.686	0.273	0.715	0.180
Stepwise	PO-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11
n	24	23	20	14	14	15	13	5	4	4	2
Direction of change											
p-value	0.855	0.236	0.332	0.463	0.363	0.394	0.249	0.893	0.068	0.465	0.655

Table. 5.3.d. Statistical significance of **CCL5** gene expression changes for each post-operative day compared to the pre-operative (PO) sample (baseline) and between successive days (stepwise) in the **live donor group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

5.4 CXCL10

The results of CXCL10 gene expression and statistical analysis are shown in Figures / Tables 5.4.a – 5.4d.
In the non-rejector group, there were significant decreases (equivalent to 3- to 4-fold change) in CXCL10 expression levels on days 1 (p=0.000) and 2 (p=0.037) post transplant compared to the baseline pre-transplant level, with a significant increase (1.5-fold) between days 1 and 2 (p=0.008) on stepwise analysis.
Subdivision of the non-rejector group into those with primary graft function (N=49) versus delayed graft function (N=11) (Appendix VI, Tables A.4.a & b) and patients with late rejection episodes (N=7) versus those without rejection episodes (N=53) (Appendix VI, Tables A.4.c & d) revealed no significantly different patterns of CXCL10 gene expression.

In the rejector group, there were significant increases (4- to 16-fold) in CXCL10 expression levels on days 2 (p=0.038), 3 (p=0.025), 4 (p=0.028), 5 (p=0.036) and 13 (p=0.025) post-transplant compared to the baseline pre-transplant level, and no significant changes on stepwise analysis. With adjustment to the time of rejection there were significant increases (16- to 32-fold) in CXCL10 expression levels on days 5 (p=0.028), 2 (p=0.046) and 1 (p=0.028) before the diagnosis of rejection when compared to the pre-transplant baseline level. On days 4 and 5 post rejection diagnosis there were also significant increases (16-fold) (p=0.028 and p=0.018 repectively) in CXCL10 expression levels compared to the baseline level. There was a significant decrease (48-fold) in CXCL10 expression from day 1 before rejection to day 1 post rejection diagnosis (p=0.036) corresponding to the commencement of anti rejection therapy.
In the live donor group, there were significant decreases (2- to 4-fold) in CXCL10 expression levels on days 1 (p=0.022) and 4 (p=0.015) post operatively compared to baseline pre-operative levels and no significant changes on stepwise analysis.
Overall, there was an increase in CXCL10 expression prior to rejection diagnosis which returned to baseline levels with anti-rejection therapy and increased again once

completed. The non-rejector group showed decreased CXCL10 expression immediately post-transplant to day 2 whereas the rejector group showed no immediate significant decrease but significant increases on days 2 to 5 post-transplant.

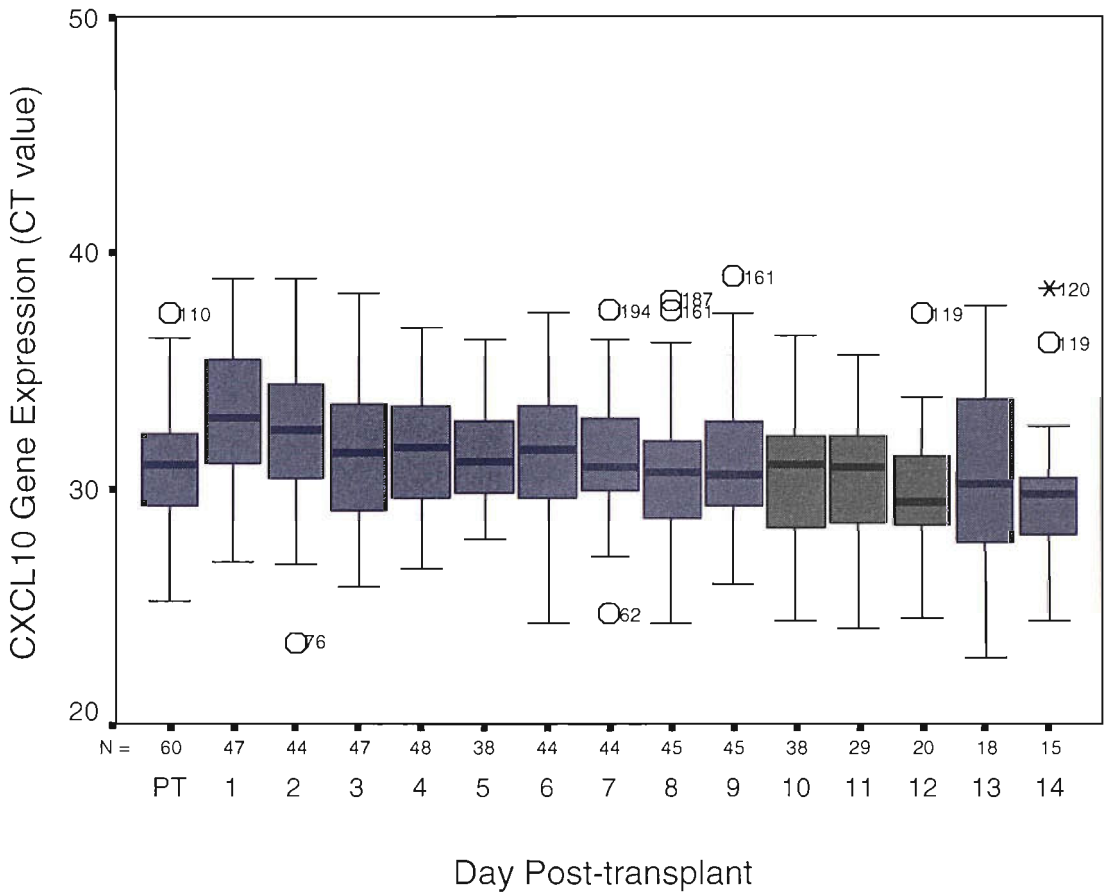

Fig. 5.4.a. Box and whisker plot showing chemokine **CXCL10** gene expression levels in the **non-rejector group**. The y-axis is CXCL10 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

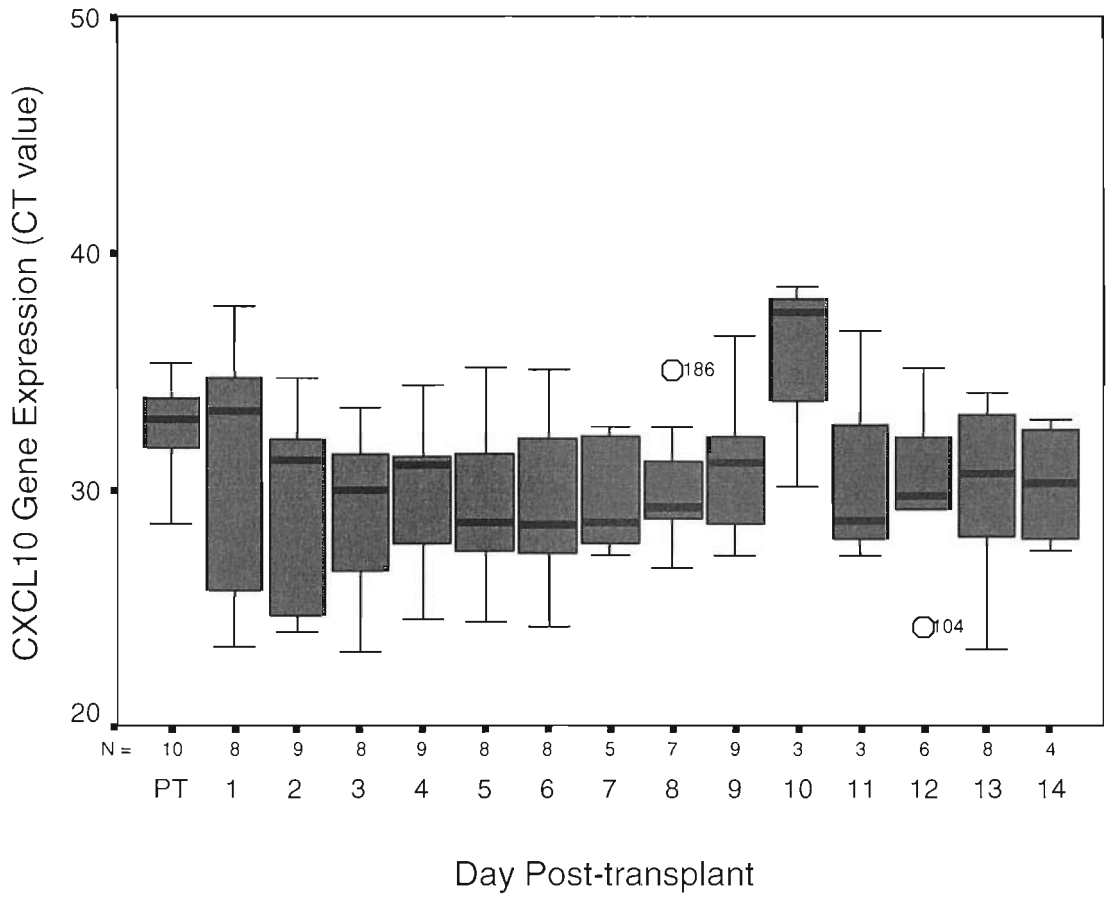

Fig. 5.4.b. Box and whisker plot showing chemokine **CXCL10** gene expression levels in the **rejector group**. The y-axis is CXCL10 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	47	44	47	48	38	44	44	45	45	38	29	20	18	15
Direction of change	↓	↓												
p-value	<0.001	**0.037**	0.379	0.160	0.919	0.214	0.203	0.739	0.697	0.673	0.880	0.351	0.396	0.875
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	47	38	38	38	31	29	33	38	39	34	22	15	15	12
Direction of change	↓	↑												
p-value	**<0.001**	**0.008**	0.455	0.919	0.710	0.125	0.531	0.556	0.622	0.369	0.082	0.410	0.496	0.099

Table. 5.4.a. Statistical significance of **CXCL10** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	8	9	8	9	8	8	5	7	9	3	3	6	8	4
Direction of change		↑	↑	↑	↑								↑	
p-value	0.575	**0.038**	**0.025**	**0.028**	**0.036**	0.123	0.080	0.128	0.214	0.285	0.276	0.116	**0.025**	0.068
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	8	7	8	7	8	7	5	4	7	3	1	2	5	3
Direction of change														
p-value	0.575	0.063	0.779	0.612	0.050	0.612	0.713	1.000	0.612	0.109	-	0.655	0.500	0.285

Table. 5.4.b. Statistical significance of **CXCL10** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

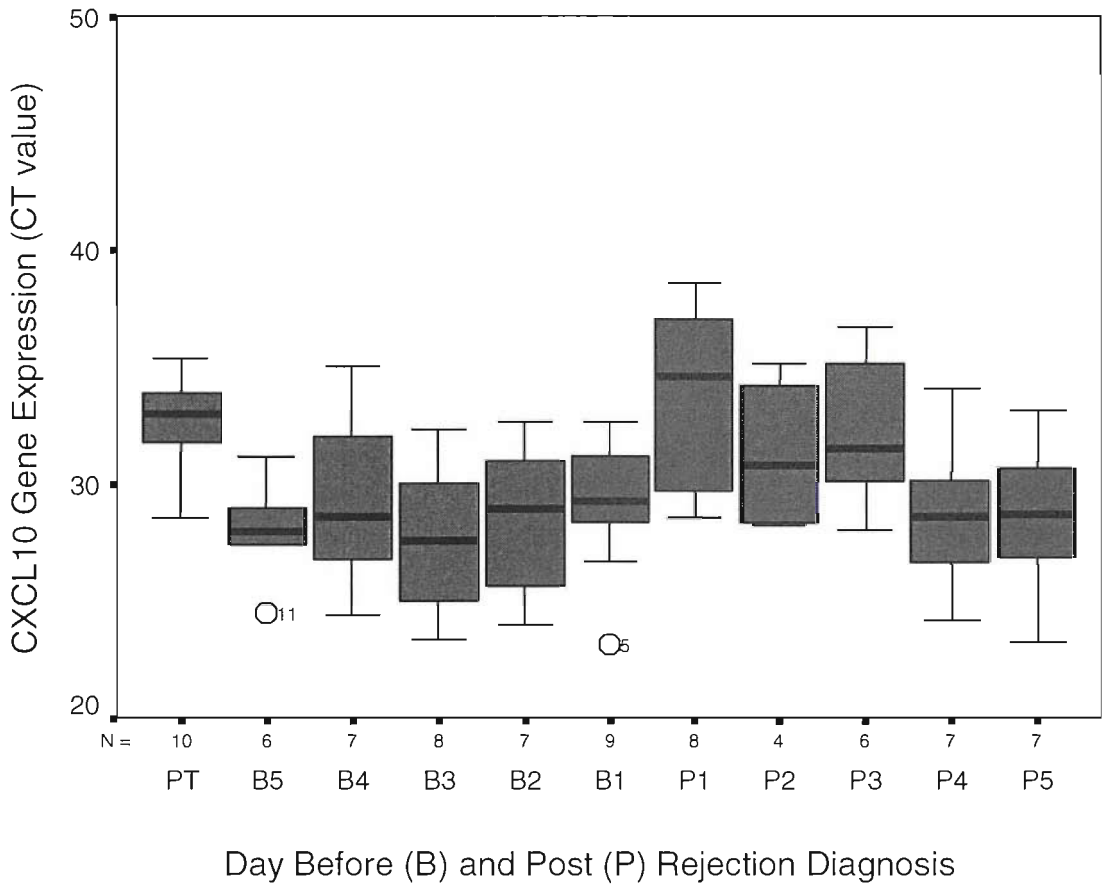

Fig. 5.4.c. Box and whisker plot showing chemokine **CXCL10** gene expression levels in the **rejector group adjusted** to time of rejection diagnosis and treatment. The y-axis is CXCL10 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day before (B) and post (P) rejection diagnosis and start of anti-rejection therapy with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

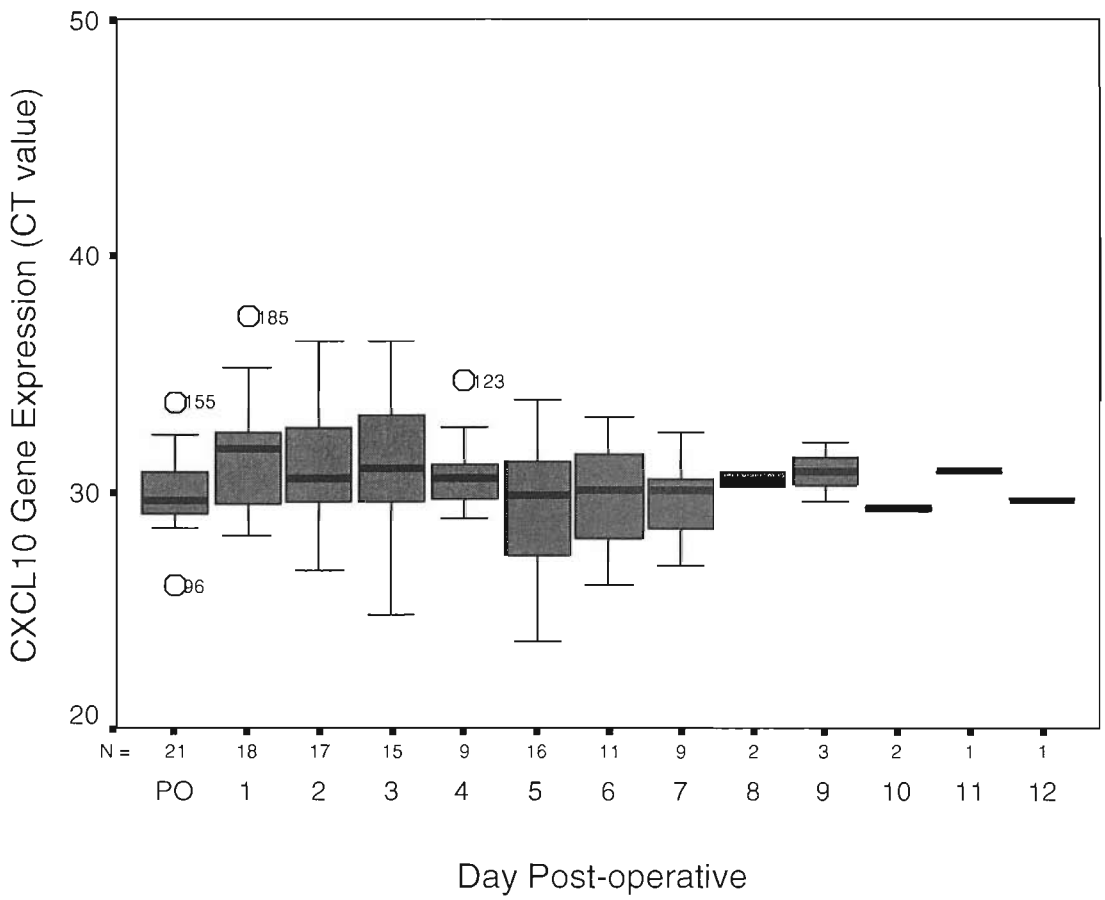

Fig. 5.4.d. Box and whisker plot showing chemokine **CXCL10** gene expression levels in the **live donor group**. The y-axis is CXCL10 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-operative with PO being the pre-operative baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-B5	PT-B4	PT-B3	PT-B2	PT-B1	PT-P1	PT-P2	PT-P3	PT-P4	PT-P5
n	6	7	8	7	9	8	4	6	7	7
Direction of change	↑		↑	↑	↑				↑	↑
p-value	**0.028**	0.128	**0.050**	**0.046**	**0.028**	0.674	0.712	0.345	**0.028**	**0.018**
Stepwise	PT-B5	B5-B4	B4-B3	B3-B2	B2-B1	B1-P1	P1-P2	P2-P3	P3-P4	P4-P5
n	6	5	5	6	7	8	3	3	5	5
Direction of change	↑					↓				
p-value	**0.028**	0.50	0.686	0.60	0.237	**0.036**	0.593	1.00	0.080	0.50

Table. 5.4.c. Statistical significance of **CXCL10** gene expression changes for each day before (B) and post (P) rejection diagnosis and treatment commencement compared to the pre-transplant (PT) sample (Baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PO-1	PO-2	PO-3	PO-4	PO-5	PO-6	PO-7	PO-8	PO-9	PO-10
n	18	17	15	9	16	11	9	2	3	2
Direction of change	↓			↓						
p-value	**0.022**	0.177	0.112	**0.015**	0.776	0.656	0.859	0.655	1.00	0.180
Stepwise	PO-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
n	18	16	14	6	6	10	7	2	2	2
Direction of change	↓									
p-value	**0.022**	0.959	0.683	0.116	0.344	0.185	0.107	0.180	0.655	0.180

Table. 5.4.d. Statistical significance of **CXCL10** gene expression changes for each post-operative day compared to the pre-operative (PO) sample (baseline) and between successive days (stepwise) in the **live donor group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

5.5 Chemokine receptor CCR1

The results of CCR1 gene expression and statistical analysis are shown in Figures / Tables 5.5.a – 5.5.d.

In the non-rejector group there were significant increases (equivalent to 3- to 6-fold change) in CCR1 gene expression levels on days 1 ($p<0.001$), 2 ($p=0.037$), 3 ($p<0.001$), 5 ($p=0.009$), 9 ($p=0.013$) and 10 ($p=0.031$) post transplant compared to the pre-transplant baseline level. A significant stepwise decrease (3-fold) in expression occurred between days 12 and 13 ($p=0.035$) post-transplant

With subdivision of the non-rejector group into those with primary graft function (N=61) versus delayed graft function (N=12) (Appendix VI, Tables A.5.a & b), both subgroups had significant increases in CCR1 expression at some time points in the first 5 days post-transplant compared to baseline levels, namely days 1 ($p<0.001$), 3 ($p<0.001$) and 5 ($p=0.017$) (6-fold) in the primary graft function subgroup, and days 2 ($p=0.015$) and 4 ($p=0.036$) (8-fold) in the delayed graft function subgroup. A difference between these two subgroups was an increase (8-fold) in CCR1 expression that occurred in the delayed graft function group on days 8 ($p=0.041$) and 9 ($p=0.008$) compared to baseline levels, which was not seen in the primary function group. Subdivision of the non-rejector group into patients with late rejection episodes (N=8) versus those without rejection episodes (N=65) (Appendix VI, Tables A.5.c & d), showed no significant changes in CCR1 expression in the former subgroup, however in the subgroup without rejection episodes increases (2- to 8-fold) in expression occurred on days 1 ($p<0.001$), 2 ($p=0.008$), 3 ($p<0.001$), 4 ($p=0.020$), 5 ($p=0.002$), 6 ($p=0.023$), 7 ($p=0.043$), 9 ($p=0.005$), 10 ($p=0.043$), 11 ($p=0.007$) and 12 ($p=0.008$) compared to baseline levels.

In the rejector group, there were significant increases (6- to 12-fold) in CCR1 expression on days 5 ($p=0.030$) and 13 ($p=0.016$) post transplant compared to the pre-transplant baseline level and a stepwise increase (12-fold) from day 4 to 5 ($p=0.005$). With adjustment to time of rejection diagnosis and commencement of treatment there were significant increases (4- to 12-fold) in CCR1 expression on days 4 ($p=0.026$), 2 ($p=0.01$) and 1 ($p=0.01$) before rejection diagnosis when comparing to the baseline

pre-transplant level. Stepwise analysis shows a significant decrease (4-fold) in CCR1 expression on day 1 following commencement of anti-rejection therapy (P1) compared to day 1 before therapy (B1) (p=0.030).
Also a significant increase (24-fold) in CCR1 expression is seen on day 4 post rejection diagnosis compared to day 3 (p=0.004) and corresponds to approximately 36 hours following the last of 3 pulses of methylprednisolone used as anti-rejection therapy.
Overall, there seems to be an increase in CCR1 expression in the days prior to rejection diagnosis which then returns to baseline levels following anti-rejection therapy.

In the live donor group, there was a significant increase (3-fold) in CCR1 expression on the first (p=0.023) and second (p=0.005) post-operative days compared to the pre-operative baseline level.

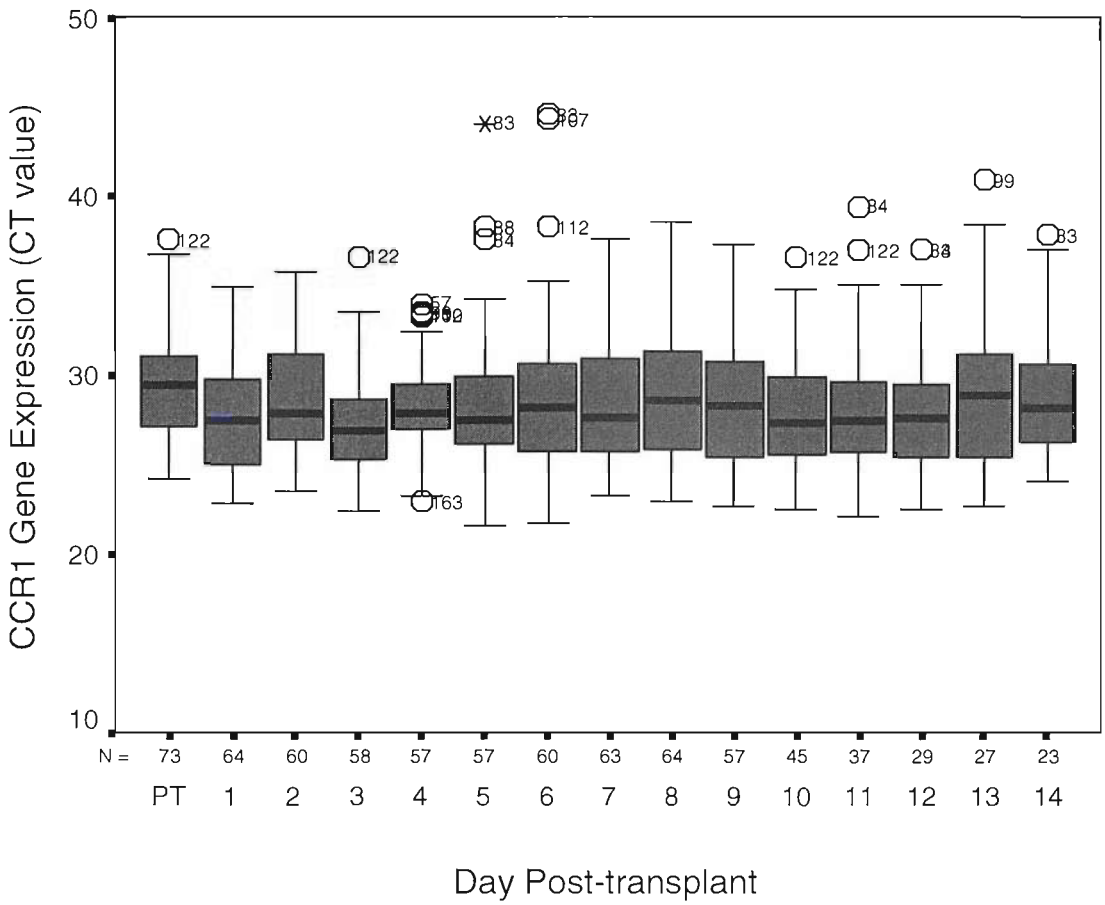

Fig. 5.5.a. Box and whisker plot showing chemokine receptor **CCR1** gene expression levels in the **non-rejector group**. The y-axis is CCR1 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

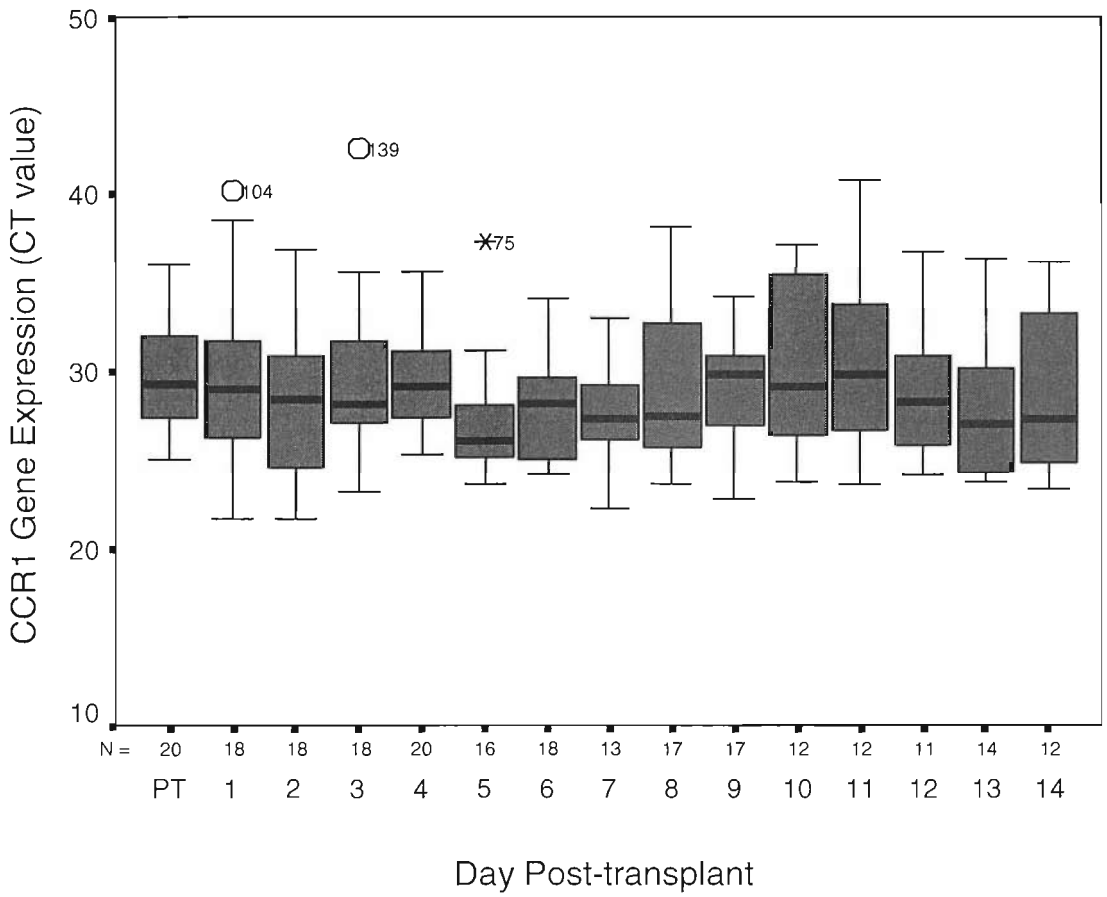

Fig. 5.5.b. Box and whisker plot showing chemokine receptor **CCR1** gene expression levels in the **rejector group**. The y-axis is CCR1 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	64	60	58	57	57	60	63	64	57	45	37	29	27	23
Direction of change	↑	↑	↑		↑				↑	↑				
p-value	**<0.001**	**0.037**	**<0.001**	0.071	**0.009**	0.065	0.121	0.165	**0.013**	**0.031**	0.053	0.050	0.990	0.693
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	64	56	51	48	44	48	53	58	55	41	28	23	21	19
Direction of change	↑												↓	
p-value	**<0.001**	0.906	0.096	0.117	0.350	0.151	0.547	0.355	0.293	0.476	0.982	0.287	**0.035**	0.469

Table. 5.5.a. Statistical significance of **CCR1** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	18	18	18	20	16	18	13	17	17	12	12	11	14	12
Direction of change					↑								↑	
p-value	0.472	0.170	0.248	0.601	**0.030**	0.094	0.055	0.276	0.653	0.388	0.937	0.248	**0.016**	0.347
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	18	17	17	18	16	14	13	13	15	12	9	7	9	10
Direction of change					↑									
p-value	0.472	0.463	0.723	0.360	**0.005**	0.140	0.701	0.463	0.910	0.136	0.515	0.499	0.066	0.721

Table. 5.5.b. Statistical significance of **CCR1** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

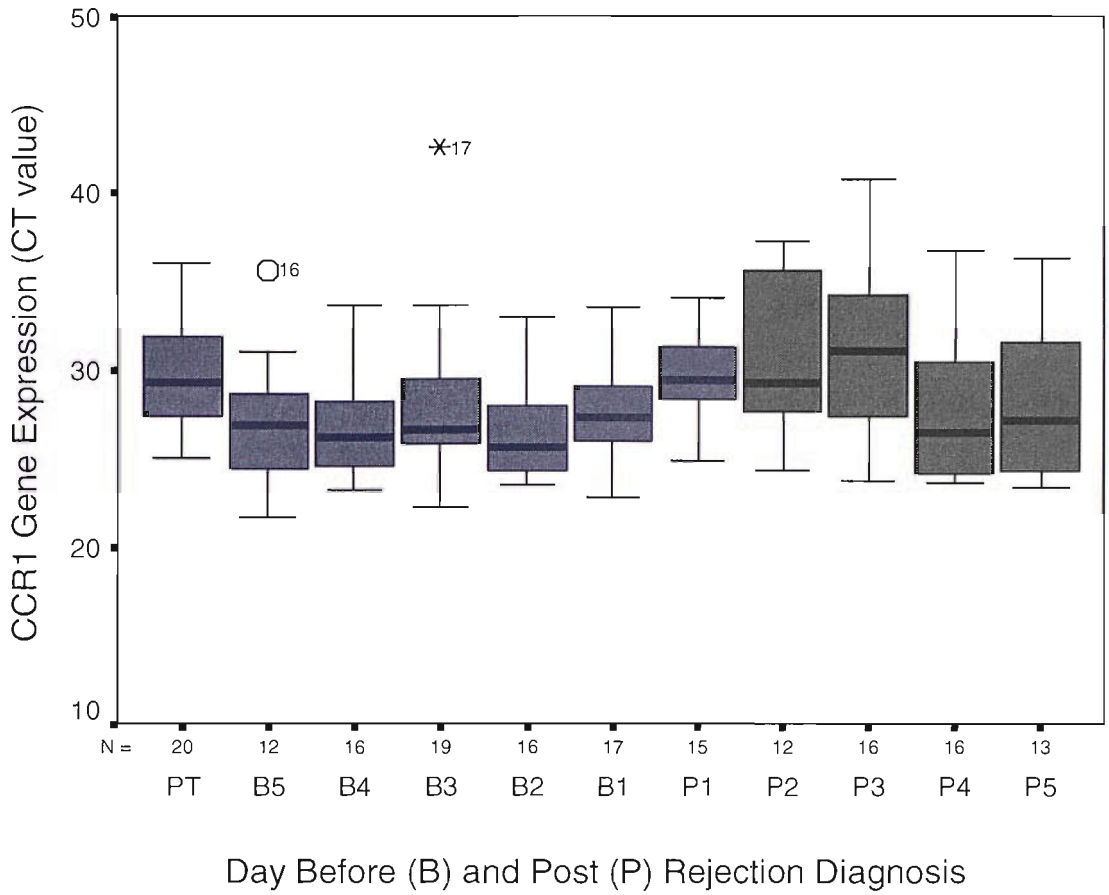

Fig. 5.5.c. Box and whisker plot showing chemokine receptor **CCR1** gene expression levels in the **rejector group** adjusted to time of rejection diagnosis and treatment. The y-axis is CCR1 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day before (B) and post (P) rejection diagnosis and start of anti-rejection therapy with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

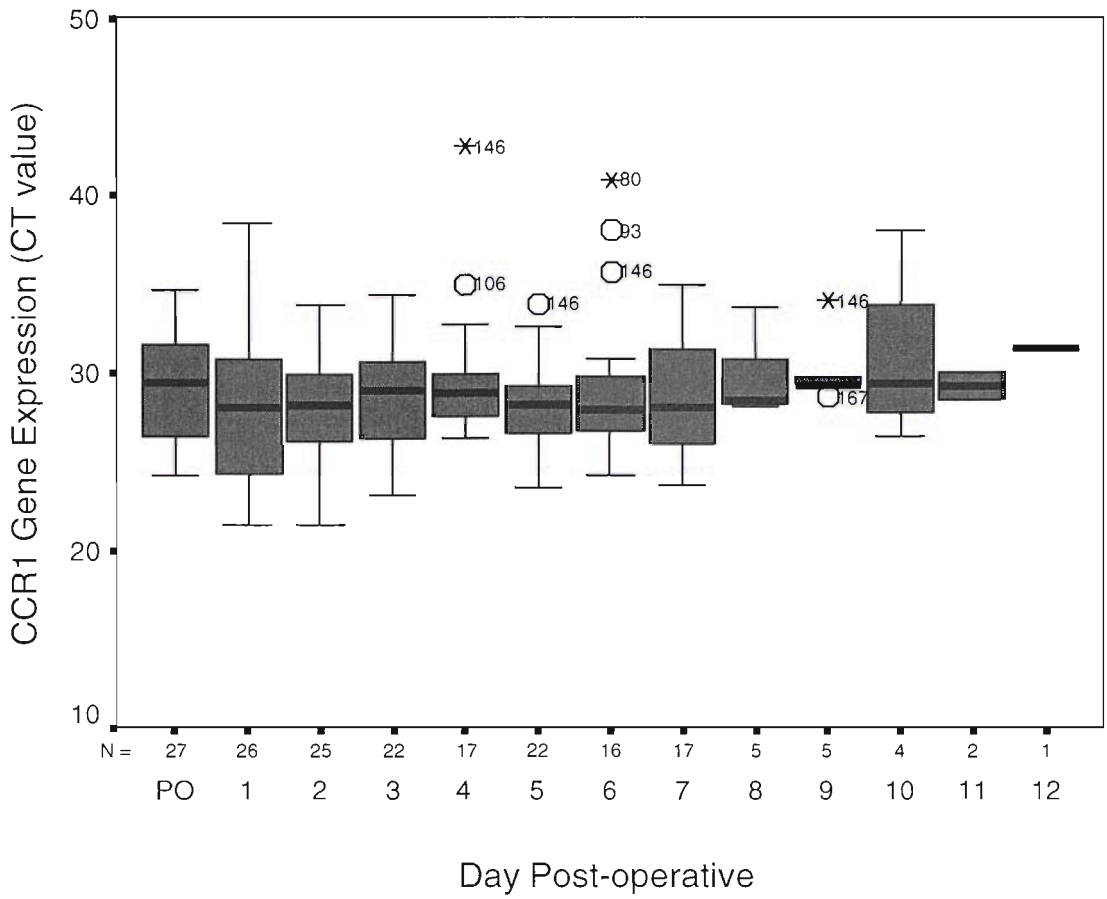

Fig. 5.5.d. Box and whisker plot showing chemokine receptor **CCR1** gene expression levels in the **live donor group**. The y-axis is CCR1 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-operative with PO being the pre-operative baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-B5	PT-B4	PT-B3	PT-B2	PT-B1	PT-P1	PT-P2	PT-P3	PT-P4	PT-P5
n	12	16	19	16	17	15	12	16	16	13
Direction of change		↑		↑	↑					
p-value	0.099	**0.026**	0.064	**0.01**	**0.01**	0.532	0.388	0.408	0.088	0.422
Stepwise	PT-B5	B5-B4	B4-B3	B3-B2	B2-B1	B1-P1	P1-P2	P2-P3	P3-P4	P4-P5
n	12	11	15	15	15	14	10	10	15	12
Direction of change						↓			↑	
p-value	0.099	0.929	0.094	0.112	0.078	**0.030**	0.646	0.799	**0.004**	0.695

Table. 5.5.c. Statistical significance of **CCR1** gene expression changes for each day before (B) and post (P) rejection diagnosis and treatment commencement compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PO-1	PO-2	PO-3	PO-4	PO-5	PO-6	PO-7	PO-8	PO-9	PO-10	PO-11
n	26	25	22	17	22	16	17	5	5	4	2
Direction of change	↑	↑									
p-value	**0.023**	**0.005**	0.140	0.605	0.085	0.301	0.569	0.893	0.686	1.00	0.180
Stepwise	PO-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11
n	26	25	21	14	13	15	13	5	4	4	2
Direction of change	↑										
p-value	**0.023**	0.382	0.398	0.594	0.422	0.496	0.875	0.138	0.068	0.715	0.655

Table. 5.5.d. Statistical significance of **CCR1** gene expression changes for each post-operative day compared to the pre-operative (PO) sample (baseline) and between successive days (stepwise) in the **live donor group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

5.6 Chemokine receptor CCR5

The results of CCR5 gene expression and statistical analysis are shown in Figures / Tables 5.6.a – 5.6.d.

In the non-rejector group there were significant decreases (equivalent to 2- to 3-fold change) in CCR5 gene expression on days 6 (p=0.006), 7 (p=0.030) and 13 (p=0.049) compared to the pre-transplant baseline level. A stepwise decrease (1.5- to 3-fold) occurred between days 5 and 6 (p=0.006), and 12 and 13 (p=0.039) post-transplant. Subdivision of the non-rejector group into those with primary graft function (N=37) versus delayed graft function (N=11) (Appendix VI, Tables A.6.a & b), showed no significant differences in their CCR5 gene expression patterns. Subdivision of the non-rejector group into patients with late rejection episodes (N=6) versus those without rejection episodes (N=42) (Appendix VI, Tables A.6.c & d), showed no significant changes in the former subgroup. However in the subgroup with no episodes of rejection, decreases (1.5- to 2-fold) in CCR5 gene expression occurred on days 6 (p=0.009) and 7 (p=0.022) compared to baseline levels and a stepwise decrease (2-fold) between days 5 and 6 (p=0.003).

In the rejector group, there was a significant increase (8-fold) in CCR5 expression on day 2 (p=0.047) post-transplant compared to the pre-transplant baseline level. With adjustment to the time of rejection diagnosis there was a significant increase (12-fold) in expression on day 5 before rejection diagnosis compared to the pre-transplant level (p=0.046). A significant decrease (1.5-fold) in expression occurred between day 1 and 2 post rejection diagnosis and start of anti-rejection therapy (p=0.017).

In the live donor group no significant changes in CCR5 gene expression occurred during the early post-operative period either by baseline or stepwise analysis.

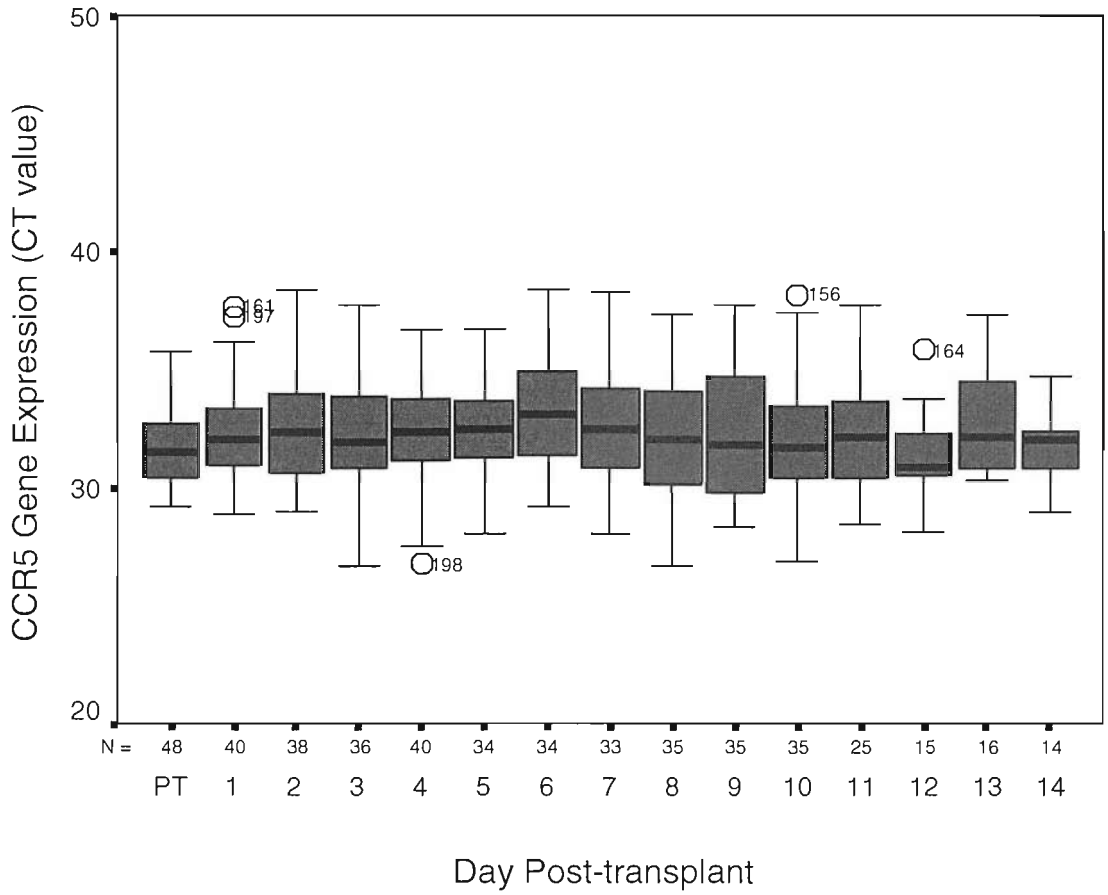

Fig. 5.6.a. Box and whisker plot showing chemokine receptor **CCR5** gene expression levels in the **non-rejector group**. The y-axis is CCR5 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

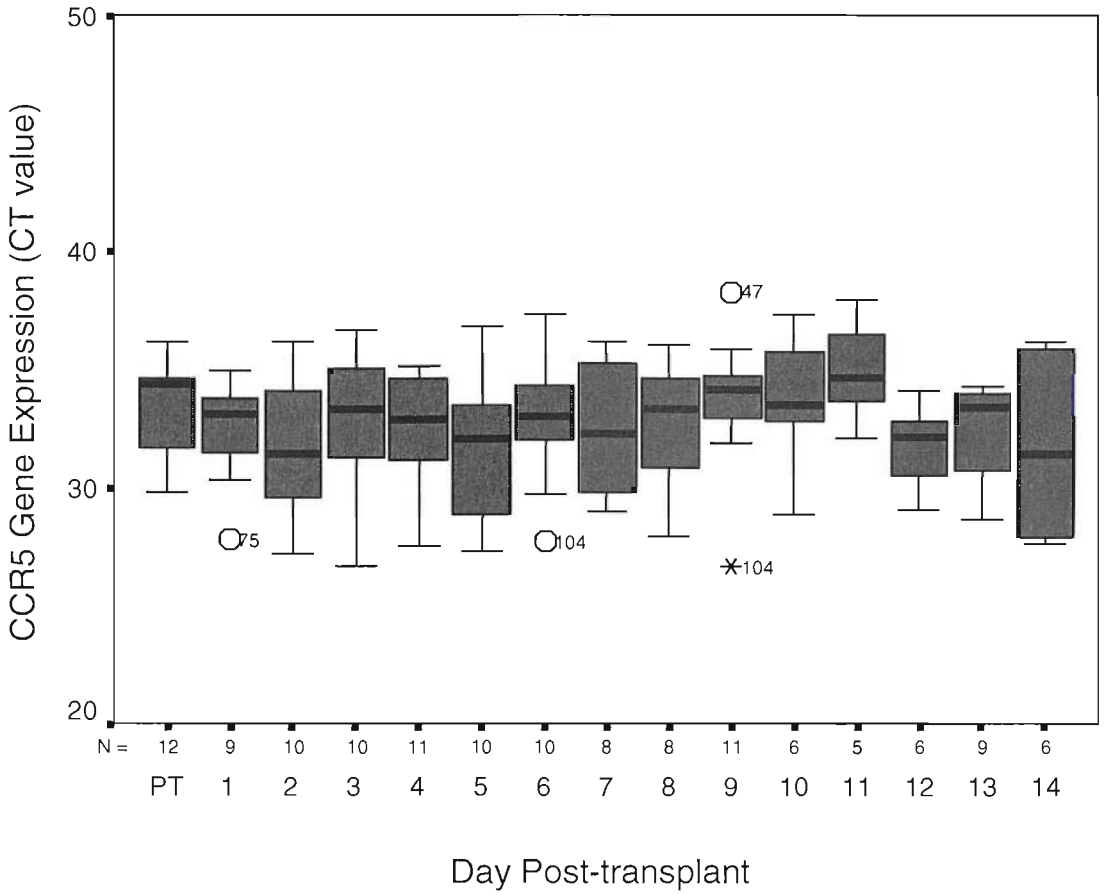

Fig. 5.6.b. Box and whisker plot showing chemokine receptor **CCR5** gene expression levels in the **rejector group**. The y-axis is CCR5 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	40	38	36	40	34	34	33	35	35	35	25	15	16	14
Direction of change						↓	↓						↓	
p-value	0.105	0.224	0.540	0.259	0.222	**0.006**	**0.030**	0.287	0.644	0.594	0.628	0.221	**0.049**	0.861
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	40	34	30	31	29	25	24	27	29	30	20	11	13	11
Direction of change						↓							↓	
p-value	0.105	0.925	0.388	0.761	0.991	**0.006**	0.063	0.589	0.880	0.439	0.970	0.534	**0.039**	0.091

Table. 5.6.a. Statistical significance of **CCR5** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	9	10	10	11	10	10	8	8	11	6	5	6	9	6
Direction of change		↑												
p-value	0.767	**0.047**	0.508	0.476	0.139	0.721	1.000	0.624	0.790	0.600	0.500	0.075	0.441	0.600
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	9	8	9	9	9	9	7	6	7	6	4	2	5	5
Direction of change														
p-value	0.767	0.123	0.236	0.722	0.260	0.050	0.866	0.753	0.249	0.916	0.461	0.180	0.080	0.893

Table. 5.6.b. Statistical significance of **CCR5** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

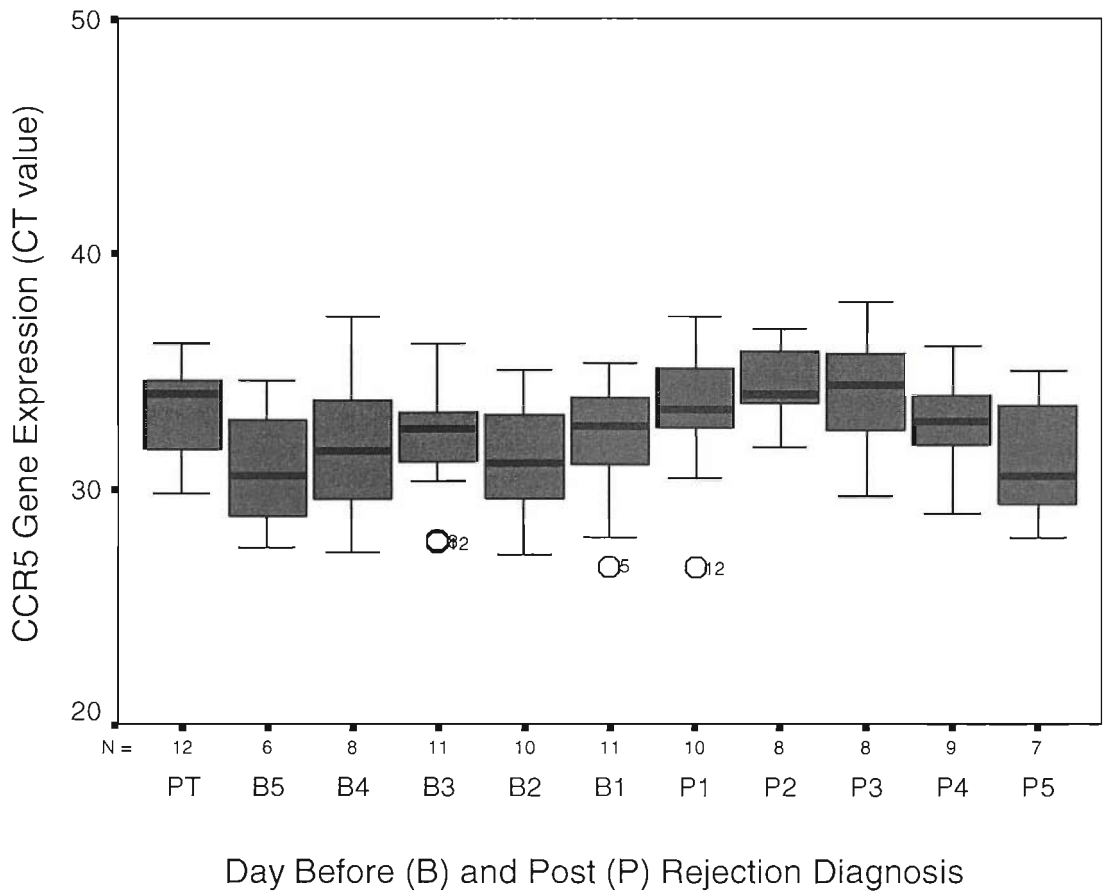

Fig. 5.6.c. Box and whisker plot showing chemokine receptor **CCR5** gene expression levels in the **rejector group adjusted** to time of rejection diagnosis and treatment. The y-axis is CCR5 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day before (B) and post (P) rejection diagnosis and start of anti-rejection therapy with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

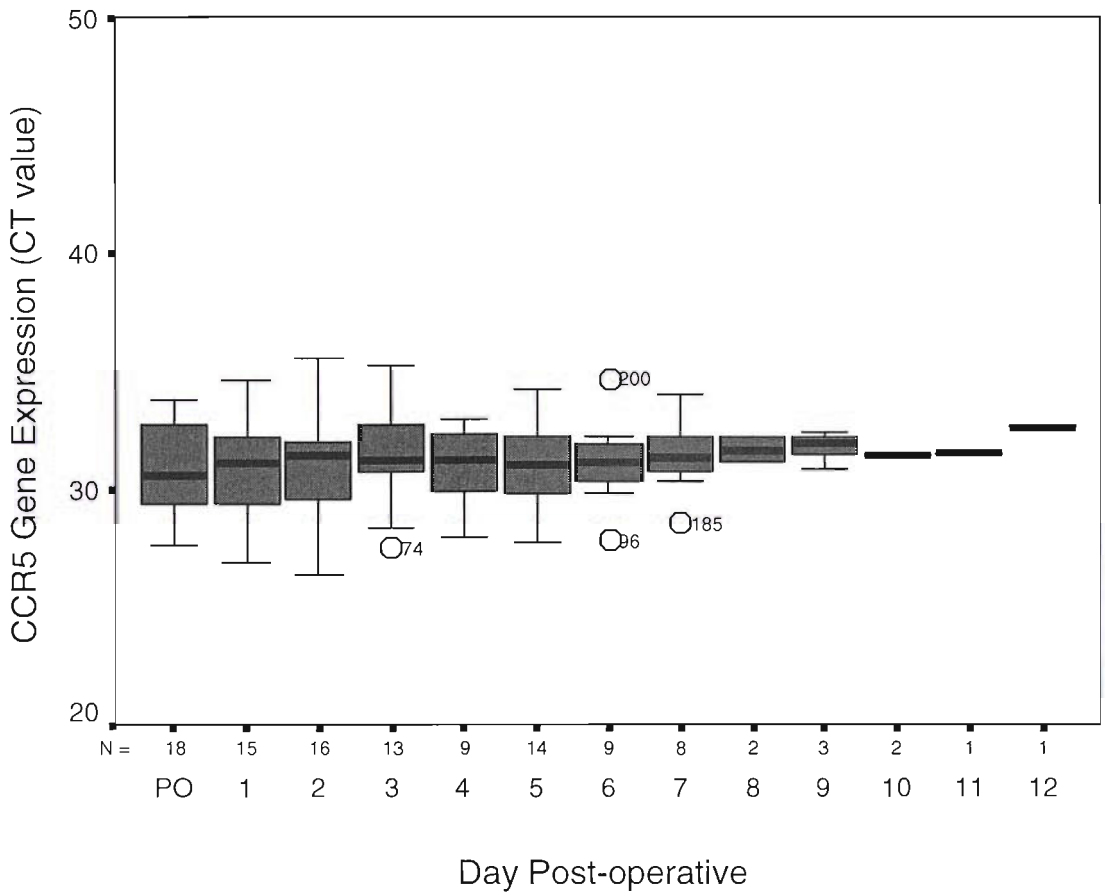

Fig. 5.6.d. Box and whisker plot showing chemokine **CCR5** gene expression levels in the **live donor group**. The y-axis is CCR5 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-operative with PO being the pre-operative baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-B5	PT-B4	PT-B3	PT-B2	PT-B1	PT-P1	PT-P2	PT-P3	PT-P4	PT-P5
n	6	8	11	10	11	10	8	8	9	7
Direction of change	↑									
p-value	**0.046**	0.263	0.374	0.139	0.230	0.721	0.092	0.575	0.441	0.063
Stepwise	PT-B5	B5-B4	B4-B3	B3-B2	B2-B1	B1-P1	P1-P2	P2-P3	P3-P4	P4-P5
n	6	5	7	9	10	9	7	6	7	6
Direction of change	↑						↓			
p-value	**0.046**	0.686	0.091	0.066	0.441	0.110	**0.017**	0.753	0.612	0.917

Table. 5.6.c. Statistical significance of **CCR5** gene expression changes for each day before (B) and post (P) rejection diagnosis and treatment commencement compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PO-1	PO-2	PO-3	PO-4	PO-5	PO-6	PO-7	PO-8	PO-9	PO-10	PO-11
n	15	16	13	9	14	9	8	2	3	2	1
Direction of change											
p-value	0.478	0.756	0.552	0.594	0.530	0.515	0.674	0.655	1.000	0.655	-
Stepwise	PO-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11
n	15	14	12	6	6	8	5	2	2	2	1
Direction of change											
p-value	0.478	0.245	0.814	0.116	0.116	0.327	0.345	0.655	0.180	1.000	-

Table. 5.6.d. Statistical significance of **CCR5** gene expression changes for each post-operative day compared to the pre-operative (PO) sample (baseline) and between successive days (stepwise) in the **live donor group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

5.7 Chemokine Receptor CXCR3

The results of CXCR3 gene expression and statistical analysis are shown in Figures / Tables 5.7.a – 5.7.d.

In the non-rejector group there were significant decreases (equivalent to 2- to 3-fold change) in CXCR3 gene expression on days 1 ($p<0.001$), 2 ($p=0.001$) and 14 ($p=0.004$) post-transplant compared to the pre-translant baseline level. A significant stepwise increase (2-fold) in expression occurred between days 2 and 3 ($p=0.005$), and a significant decrease between days 5 and 6 ($p=0.044$) post-transplant. Subdivision of the non-rejector group into those with primary graft function (N=50) versus delayed graft function (N=11) (Appendix VI, Tables A.7.a & b), showed no significant changes in CXCR3 expression in the latter subgroup. However in the the subgroup with primary function, decreases (2- to 3-fold) were seen on days 1 ($p=0.001$), 2 ($p=0.003$) and 13 ($p=0.017$) compared to baseline levels and a stepwise increase (3-fold) between days 2 and 3 ($p=0.005$). Subdivision of the non-rejector group into patients with late rejection episodes (N=7) versus those without rejection episodes (N=54) (Appendix VI, Tables A.7.c & d), showed no significant differences in their CXCR3 gene expression patterns.

In the rejector group a significant decrease (1.5-fold) in CXCR3 expression occurred on day 10 ($p=0.043$) post-transplant compared to the pre-transplant base line level. With adjustment to the time of rejection diagnosis a significant increase (6-fold) in expression occurred on day 5 before rejection diagnosis ($p=0.028$), and decrease (1.5-fold) on day 2 post rejection diagnosis ($p=0.028$) compared to the pre-transplant baseline level.

In the live donor group there were no significant changes in CXCR3 expression on baseline or stepwise analysis.

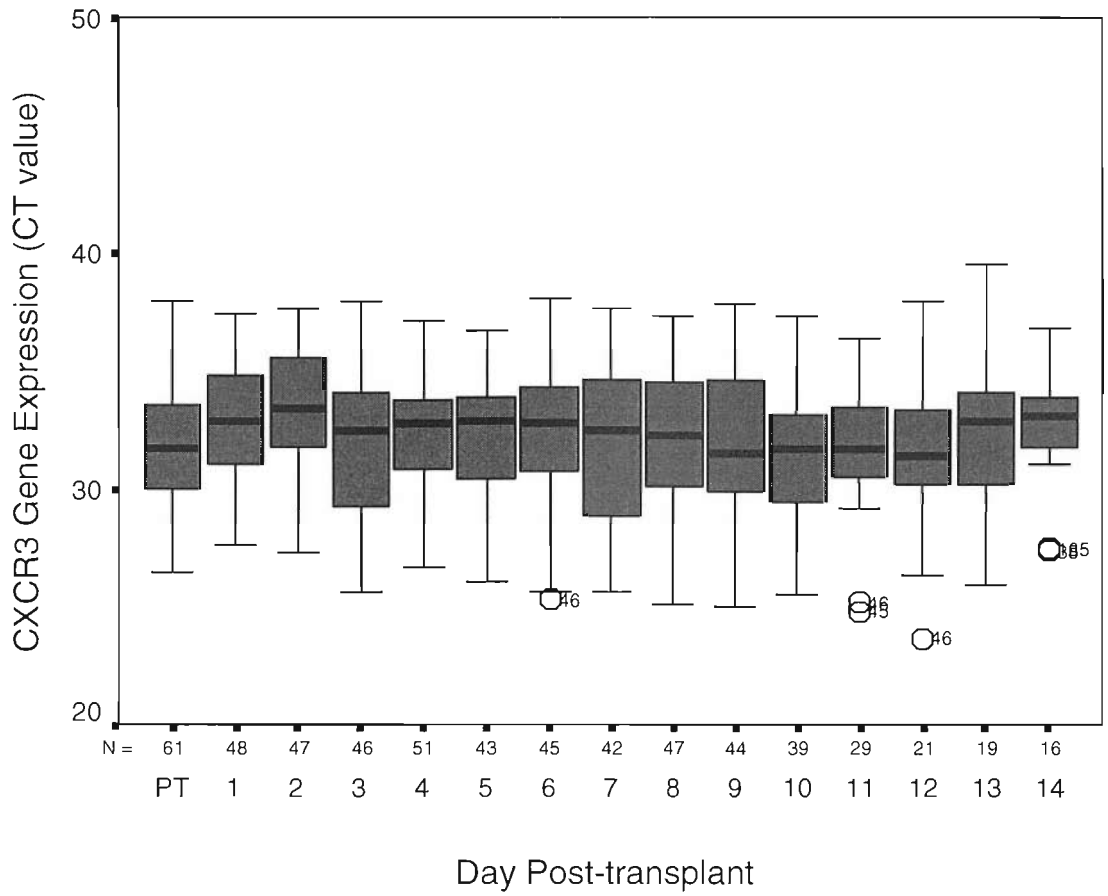

Fig. 5.7.a. Box and whisker plot showing chemokine receptor **CXCR3** gene expression levels in the **non-rejector group**. The y-axis is CXCR3 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

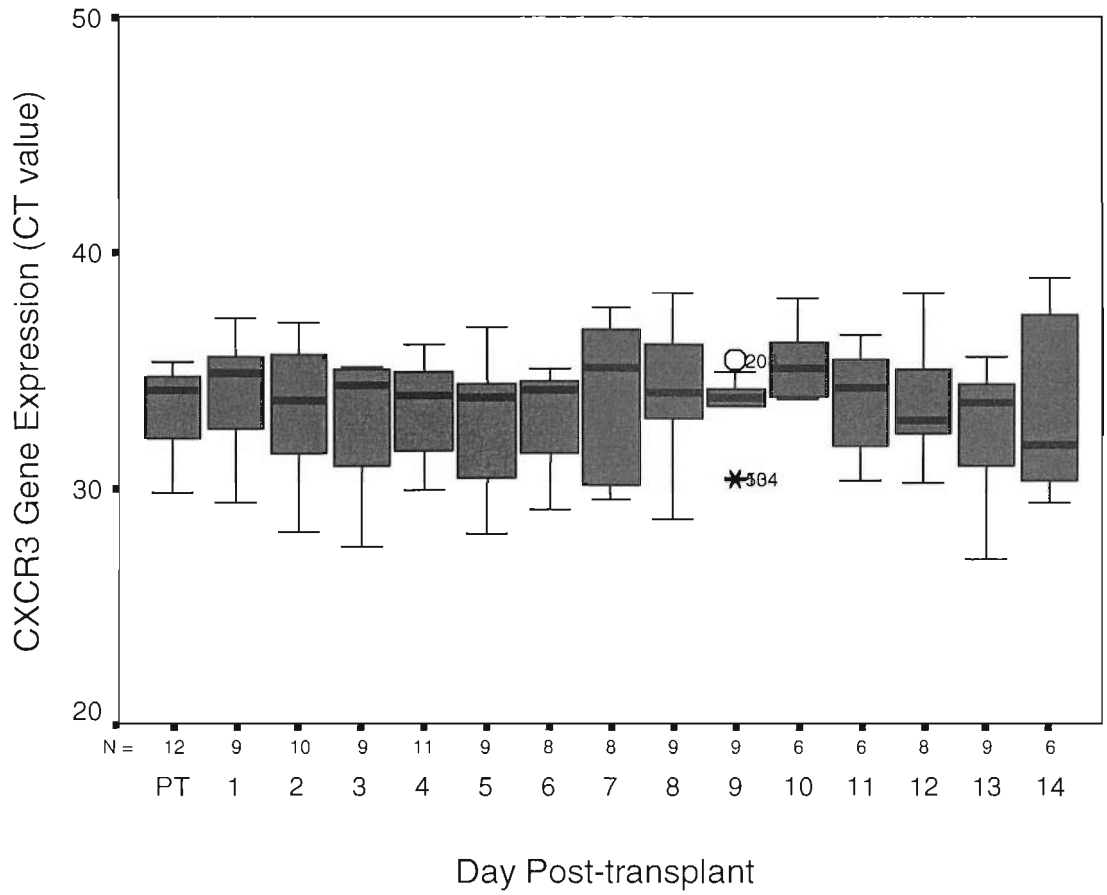

Fig. 5.7.b. Box and whisker plot showing chemokine receptor **CXCR3** gene expression levels in the **rejector group**. The y-axis is CXCR3 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-transplant with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	48	47	46	51	43	45	42	47	44	39	29	21	19	16
Direction of change	↓	↓											↓	
p-value	**<0.001**	**0.001**	0.956	0.128	0.629	0.138	0.191	0.184	0.898	0.494	0.957	0.876	**0.004**	0.255
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	48	40	40	40	36	32	33	39	38	33	23	16	16	12
Direction of change	↓		↑			↑								
p-value	**<0.001**	0.354	**0.005**	0.973	0.306	**0.044**	0.557	0.491	0.983	0.748	0.584	0.836	0.326	0.480

Table. 5.7.a. Statistical significance of **CXCR3** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	9	10	9	11	9	8	8	9	9	6	6	8	9	6
Direction of change										↓				
p-value	0.138	0.799	0.859	0.657	0.859	0.401	0.128	0.441	0.594	**0.043**	0.753	0.575	0.110	0.917
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	9	8	8	8	9	7	7	7	7	5	4	4	7	5
Direction of change														
p-value	0.138	0.176	1.000	0.889	0.515	0.176	0.612	0.866	0.866	0.225	0.068	0.144	0.498	0.345

Table. 5.7.b. Statistical significance of **CXCR3** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

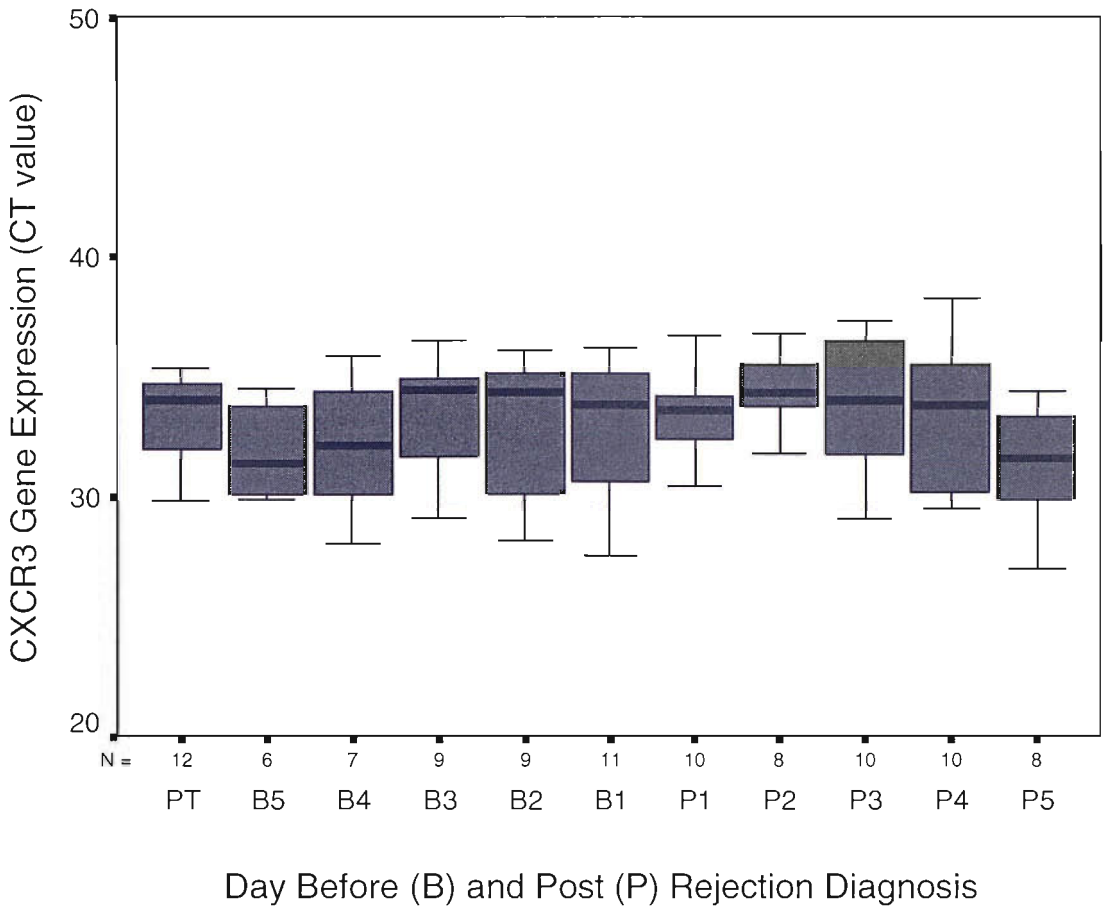

Fig. 5.7.c. Box and whisker plot showing chemokine receptor **CXCR3** gene expression levels in the **rejector group adjusted** to time of rejection diagnosis and treatment. The y-axis is CXCR3 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day before (B) and post (P) rejection diagnosis and start of anti-rejection therapy with PT being the pre-transplant baseline sample. The N number on the x-axis is the number of patient samples for each day.

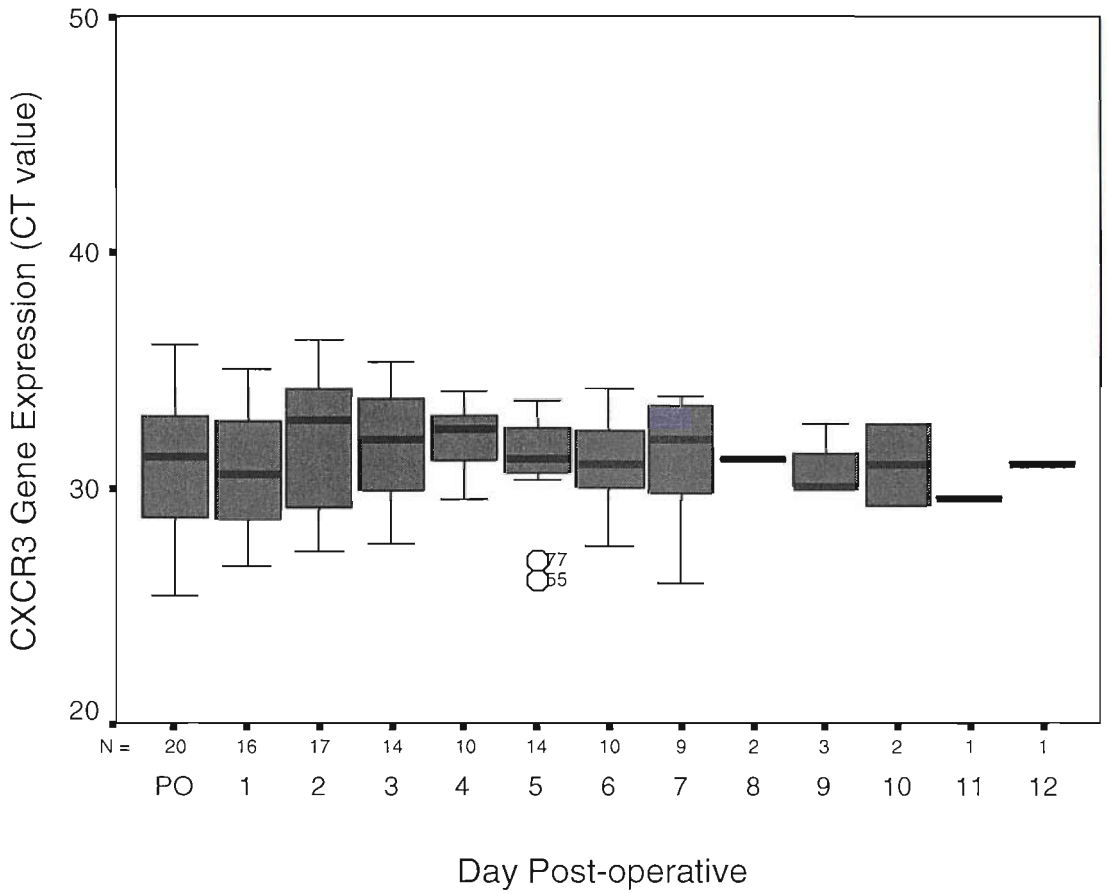

Fig. 5.7d. Box and whisker plot showing chemokine **CXCR3** gene expression levels in the **live donor group**. The y-axis is CXCR3 gene expression as the PCR threshold cycle (CT) value. The x-axis is the sample day post-operative with PO being the pre-operative baseline sample. The N number on the x-axis is the number of patient samples for each day.

Baseline	PT-B5	PT-B4	PT-B3	PT-B2	PT-B1	PT-P1	PT-P2	PT-P3	PT-P4	PT-P5
n	6	7	9	9	11	10	8	10	10	8
Direction of change	↑						↓			
p-value	**0.028**	0.310	0.594	0.889	0.859	0.221	**0.028**	0.445	0.444	0.080
Stepwise	PT-B5	B5-B4	B4-B3	B3-B2	B2-B1	B1-P1	P1-P2	P2-P3	P3-P4	P4-P5
n	6	4	6	8	9	10	8	6	9	7
Direction of change	↑									
p-value	**0.028**	0.715	0.074	0.889	0.594	0.221	0.069	0.345	0.441	0.499

Table. 5.7.c. Statistical significance of **CXCR3** gene expression changes for each day before (B) and post (P) rejection diagnosis and treatment commencement compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **rejector group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Baseline	PO-1	PO-2	PO-3	PO-4	PO-5	PO-6	PO-7	PO-8	PO-9	PO-10	PO-11
n	16	17	14	10	14	10	9	2	3	2	1
Direction of change											
p-value	0.733	0.107	0.090	0.959	0.300	0.169	0.214	0.180	0.285	0.655	-
Stepwise	PO-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11
n	16	15	12	6	5	9	6	2	2	2	1
Direction of change											
p-value	0.733	0.057	0.906	0.345	0.500	0.484	0.753	0.655	0.180	0.655	-

Table. 5.7.d. Statistical significance of **CXCR3** gene expression changes for each post-operative day compared to the pre-operative (PO) sample (baseline) and between successive days (stepwise) in the **live donor group** using the Wilcoxon rank signed test. A p-value of significance (<0.05) is highlighted and the direction of significant change shown.

Chapter 6

Discussion of Chemokine and Chemokine Receptor Gene Expression Analysis

In this chapter the results of the chemokine and chemokine receptor gene expression analysis outlined in chapter 5 are discussed, firstly individually, then followed by a general discussion.

6.1 CCL3

The CCL3 gene expression analysis results are outlined in section 5.1.
The β chemokine CCL3 has been shown to be a chemoattractant for monocytes and T lymphocytes [Wang et al, 1993; Schall et al, 1993] and expressed by macrophages, lymphocytes and endothelial cells [Adams et al, 1996; Lukacs et al, 1996; Schall et al, 1991; Zipfel et al, 1989]. A potential role in allograft rejection has been demonstrated by increased levels of expression and association with mononuclear cell infiltration during rejection [Belperio et al, 2000; Grau et al, 2000; Adams et al, 1996; Robertson et al, 2000; Segerer et al, 2001].
This study showed that CCL3 gene expression in peripheral blood mononuclear cells remained relatively constant over the immediate post-transplant and post-operative period. In the live donor and non-rejector groups, no significant changes in CCL3 expression occurred in the 14 days post-transplant compared to pre-operative / transplant baseline levels. Two significant separate stepwise changes occurred in the non-rejector group and one in the live donor group, but no clear pattern could be discerned. It seems, therefore, that CCL3 expression in PBMCs is not affected by surgery or immunosuppression in the early post transplant period.

Subdivision of the non-rejector group into primary function versus delayed graft function, and patients with late rejection episodes versus those without rejection episodes, revealed no significantly different patterns of CCL3 gene expression.

In the rejector group, no significant changes occurred on baseline or stepwise analysis, however, with adjustment to time of rejection a significant stepwise decrease in expression occurred between days 2 and 1 before rejection and a decrease on day 1 post rejection diagnosis compared to baseline. The latter decrease may reflect the anti-inflammatory effect of the first pulse of methylprednisolone. Overall no distinct pattern was discernable in the rejector group, and therefore it seems that CCL3 expression in PBMCs is not affected by allograft rejection or if there are changes in expression they are not detectable.
These results may be explained by the fact that the increased expression of CCL3 during acute allograft rejection reported in the literature may come fom the epithelial and/or endothelial cells of the allograft itself rather than infiltrating mononuclear cells. Or possibly if the mononuclear cells were a significant source then increased expression may be the result of monocyte-endothelial cell interaction as demonstrated by Lukacs et al [Lukacs et al, 1994].

6.2 CCL4

The CCL4 gene expression analysis results are outlined in section 5.2.
The β chemokine CCL4 has been shown to be a chemoattractant in vitro for T lymphocytes and monocytes [Wang et al, 1993; Schall et al, 1993] and expressed by a variety of cells including lymphocytes, endothelial cells and renal tubular epithelial cells [Zipfel et al, 1989; Robertson et al, 2000; Adams et al, 1996]. Increased expression at the mRNA and protein level has been demonstrated in rejecting allografts and following allograft reperfusion [Adams et al, 1996; Morita et al, 2001; Segerer et al, 2001; Robertson et al, 2000].

No significant changes were seen in CCL4 expression levels in the live donor group on baseline or stepwise analysis, suggesting that surgery alone does not influence PBMC CCL4 gene expression.

In the non-rejector group there was a significant decrease in CCL4 gene expression on days 6,7 and 8 post-transplant compared to pre-transplant levels and a stepwise decrease from day 5 to 6 post-transplant. A decrease in expression is also seen in the rejector group on days 8 and 9 post-transplant, however, this does not quite reach statistical significance ($p=0.055$, $p=0.050$ respectively), which may be due to the lower numbers in this group. This may reflect a subclinical immunological process. It is at around this time that acute rejection can often manifest itself and it may be, therefore, the result of a subclinical rejection process. However this is not supported by the findings in the rejector group where no significant changes in CCL4 expression were demonstrated at the time of rejection. With subdivision of the non-rejector group into patients with late rejection outside the study period and those with no episodes of rejection, it was shown that the latter group had significantly decreased CCL4 expression at this time (days 6-9 post-transplant). The late rejector subgroup had no significant changes in CCL4 expression, although this may reflect the smaller numbers in the group. It therefore seems unlikely that these changes indicate a subclinical rejection process that manifests itself clinically outside the 14 day study period.

Subdivision of the non-rejector group into patients with primary graft function and those with delayed graft function, revealed that the latter group had significant decreases in CCL4 expression on days 8, 9 and 10 post-transplant compared to pre-transplant levels, which did not occur in those with primary graft function. This suggests that delayed graft function may influence PBMC CCL4 expression, or vice versa, and may have some role in the changes in CCL4 expression seen in the non-rejector group. However the timing of the decreased CCL4 expression seen in the delayed graft function subgroup and non-rejector group do not mirror each other exactly (days 8,9,10 and 6,7,8 respectively).

This decrease in CCL4 expression may reflect sequestration of CCL4 expressing lymphocytes or monocytes into the allograft as Grim et al showed that CCL4 was

expressed by graft infiltrating cells in asymptomatic human renal allograft rejection [Grim et al, 1995].
CCL4 may have a role in T cell activation and proliferation as it has costimulatory activity in the presence of CD3 ligation, which in part seems to function by induction of CD25 expression and IL-2 production [Taub et al, 1996]. Cyclosporin inhibits IL-2 production and it may be that it also decreases CCL4 expression in PBMCs, as the majority (80%) of patients were taking cyclosporin.

This study has demonstrated no significant pattern of changes in PBMC CCL4 gene expression levels leading up to rejection and following treatment compared to baseline pre-transplant levels. There was a significant stepwise decrease in expression from day 2 to day 1 before the diagnosis and treatment of rejection, however with no significant difference with each day compared to baseline and being a single time point change, there is little one can interpret from it. From this it seems that CCL4 PBMC gene expression is not influenced by renal allograft rejection or the anti-inflammatory effects of methylprednisolone. As with CCL3, the increased expression of CCL4 during acute rejection reported in the literature may come from the epithelial and/or endothelial cells of the allograft itself rather than infiltrating mononuclear cells. Or if infiltrating lymphocytes are a prominent source of CCL4 then its up-regulation may occur at the time of infiltration into the allograft.

6.3 CCL5

The CCL5 gene expression analysis results are outlined in section 5.3.
CCL5 is a potent lymphocyte and macrophage chemoattractant and thought to play a part in lymphocyte recruitment during allograft rejection [Uguccioni et al, 1995; Schall et al, 1990; Belperio et al, 2000; Robertson et al, 2000].

In this study no significant changes were observed in CCL5 gene expression in PBMCs in the live donor or non-rejector groups comparing each time point to baseline

pre-operative or pre-transplant levels and sequentially. This suggests that surgery alone or immunosuppression does not influence PBMC CCL5 expression in this early post-transplant period.

Subdivision of the non-rejector group into primary graft function versus delayed graft function revealed no significant differences in the pattern of CCL5 expression. However, with subdivision into patients with late rejection episodes versus no rejection episodes, a significantly different pattern of CCL5 expression was demonstrated. In the subgroup with an episode of rejection outside the study period, significant decreases in expression occurred on days 2 to 6, 8 and 11 post-transplant compared to pre-transplant levels, which was not seen in the subgroup with no episodes of rejection. There seems to be a particular pattern of CCL5 expression in PBMCs in those patients that have late rejection episodes, however as we see below, no discernable pattern is evident in those under going rejection within the study period. This may represent two distinct molecular mechanisms of rejection which are also differentiated by their timing. Using microarray profiling Sarwal et al. demonstrated molecular heterogeneity in acute rejection in renal allografts [Sarwal et al, 2003]. Or maybe this subgroup represents a specific genetic population whose PBMCs are sensitive to immunosuppressive therapy and also predisposes them to late rejection episodes. It may also be that CCL5 expressing PMBCs are sequestered into the allograft leading to the decrease in PBMC CCL5 gene expression detected in this subgroup which may then influence the molecular mechanisms of late rejection. Song et al showed that in a rat renal allograft model early inhibition of CCL5 ameliorated chronic allograft nephropathy, of which infiltration by mononuclear inflammatory cells and rejection are contributing factors [Song et al, 2002].

In the non-adjusted rejector group no significant pattern of CCL5 expression was discernable, however, when the data were adjusted to the time of rejection diagnosis and anti-rejection therapy started, a significant decrease in CCL5 expression occurred on days 2 and 3 post-rejection diagnosis which correlated with the timing of the 2nd and 3rd pulses of methylprednisolone.

Glucocorticoids have been shown to decrease expression levels of other chemokines. For example, CCL2 in vitro in vascular smooth muscle cells and in vivo in ischaemic rat kidneys [Poon et al, 1991], and CXCL8 and CCL2 in rheumatoid arthritis synovial cells [Loetscher et al, 1994]. Glucocorticoids are potent anti-inflammatory agents which decrease the number of circulating monocytes in blood as well as inhibiting accumulation of monocytes and macrophages at sites of inflammation [Parillo et al, 1979; MacDonald et al, 1987]. Increased levels of CCL5 have been demonstrated in rejecting allografts and shown by immunocytochemistry to be localized to graft infiltrating mononuclear cells as well as certain graft cells [Robertson et al, 2000; Belperio et al, 2000]. Expression of CCL5 by an allograft attracts activated mononuclear cells into the graft contributing to the process of rejection. These infiltrating mononuclear cells may well produce CCL5 which contributes further to the rejection process. Here we have demonstrated a reduction in CCL5 expression in peripheral mononuclear cells on exposure to methylprednisolone and therefore this may well reduce CCL5 expression in graft infiltrating mononuclear cells and also graft cells which may be part of its anti-rejection mechanism.
As with CCL3 and CCL4, the increased expression of CCL5 during acute rejection reported in the literature may come from the epithelial and/or endothelial cells of the allograft rather than the infiltrating mononuclear cells. Or if infiltrating lymphocytes are a prominent source of CCL5 then its up-regulation may occur at the time of infiltration into the allograft.

6.4 CXCL10

The CXCL10 gene expression analysis results are outlined in section 5.4.
The α chemokine CXCL10 has chemoattractant activity for monocytes, CD4+ cells and NK cells [Taub et al, 1993; Taub et al, 1995], and evidence suggests involvement in eliciting Th1 responses [Qin et al, 1998] including acute allograft rejection [Koga et al, 1999; Hancock et al, 2000; Hancock et al, 2001; Agostini et al, 2001; Melter et al,

2001]. CXCL10 is expressed by monocytes, endothelial cells and fibroblasts [Luster et al, 1987].

In this study we demonstrated significant changes in PBMC CXCL10 gene expression in the early post-transplant period correlating with clinical events.

In the live donor group a significant decrease in CXCL10 gene expression on the 1st and 4th post-operative days occurred compared to pre-operative levels. The decrease on the 1st post-operative day may reflect the immunosuppressive effect from the stress of surgery . No changes though were seen with the other chemokines CCL3, CCL4 and CCL5, though it may be that CXCL10 expression is more sensitive to such influences. Englehardt et al demonstrated increased CXCL10 expression correlating with lymphocyte accumulation, in human wound healing in vivo, which occurred at day 4 after wounding. There was no significant increased expression of CCL3, CCL4 or CCL5 during wound healing [Englehardt et al, 1998]. It may be that the decrease in PBMC expression of CXCL10 on day 4 in this study correlates to changes observed by Englehardt et al occurring in the surgical wound at day 4. One hypothesis, if there is a correlation, is that CXCL10 expressing lymphocytes are sequestered into the post-operative wound resulting in the observed decrease in expression in the PBMCs.

In the non-rejector group there was a significant decrease in expression of CXCL10 on the 1st and 2nd day post-transplant. As well as the possible effect of surgery, this may reflect the anti-inflammatory effect of methylprednisolone given at the time of transplantation. No other significant changes were seen during the study period in this group. Subdivision of the non-rejector group into those with primary graft function versus delayed graft function and patients with late rejection episodes versus those without rejection episodes revealed no significantly different patterns of CXCL10 gene expression. There seems to be no correlation therefore between PBMC CXCL10 expression and delayed graft function, or with its early expression and late episodes of rejection.

In the rejector group, significant increases in expression were seen on days 2 to 5 and 13 post-transplant. With adjustment to the time of rejection there was an increase in

expression in the five days prior to rejection diagnosis, 3 of which were significant and 1 was borderline significant (p=0.050), compared to baseline. Anti-rejection therapy significantly decreased CXCL10 expression bringing levels back to baseline values on the 3 days following pulse treatment, but then significantly increased again compared to baseline. These results show a significant difference between the rejector and non-rejector groups, with a decrease in CXCL10 expression in the latter group immediately following transplantation, and the rejector group with increases in expression following transplantation and prior to rejection diagnosis.

CXCL10 expression is induced by the Th1 cytokine IFN-γ [Farber et al, 1997; Vaguri et al, 1990; Ohmori et al, 1993] whose expression is associated with allograft rejection [Thai et al, 1995; Zuo et al, 1995; McLean et al, 1997; Kaminski et al, 1995]. It seems possible therefore, to suggest that Th1 infiltrating cells producing IFN-γ during allograft rejection may influence CXCL10 expression in circulating PBMCs. Kaminski et al investigated IFN-γ expression in mononuclear cells taken from patients prior to renal transplantation. They showed that mononuclear cells from patients who subsequently developed acute cellular rejection secreted higher levels of IFN-γ than those who had no rejection episodes, by stimulation with anti-CD3 monoclonal antibody in vitro. They also showed that although cyclosporin suppressed IFN-γ secretion, there was no difference in sensitivity to suppression between rejectors and non-rejectors [Kaminski et al, 1995]. This higher level of IFN-γ expression in mononuclear cells from patients who subsequently have rejection episodes may also explain the increased CXCL10 expression levels observed in the rejector group following transplantation and when adjusted to time of rejection diagnosis.

Anti-rejection therapy with methylprednisolone influenced CXCL10 gene expression bringing levels back to baseline values however, levels significantly increased again, showing only a transient association.

The exact causation and location of CXCL10 upregulation remains speculative. It seems likely that the changes seen prior to rejection diagnosis occur at the time of T lymphocyte activation by alloantigen in the grafts and draining lymph nodes with Th1 polarisation and IFN-γ upregulation. The increased expression seen from day 2 post-transplant in the rejector group may result from local exposure to IFN-γ, produced by

graft infiltrating cells, as the PBMCs pass through the graft or alternatively this may reflect a more systemic effect.
However, in our laboratory, Tan et al did not demonstrate an increase in IFN-γ gene expression in T cells in patients experiencing acute rejection. In fact they found an early reduction in expression in patients who subsequently went on to experience acute rejection [Tan et al, 2001]. It may be that PBMC IFN-γ expression is not a significant inducer of CXCL10, but rather the mononuclear cells (or the mediators produced by them) infiltrating the graft are. It may also be that IFN-γ is not the major influence on PBMC CXCL10 expression.

These results show that PBMC CXCL10 gene expression has potential for use in immunomonitoring of renal allografts as significant differences were demonstrated between rejectors and non-rejectors and temporally, at the time of rejection.

6.5 CCR1

CCR1 is expressed on a large number of leukocyte populations [Su et al, 1996], its expression is influenced by cytokines [Bonecchi et al, 1999; Loetscher et al, 1996; Polentarutti et al, 1997; Sozzani et al, 1998] and has been shown to be associated with mononuclear cell infiltration and allograft rejection in animal models [Belperio et al, 2000; Gao et al, 2000; Horak et al, 2001].
In this study, significant changes in CCR1 gene expression in peripheral blood have been demonstrated correlating with clinical events in the early post transplant period.

In the live donor group a significant increase in PBMC CCR1 gene expression occurred on days 1 and 2 post-operatively compared to baseline pre-operative levels. This demonstrates that surgery alone may increase PBMC CCR1 gene expression or increase the proportion of mononuclear cells in the peripheral blood that are CCR1 expressors.

In the non-rejector group there was also an increase in CCR1 gene expression in the few days immediately following surgery. This may reflect the effect of surgery or the initial influence of immunosuppressive therapy. This immediate post-transplant rise in CCR1 expression is more likely to be due to surgery than result from immunosuppressive therapy though, as levels soon return to pre-transplant levels, and the same immediate change is seen in the live donor group in the absence of immunosuppression. Also the immunosuppressive, methylprednisolone, which has been shown to reduce CCR1 expression (see adjusted rejector group, section 5.5 and below), and is given at the time of transplantation, does not seem to counteract this rise. However, in the rejector group, there is no significant immediate change in CCR1 expression following surgery. This may be a reflection of the smaller number in the rejector group, and therefore ability to reach statistical significance, as on visual inspection of the graph there is a small increase at this time.

The significant increase in CCR1 expression following surgery is prolonged in the non-rejector group up to day 5 compared to the live donor group where levels return to baseline on day 3 post surgery. This may reflect the immunological influence of re-perfusion injury of the allograft and possibly delayed graft function. Neutrophils have a crucial role in ischaemic reperfusion injury of organs [Romson et al, 1983] and their expression of CCR1 is an important factor in this. Experiments using knock-out mice lacking CCR1, showed that they were completely protected against ischaemic reperfusion injury to kidney and liver. There was a lack of neutrophils in the ischaemic tissue preventing injury and also infiltration by T cells and macrophages [Pratschke et al, 1999]. However, this increase in CCR1 expression observed is not due to neutrophils as this study was carried out on PBMCs, but it might be that there is also an influence on T cell and macrophage CCR1 expression due to ischaemic reperfusion injury.

Subdivision of the non-rejector group into those with primary function and those with delayed graft function, revealed both subgroups had significant increases in CCR1 expression in the immediate days post-transplant. The primary function subgroup had significant increases on days 1, 3 and 5 post-transplant and the delayed graft function

subgroup on days 2 and 4 post-transplant, compared to baseline. Delayed graft function does not seem to influence this early increase in CCR1 expression.
In the non-rejector group, there were significant increases in CCR1 expression on days 9 and 10 as well as the immediate increases post-transplant. This may reflect a subclinical immunological process occurring at this time, and it can be hypothesised whether this correlates with initial graft function or rejection outside of the 14 day period studied. To partly address this, the non-rejector group was subdivided into those with rejection episodes outside of the 14 day study period and those without any rejection episodes. The late rejector subgroup showed no significant changes in CCR1 expression post-transplant, but this may reflect the smaller number in this group. However, with subdivision into those with primary versus delayed graft function, those with delayed graft function showed significant increases in CCR1 expression on days 8 and 9 post-transplant compared to the pre-transplant baseline, that were not seen in the primary function subgroup. Therefore there seems to be a correlation between delayed graft function and PBMC CCR1 expression at this time.

In the non-adjusted rejector group, there were significant increases in CCR1 expression on days 5 and 13 compared to pre-transplant levels. However when the data were adjusted to the time of rejection diagnosis and commencement of anti-rejection therapy, a clear pattern emerged. There were significant increases in CCR1 expression in days 4, 3 and 1 prior to rejection diagnosis compared to pre-transplant baseline levels, which returned back to baseline levels with anti-rejection pulse therapy (methylprednisolone). Stepwise analysis showed a significant decrease in CCR1 expression after the first methylprednisolone pulse and significant increase in expression following the 3rd and last pulse. This shows a clear correlation between increased PBMC CCR1 expression and rejection which responds to anti-rejection therapy by returning to pre-transplant levels.
This increased gene expression of CCR1 at the time of allograft rejection may reflect an actual increase in gene expression by a proportion of the peripheral mononuclear cells or an increase in a particular subset that are higher expressors of CCR1 compared to other subsets, or both.

Monocytes which represent the precursors of macrophages and dendritic cells express CCR1 and are sequestered into areas of delayed type hypersensitivity inflammation, such as allograft rejection [Sallusto et al, 1998]. It may be that increased numbers are released into the blood from the bone marrow in response to the rejection process, influencing the overall PBMC CCR1 gene expression level.

Allograft rejection is associated with Th1 lymphocyte activation and infiltration into the graft [Strom et al, 1996]. However, CCR1 is expressed equally in both Th1 and Th2 cells [Bonecchi et al, 1998]. It has been demonstrated that with addition of IFN-α at the time of T cell polarisation, a dramatic increase in CCR1 expression occurs [Sallusto et al, 1998]. Therefore, Th1 cells polarised in the presence of IFN-α are able to respond to CCR1 ligands. CCL3 and CCL5, two ligands for CCR1, are produced by rejecting allografts. It may be the influence of cytokines, such as IFN-α, produced by the immunological processes of rejection that contribute to this observed increase in CCR1 expression.
Addition of transforming growth factor β (TGF-β) at the time of T cell polarisation had the opposite effect reducing CCR1 expression [Sallusto et al, 1998]. TGF-β is an anti-inflammatory cytokine and increased levels have been associated with acute rejection in transplanted kidneys [Cohen et al, 1998]. Gibbs et al using the same method as this study demonstrated an increase in TGF-β expression in PBMC at the time of rejection in human renal transplant patients [Gibbs et al, 2001]. TGF-β therefore, seems unlikely to be a major influence on CCR1 expression in PBMCs in vivo, unless it is acting to reduce elevated CCR1 expression levels.

The Th1 cytokine IL-2 can also influence CCR1 expression. Loetscher et al demonstrated an increase in CCR1 expression in CD45RO+ memory lymphocytes on exposure to IL-2 [Loetscher et al, 1996]. Tan et al demonstrated a reduction in IL-2 gene expression in T cells using RT-PCR ELISA at the time of rejection in renal transplant patients [Tan et al, 2001]. However, Loetscher et al showed that increased CCR1 expression was associated with an increase in IL-2 receptor α subunit

expression. The α subunit receptor is a high affinity receptor and therefore changes in this may influence CCR1 expression rather than the IL-2 expression level per se.

These results provide evidence for a role of CCR1 in human allograft rejection. Most evidence previously has come from animal allograft models and in humans indirectly from the correlation between increased expression of its ligands CCL3 and CCL5 in biopsy material from rejecting allografts compared to normal controls [Pattison et al, 1994; Yun et al, 2001; Belperio et al, 2000; Robertson et al, 2000; Segerer et al, 2001; Oliveira et al, 1997].
Segerer et al, in fact, showed no difference in CCR1 expression in rejecting human renal allografts compared to normal kidneys. However they used GAPDH as a house keeping gene and we have shown in this study that GAPDH gene expression increases in PBMCs at the time of rejection demonstrating it should not be used for this purpose in this model. Using the ribonuclease protection assay as the method of gene expression analysis, Segerers' group did not identify the cellular source of CCR1 expression.

Increased CCR1 expression in PBMCs at the time of rejection is consistent with the evidence that its ligands CCL3 and CCL5 are expressed at elevated levels in a rejecting allograft. This may result from the influence of inflammatory cytokines present at the time of activation or polarisation to Th1, allowing the CCR1 expressing peripheral mononuclear cells to enter the allograft enhancing the immunological process of rejection.
Anti-rejection therapy with methylprednisolone correlates with reduced CCR1 expression in PBMCs back to baseline values. This may be one of its anti-inflammatory modes of action, since by reducing CCR1 expression, there may be less mononuclear cell infiltration into the graft with the effect of dampening down the rejection process.
Penton-Rol et al showed that the glucocorticoid, dexamethasone, did not substantially affect CCR1 or CCR5 mRNA expression levels in mononuclear cells unlike CCR2 expression, which was upregulated. This correlated with increased responsiveness of

dexamethasone treated monocytes to CCL2 (CCR2 ligand) and no change in responsiveness to CCL4 (CCR5 ligand) [Penton-Rol et al, 1999]. However these workers measured mRNA expression by Northern blot analysis which is a lot less sensitive than quantitative RT-PCR and therefore may not detect the changes observed in this work. Also their in vitro work only exposed the monocytes to four hours of dexamethasone whereas our patients received three doses over three days.

These results show that CCR1 gene expression in PBMCs has the potential for use in immunomonitoring due to significant rises at the time of rejection which subsequently fall with anti-rejection therapy.

6.6 CCR5

The chemokine receptor CCR5 is expressed on peripheral blood-derived dendritic cells [Granelli-Piperno et al, 1996; Rubbert et al, 1998] and activated / memory Th1 lymphocytes [Bleul et al, 1997; Loetscher et al, 1998], and is associated with Th1 inflammatory reactions [Qin et al, 1998] including allograft rejection [Belperio et al, 2000; Goddard et al, 2001; Segerer et al, 1999; Eitner et al, 1998; Segerer et al, 2001; Fischereder et al, 2001].
In this study we demonstrated few significant changes in PBMC CCR5 gene expression in the early post-transplant period, while in the live donor group there were no significant changes in CCR5 gene expression showing that surgery does not seem to have any influence.

In the non-rejector group a significant decrease in CCR5 expression occurred on days 6,7 and 13 post-transplant compared to the pre-transplant baseline value. A similar phenomenon was observed with CCL4, which showed a decrease in expression on days 6,7 and 8 post-transplant in the non rejector group and may also reflect a subclinical immunological process. On subdividing the non-rejector group into those with and without delayed graft function, a decrease at the same time period occurred

in the primary function group but not in the group with delayed graft function. This may reflect the smaller numbers in the delayed graft function group. The same occurred when subdividing into those patients who rejected late outside the study period and those who did not, with those in the latter group being smaller in number and not showing decreased CCR5 expression at days 6 and 7 post-transplant. It seems therefore that the changes in CCR5 expression seen at this time do not correlate with delayed graft function or late episodes of rejection.

In the rejector group, no significant pattern of CCR5 gene expression occurred sequentially post-transplant or when adjusted to time of rejection. Single time point changes occurred at day 2 post-transplant and day 5 before rejection with increases in expression compared to baseline, but their p values only just reached significance (0.047 and 0.046 respectively). A significant stepwise decrease occurred between day 1 and 2 post-rejection diagnosis and may reflect the effect of anti-rejection therapy given at that time.
Although CCR5 is expressed on Th1 lymphocytes and is associated with allograft rejection, no pattern of PBMC expression was observed in this work, particularly at the time of rejection. It may be that CCR5 expression by infiltrating mononuclear leukocytes within the graft during rejection [Eitner et al, 1998; Segerer et al, 2001] is not reflected in PBMC expression.

6.7 CXCR3

The chemokine receptor CXCR3 is expressed by activated Th1 lymphocytes [Sallusto et al, 1998] and associated with Th1 inflammatory reactions [Qin et al, 1998] including allograft rejection [Miura et al, 2001; Hancock et al, 2000; Goddard et al, 2001; Agostini et al, 2001; Melter et al, 2001].
Few significant changes in PBMC CXCR3 gene expression occurred in the early post-transplant period or showed correlation with clinical events.
In the live donor group, no significant changes in CXCR3 expression occurred, indicating surgery has little effect on expression. In the non-rejector group there was a

significant decrease in CXCR3 expression on days 1 and 2 post-transplant on pre-transplant baseline analysis and this may reflect the effect of immunosuppression, as surgery itself seems to have little effect. The expression profile in this group has a similar pattern to its ligand CXCL10 which also showed decreased expression on days 1 and 2 post-transplant. It may be that their expression levels are linked, however this was not seen in the rejector group. On subdividing the non-rejector group, a decrease in CXCR3 expression occurred on days 1 and 2 post-transplant in the primary graft function subgroup but not in the delayed graft function subgroup, however this may reflect the smaller numbers in the latter subgroup. No significant difference in pattern was seen between the late rejector subgroup and the subgroup with no episodes of rejection.

In the rejector group, no significant pattern of changes in CXCR3 gene expression occurred sequentially post-transplant or when adjusted to time of rejection. A stepwise decrease in expression occurred between days 1 and 2 post rejection diagnosis and this may reflect the effect of anti-rejection therapy given at this time.
The expression pattern of CXCR3 in the rejector group was similar to that of CCR5, another Th1 cell associated chemokine receptor. As with CCR5, the expression of CXCR3 within an allograft may not be reflected in PBMC expression.

6.8 General Discussion

This work has demonstrated significant changes in selected chemokine and chemokine receptor gene expression in PBMCs by daily sequential measurement in the early renal post-transplant period, that correlate with allograft rejection. Table 6.1 summarises the significant findings for the changes in expression levels of the chemokines and their receptors in the rejector group adjusted to the time of rejection. Both CXCL10 and CCR1 have shown increased expression prior to rejection diagnosis and therefore may have a predictive role in detecting rejection. The magnitude of change, in the median average, seen in the five days prior to rejection diagnosis

Time Point	B5	B4	B3	B2	B1	P1	P2	P3	P4	P5
CCL3	NS	NS	NS	NS	NS	↓0.041	NS	NS	NS	NS
CCL4	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
CCL5	NS	NS	NS	NS	NS	NS	↓0.013	↓0.015	NS	NS
CXCL10	↑0.028	NS	↑0.050	↑0.046	↑0.028	NS	NS	NS	↑0.028	↑0.018
CCR1	NS	↑0.026	NS	↑0.01	↑0.01	NS	NS	NS	NS	NS
CCR5	↑0.046	NS	NS	NS	NS	NS	NS	NS	NS	NS
CXCR3	↑0.028	NS	NS	NS	NS	NS	↓0.028	NS	NS	NS

Table 6.1 Changes in chemokine and chemokine receptor gene expression in the days before (B) and post (P) rejection diagnosis. P value compares each time point with pretransplant baseline (Wilcoxon matched-pairs signed-ranks test). NS- Not significant (p>0.05)
↓decreased expression ↑increased expression

compared to pre-transplant levels, was an increase in CCR1 expression in the range of 4 to 12-fold, and 16 to 32-fold increase for CXCL10. The sensitivity (true positive rate) in detecting an increase in CCR1 expression post-transplant compared to pre-transplant levels, prior to rejection, was 66% on day 5 (i.e. 66% of changes detected in those with rejection were increases in expression), and between 73% and 87% on days 4 to 1 before rejection. With CXCL10, the sensitivity in detecting an increase in its expression post-transplant compared to pre-transplant levels, in the five days prior to rejection was between 71% and 100%. However, the specificity (100 minus false positive rate in non rejector group) for CCR1 expression predicting rejection was low, at between 28% and 48% (i.e. only 28-48% of CCR1 changes detected in the non rejector group were decreases in expression i.e. true negatives), when looking at the 14 days post-transplant compared to pre-transplant levels in the non-rejector group. For CXCL10 this specificity was between 45% and 74%, looking at the 14 days post-transplant compared to pre-transplant levels in the non-rejector group. This shows a similar sensitivity in detecting these changes prior to rejection for both CXCL10 and CCR1 but poor specificity, especially with CCR1.

The magnitude of increase from baseline seen in some of the non-rejector group was of the same order as that seen in the rejector group prior to rejection for both CXCL10 and CCR1. This indicates that the rejectors do not have uniformly greater rises in CXCL10 and CCR1 expression than the non-rejectors.

Other studies have also found changes in peripheral blood cellular gene expression, that have correlated with rejection episodes in human renal transplantation. In this laboratory Tan et al and Gibbs et al have shown changes in cytokine gene expression in PBMCs that have correlated with acute rejection episodes [Tan et al, 2001; Gibbs et al, 2001]. Rukavina et al demonstrated increased perforin protein (a cytolytic molecule expressed in granules of cytolytic T cells and NK cells) expression in lymphocytes which correlated with rejection in human kidney transplant recipient [Rukavina et al, 1996].

Changes in gene or protein expression levels may correlate with immunological events within a graft, but how much do they reflect the cellular events within the graft infiltrating cells? A detected decrease in PBMC gene expression of a particular gene at the time of allograft rejection can be interpreted as sequestration of cells that are high expressors of that gene into the allograft at the time of rejection, or as an actual overall decrease in PBMC expression.

We have shown increased expression of CXCL10 and CCR1 at the time of rejection in PBMCs which correlates well with other studies showing increased expression within a rejecting allograft [Koga et al, 1999; Hancock et al, 2000; Hancock et al, 2001; Agostini et al, 2001; Melter et al, 2001; Belperio et al, 2000; Gao et al, 2000; Horak et al, 2001]. However, we showed no major changes in CCL3, CCL4, CCL5 or CXCR3 and CCR5 expression patterns at the time of rejection but other studies have shown increased expression within a rejecting allograft [Belperio et al, 2000; Grau et al, 2000; Adams et al, 1996; Robertson et al, 2000; Segerer et al, 2001; Miura et al, 2001; Agostini et al, 2001; Hancock et al, 2000]. The cellular source of the gene expression in these studies is often not identified. As far as CCL3, CCL4 and CCL5 are concerned, when the cellular source was identified it was the endothelium or epithelium of the allograft, and is associated with an inflammatory cell infiltrate. It may well be that the graft itself is the major source of these chemokines seen at the time of rejection rather than the inflammatory cell infiltrate.

The receptors CXCR3 and CCR5 are both preferentially expressed on Th1 cells and shown to be expressed by infiltrating lymphocytes in allograft rejection [Qin et al, 1998;

Miura et al, 2001; Agostini et al, 2001; Hancock et al, 2000; Belperio et al, 2000; Goddard et al, 2001; Segerer et al, 1999; Fischereder et al, 2001]. A change in expression levels of these receptors would therefore have been predicted, either due to sequestration of the receptor expressing lymphocytes into the graft or increased expression reflecting events within the graft. Both CXCR3 and CCR5 have similar expression patterns in the rejector group, with little significant change around the time of rejection other than an increase at day 5 before rejection diagnosis, which was not highly significant ($p=0.028$ and $p=0.046$ respectively). However, the number in the CCR5 and CXCR3 rejector groups were small (12 pre-transplant samples compared to 20 in the CCR1 group) which may be a factor in the lack of demonstrated significant change. Visually, there is an increase in CCR5 expression (corresponding to decreased median CT values) before rejection diagnosis that returns to baseline values with anti-rejection therapy. With CXCR3, visually an increase in expression occurred on days 4 and 5 before rejection diagnosis and equal to the baseline values in the 3 days before rejection diagnosis (reflected by changes in the median CT values). Therefore it may be that actual changes were detected but these did not achieve statistical significance due to the small numbers of samples analysed or a failure of using an approach without a means of accurate standardisation.

As previously mentioned we have shown some association with PBMC chemokine and chemokine receptor gene expression in this study, and their intragraft expression demonstrated in other studies, but how much does PBMC gene expression reflect infiltrating mononuclear cell gene expression? Gorezynski et al went some way to answering this question by comparing cytokine gene expression simultaneously in peripheral blood and in biopsies from orthotopic liver transplants with rejection. They found that although some differences were seen in the frequency of cytokine gene expression, a good correlation was found between their intragraft and PBMC cytokine expression profiles. The Th1 cytokines IL-2 and IFN-γ were found in PBMC and biopsies from patients with acute rejection. They concluded that this suggested that similar lymphocyte subpopulations regulating graft rejection were predominant both locally (intragraft) and peripherally (PBMC) [Gorezynski et al, 1996]. However the

biopsy samples contain allograft cells and maybe inflammatory cells other than mononuclear cells eg.neutrophils which may also be a source of cytokine mRNA transcripts.

In the live donor group significant changes in expression were seen only with CCR1 and CXCL10, and that the constant expression seen with CCL3, CCL4, CCL5, CCR5 and CXCR3 suggests that changes seen are real and not an artefact of the method used. The significant increase in expression seen with CCR1 on the first two post-operative days and decrease in CXCL10 expression on the first post-operative day in the live donor group is also reflected in the non-rejector group with increases in CCR1 and decreases in CXCL10 expression in the immediate post-transplant days. As previously mentioned the decrease in CXCL10 may be due to immunosuppression (from the surgery and immunosuppressive therapy) and increase in CCR1 due to monocyte and dendritic cell response to the inflammation of surgery and more prolonged with ischaemic/reperfusion injury to the allograft.
Anti-rejection therapy with methylprednisolone given at the time of rejection seems to decrease expression in the PBMCs of all the chemokines and chemokine receptors in this study except for CCL4. This is in keeping with the general anti-inflammatory effect that steroids have, as the chemokines and chemokine receptors studied in this work are considered to be "inflammatory" as they are involved in inflammatory responses, in contrast to the constitutive chemokines and their receptors (eg. CXCL12 / CXCR4 and CCL21 / CCR7).

We have also demonstrated in this work that changes in chemokine and chemokine receptor gene expression in PBMCs may have some correlation with delayed graft function. Significant decreases in CCL4 expression on days 8, 9 and 10, and increases in CCR1 expression on days 8 and 9 post-transplant were seen in patients with delayed graft function, which did not occur in those with primary graft function. This suggests that delayed graft function may influence PBMC CCL4 and CCR1 expression, or vice versa.

Chapter 7

Summary and Conclusion

7.1 Summary

The principal aim of this work was to determine whether sequential monitoring of PBMC chemokine ligand/receptor gene expression in a renal transplant recipient reflects the clinical effects of the immunological processes within the allograft, the main immunological process of interest being rejection.

The chemokine ligands and their receptors investigated in this work, namely the ligands CCL3, CCL4, CCL5 and CXCL10 with their receptors CCR1, CCR5 and CXCR3, were chosen due to evidence for their role, and demonstrated presence in, allograft rejection. They have all been shown to be involved in chemoattractant activity of lymphocytes or monocytes or both, and are thought to influence migration of these cells into rejecting allografts. However this may not be their predominant activity as some have been shown to influence T-cell activation, proliferation and angiogenesis which may influence allograft rejection and function.

Renal transplant recipients and donor nephrectomy patients were recruited into the study and their PBMC target gene expression levels determined on a daily sequential basis for up to 14 days. Gene expression levels were measured using real-time PCR TaqMan technology. When considering groups of patients (i.e. rejectors and non-rejectors) statistically significant changes in some of the chemokine ligand/receptor gene expression levels occurred which correlated closely with clinical events. In the live donor group, increased expression of CCR1 and decreased CXCL10 expression occurred in the immediate days post-operatively. This increased CCR1 expression was prolonged in the non-rejector group, suggesting CCR1 PBMC gene expression is influenced by surgery and possibly ischaemic/reperfusion injury. Anti-rejection therapy in the form of methylprednisolone decreased CCR1 expression as

demonstrated in the 'adjusted' rejector group. Methylprednisolone is given at the time of transplant but this does not negate the increase seen post-transplant in the non-rejector group. The smaller number in the rejector group may be the reason no significant change is seen in this group immediately post-transplant, as on graph inspection there is a small increase in expression at this time. The decrease in CXCL10 in the first post-operative day in the live donor group is also reflected in the non-rejector group but lasting until day 2 post-transplant and may reflect the immunosuppression effects of surgery and initial immunosuppressive therapy. The CXCL10 expression profiles showed a clear difference between the rejector and non-rejector groups with significant decreases in expression in the first 2 post-transplant days in the non-rejector group, compared to significant increases in CXCL10 expression in the 2^{nd} to 5^{th} days post-transplant in the rejector group. As aluded to in chapter 6 this may reflect higher expression of CXCL10 (or IFN-γ) in a patient group, predisposing them to rejection.

In the rejector group significant increases in gene expression of both CCR1 and CXCL10 occurred at the time of rejection, which responded to anti-rejection therapy by returning to baseline pre-transplant levels. This suggests that these two genes may be useful in PBMC gene expression immunomonitoring to detect allograft rejection. To improve on the sensitivity and specificity of these PBMC gene expression markers in detecting allograft rejection, their analysis could be combined together with the cytokine genes, identified by Tan et al and Gibbs et al in this laboratory, with potential in PBMC immunomonitoring, namely IL-4, IL-5, IL-13 and TNF-α [Tan et al, 2001; Gibbs et al, 2001]. A select panel of PBMC gene expression markers may then be used for immunomonitoring in this early human renal post-transplant period.

The second aim of this work was to test a selection of possible candidate genes that could be used as endogenous controls for this gene expression analysis work. This would help standardise against the variability within the method, for example the reverse transcription reaction, making the method more robust. Making adjustments for this variability would improve the chances of picking up any significant changes in

gene expression levels. This may allow achievement of inter-sample comparisons in a series of samples from a single patient, or from between patients.

Five genes were selected due to studies showing that they were stably expressed in leukocytes or stably expressed during T cell differentiation, namely GAPDH, MLN51, YWHAZ, EF-1α and UbcH5B. The results of the endogenous control gene expression analysis showed that there were significant changes in expression levels of all five genes at various points post-transplantation when considering groups of patients. They therefore seemed to be influenced by either renal transplantation, rejection or both and some by surgery itself. This makes them unsuitable for use as endogenous control genes in this gene expression model.

Interestingly, GAPDH and UBCH5B expression correlated well with rejection, with increased expression several days before rejection diagnosis. Treatment with anti-rejection therapy then reduced their expression levels back to baseline. This suggests that they may have a potential role in immunomonitoring over the early transplant period, rather than as endogenous control genes, and should not be used as such despite reports in the literature.

However, despite not identifying and using an endogenous control gene to standardise this work, significant patterns of change in some target genes were identified that correlated with clinical events. The greater the variability within the method, the less likely the chance of changes detected being statistically significant, especially with limited numbers. Significant changes were still detected despite the 'noise' in the system and actual correlates with clinical events may be stronger than those demonstrated in this study. Also the results of 3 out of 5 of the endogenous control genes in this study and 3 from the study by Gibbs et al [Gibbs et al, 2003], which showed no significant changes in gene expression in the live donor group, gives added evidence for the robustness of this method and validity for use in post-transplant monitoring. However the variability within the method, as outlined in chapter 4, must have a limiting affect on the detection of target gene expression changes and their reaching statistical significance. This variability will also influence the specificity and sensitivity of any changes being correlated to clinical events.

Therefore identification and use of a method of standardisation in this gene expression analysis model would ultimately improve its validity.

7.2 Conclusion

This work on sequential monitoring of chemokine and chemokine receptor gene expression in human PBMCs in the early renal post-transplant period, has demonstrated changes in CCR1 and CXCL10 gene expression that closely correlate with clinical events. This correlation of expression with rejection and response to anti-rejection therapy suggests that CCR1 and CXCL10 are potential candidates for PBMC gene expression immunomonitoring over the transplant period. This work also may provide further understanding of the complex role of chemokines and their receptor expression around the time of allograft transplantation and rejection.

The search for an endogenous control gene for use in the gene expression analysis model used in this work was unsuccessful, with significant changes in expression of all five genes tested at some point post-transplantation. In fact, the close correlation of GAPDH and UbcH5B gene expression with rejection and their response to anti-rejection therapy, suggests that they may be potential candidates for PBMC gene expression immunomonitoring over the transplant period.

7.3 Suggestions for further work

This work has demonstrated, as has previous work in this laboratory, the validity of sequential monitoring of PBMC gene expression in the early post-transplant period. It has also shown gene expression changes which may be of immunological and clinical relevance that would not be readily detectable in single time point studies often described in the literature.

A drawback with this work was the limited number of patients who experienced rejection within the study period. To substantiate this work further, greater numbers of patients with rejection need to be analysed. A way to achieve this would be to extend the study period up to six weeks post-transplant, in which time period the majority of rejection episodes occurred.

Further analysis of the chemokine and chemokine receptor gene expression levels, correlating them with the patients' immunosuppressive regimes may show some correlation. This may have a potential in immunosuppressive monitoring and may identify particular patient groups whose gene expression levels are positively or negatively correlated to a particular immunosuppressive regime that predisposes them to clinical events, for example, rejection.

Simultaneous monitoring of gene expression within the allograft and in the peripheral blood would give a greater understanding of the relationship between gene expression in the sequestered inflammatory cells and the those circulating in the peripheral blood. A confounding factor with this however, is the allograft tissue present in the biopsy with its gene transcripts, unless the infiltrating inflammatory cells can somehow be separated or gene expression targeted to be occurring within them. Also extracting mRNA from paraffin embedded tissues, while achievable, is more problematic, and tissues must be fixed quickly if mRNA degradation is to be avoided. This simultaneous monitoring would greatly improve our ability to interpret peripheral blood gene expression changes.

It is possible to obtain a target gene expression level from a peripheral blood sample within the 8 hour period of a working day with the appropriate laboratory set up. This would enable clinical action to be taken, if necessary, upon the result of a gene expression analysis from blood taken the same day. Therefore identifying a panel of markers whose change or relative change in expression levels had the specificity and sensitivity to pick up the immunological changes of rejection would be highly desirable.

This would allow diagnosis by taking a peripheral blood sample rather than invasive core biopsy of an allograft with its risks, as is done at present.
To shorten the laboratory time and simplify the procedure used in this work, a one step RNA extraction method from peripheral blood could be employed (Quiagen). However it must be noted that this would also extract RNA from neutrophils in addition to the mononuclear cells and may therefore alter expression patterns.

An important improvement in the reliability of gene expression analysis, is to be able to standardize the process. Endogenous control genes are often used, however this work and previous work in this laboratory have been unable to identify a suitable candidate so far. Two other possible candidates genes that could be tested in this gene expression model are RNA polymerase II and G6PDH (glucose 6 phosphate dehrdrogenase). Radonic et al. tested a selection of putative reference genes in a human T-cell line treated with TPA and ionomycin, which increased IL-2 transcription, but found RNA polymerase II and G6PDH transcription to be stable [Radonic et al, 2004].
It may be that a suitable endogenous control gene does not exist for this gene expression model, and therefore another method of standardisation is required. One such method, may be by using real-time PCR and expressing target gene expression as the copy number per microgram of cDNA, as described by Whelan et al. Real-time PCR products are cloned into plasmids and then used to calibrate unknown samples avoiding the use of endogenous control genes [Whelan et al, 2003].

Using DNA microarray technology, which is able to study the simultaneous gene expression of thousands of genes, Sarwal et al showed molecular heterogeneity in acute renal allograft rejection by profiling allograft biopsies [Sarwal et al, 2003]. It would be interesting to use this technology to profile PBMC gene expression in the post transplant period, and may in the future be a routine test in immunomonitoring and predicting or making the diagnosis of allograft rejection.

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Appendix I

Consent Form

I agree to be a patient in the MD project being carried out by Mr Richard Dalton and Miss Joanne Webber at Queen Alexandra Hospital, Portsmouth.

I understand that this will involve me having extra blood samples taken, on top of the routine ones, both before and after my operation.

I have agreed to participate in the study of my own free will and have had all my questions answered to my satisfaction.

I understand that I can withdraw at any stage without my subsequent treatment and care being affected in any way.

Signed: .....

Date: .....

Appendix II

Blood collection, Cell separation and RNA stabilisation.

Consumables:

1. Vacutainer Tubes Sodium Citrate 4.5ml
2. Phosphate buffered saline [HMS, UK] (PBS) without calcium and magnesium (IOx) [Life Technologies UK]
3. Lymphoprep, specific gravity 1.077g/l [Nycomed UK]
4. Sterile 50ml skirted conical-based polypropylene tubes [Greiner Labortechnik UK]
5. Sterile 15ml conical-based polypropylene tubes [Greiner Labortechnik UK]
6. Sterile 3.5ml Pasteur pipettes [Greiner Labortechnik UK]
7. Sterile 10ml serological pipettes [Greiner Labortechnik UK]
8. RNAzolB [Biogenesis UK]
9. Sterile 1.5ml Eppendorfs [Greiner Labortechnik UK]

Equipment:

1. MSE Mistral 3000i centrifuge [Sanyo UK]
2. Microflow pathfinder laminar flow cabinet [MDH UK]

Appendix III

RNA extraction, quantification, DNase treatment and standardisation

Consumables

1. Chloroform [Merck UK]
2. Isopropanol [Merck UK]
3. RNase free waters [Sigma]
4. 75% ethanol – prepared by dilution of absolute ethanol [Jones Burroughs UK] with RNase free water
5. Sterile glass Pasteur pipettes [Greiner Labortechnik UK]
6. Sterile 1.5ml Eppendorfs [Greiner Labortechnik UK]
7. Sterile pipette tips of various sizes [Greiner Labortechnik UK]
8. Deoxyribonuclease 1, RNase-free [Sigma].

Equipment:

1. Heraeus Sepatec contifuge 17RS centrifuge [Heraeus UK]
2. Windsor incubator set at 37°C [Sandrest UK]
3. Whirlimixer [Jencons Scientific UK]
4. Water bath, set at 55°C and 65°C [Grant Instruments UK]
5. Gene Quant RNA/DNA Calculator [Pharmacia UK]
6. Ultramicrovolume cell [Pharmacia UK]
7. Pipettes of various ranges [Gilson, Anachem, UK; Eppendorf, Merck, UK; Biokit, Alpha Laboratories UK].

Appendix IV

First-strand cDNA synthesis.

Consumables:

1. First-strand cDNA Synthesis [Pharmacia Biotech UK]
2. RNase-Free water [Sigma]
3. Sterile 1.5ml and 0.75ml Eppendorfs [Greiner Labortechnik UK].

Equipment:

1. Amplirad UV cabinet [Gene Research Instrumentation UK]
2. Laminar airflow cabinet [MDH UK]
3. Pipettes of various ranges [Gilson, Anachem UK; Eppendorf, Merck; UK, Biokit, Alpha Laboratories UK]
4. Hybaid thermal reactor [Hybaid UK]
5. Whirlimixer [Jencons Scientific UK]
6. Water bath, set at 37°C [Grant Instruments UK].

Appendix V

TaqMan gene expression analysis.

Consumables:

1. 384-Well Clear Optical Reaction Plate [Applied Biosystems UK]
2. Optical Adhesive covers [Applied Biosystems UK]
3. Sterile pipette tips of various sizes [Greiner Labortechnik UK]
4. Sterile 1.5ml and 5ml Eppendorfs [Greiner Labortechnik UK]
5. TaqMan Universal PCR Master Mix [Applied Biosystems UK]
6. RNase-Free water [Sigma]

Equipment:

1. ABI Prism 7900 HT Sequence Detector [Applied Biosystems UK] previously calibrated using Sequence Detection System Spectral Calibration Kit [Applied Biosystems UK]
2. Pipettes of various ranges [Gilson, Anachem, UK; Eppendorf, Merck UK; Biokit, Alpha Laboratories UK]
3. Automated Multidispense pipette [Rainin Instrument Co UK]
4. Sorval® Legend T Centrifuge [Kendro Laboratory Products UK]

Appendix VI

Tables showing statistical analysis of non-rejector group subdivided into patients with primary function and delayed graft function (Tables a and b), and patients with an episode of rejection outside the study period and those that did not (Tables c and d), for each chemokine and chemokine receptor

Table 1 - CCL3
Table 2 - CCL4
Table 3 - CCL5
Table 4 - CXCL10
Table 5 - CCR1
Table 6 - CCR5
Table 7 - CXCL10

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	46	47	46	46	44	44	43	40	39	32	27	17	17	14
Direction of change														
p-value	0.068	0.204	0.563	0.657	0.197	0.192	0.438	0.202	0.161	0.688	0.394	0.796	0.136	0.258
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	46	40	39	39	35	36	34	34	33	27	19	15	11	11
Direction of change						↓		↑						
p-value	0.068	0.819	0.151	0.302	0.719	**0.017**	0.602	**0.014**	0.411	0.191	0.421	0.865	0.477	0.824

Table. A.1.a Statistical significance of **CCL3** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with primary graft function (n=57).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	10	11	9	8	5	10	11	10	11	10	6	8	6	5
Direction of change														
p-value	0.767	0.859	0.767	1.000	0.893	0.202	0.424	0.153	0.155	0.214	0.463	0.401	0.116	0.345
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n														
Direction of change														
p-value	0.068	0.507	0.953	0.398	0.593	0.068	0.260	0.441	0.575	0.678	0.753	0.715	0.345	0.068

Table. A.1.b Statistical significance of **CCL3** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the non-**rejector group with delayed graft function (n=12)**. The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	51	53	51	48	45	50	50	44	47	37	29	24	20	15
Direction of change														
p-value	0.114	0.332	0.373	0.492	0.238	0.072	0.301	0.107	0.089	0.588	0.449	0.648	0.097	0.865
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	51	46	44	42	35	36	40	40	40	33	22	18	16	12
Direction of change						↓		↓						
p-value	0.114	0.840	0.236	0.291	0.617	**0.008**	0.510	**0.006**	0.214	0.197	0.527	0.744	0.326	0.433

Table. A.1.c Statistical significance of **CCL3** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with no episodes of rejection outside the study period (n=62).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	5	5	4	6	4	4	4	6	3	5	4	1	3	4
Direction of change														
p-value	0.225	0.225	0.465	0.465	1.000	0.715	0.715	0.600	1.000	0.500	0.273	-	0.285	0.715
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	5	4	4	4	3	4	3	3	3	3	3	1	1	3
Direction of change														
p-value	0.225	0.465	0.715	0.715	1.000	0.465	0.109	1.000	0.285	0.285	0.109	-	-	0.109

Table. A.1.d Statistical significance of **CCL3** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with an episode of rejection outside the study period (n=7).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	47	46	45	46	48	45	47	46	42	31	27	18	18	15
Direction of change													↓	
p-value	0.362	0.149	0.857	0.270	0.701	0.071	0.061	0.057	0.193	0.439	0.829	0.943	**0.045**	0.069
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	47	41	39	38	39	39	37	42	38	30	17	15	13	12
Direction of change						↓								
p-value	0.362	0.379	0.414	0.243	0.451	**0.018**	0.820	0.317	0.528	0.141	0.356	0.198	0.133	0.209

Table. A.2.a Statistical significance of **CCL4** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with primary graft function (n=57).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	10	11	9	8	5	10	11	10	11	10	6	8	4	3
Direction of change								↓	↓	↓				
p-value	0.285	0.477	0.086	0.093	0.500	0.086	0.286	**0.007**	**0.004**	**0.009**	0.249	0.063	0.273	0.285
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	10	10	9	7	3	4	9	9	10	9	6	4	4	2
Direction of change														
p-value	0.285	0.646	0.109	0.866	1.000	0.273	0.594	0.374	0.594	0.859	0.463	0.715	0.465	0.180

Table. A.2.b Statistical significance of **CCL4** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with delayed graft function (n=12)**. The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	52	52	50	48	47	51	53	49	49	36	29	24	19	14
Direction of change						↓	↓	↓	↓					
p-value	0.356	0.377	0.919	0.246	0.430	**0.012**	**0.022**	**0.023**	**0.044**	0.106	0.681	0.615	0.019	0.048
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	52	47	44	41	37	39	43	46	44	35	20	17	15	11
Direction of change						↓								
p-value	0.356	0.731	0.972	0.268	0.126	**0.006**	0.965	0.388	0.587	0.355	0.823	0.215	0.036	0.110

Table. A.2.c Statistical significance of **CCL4** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with no episodes of rejection outside the study period (n=62).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	5	5	4	6	6	4	5	7	4	5	4	2	3	4
Direction of change														
p-value	0.080	0.138	0.273	0.249	0.249	0.715	0.893	0.310	1.000	0.500	0.273	0.655	1.000	1.000
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	5	4	4	4	5	4	3	5	4	4	3	2	2	3
Direction of change														
p-value	0.080	0.465	0.715	1.000	0.080	0.465	0.109	0.500	0.068	0.273	0.109	0.655	0.655	0.593

Table. A.2.d Statistical significance of **CCL4** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with an episode of rejection outside the study period (n=7).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	61	58	58	56	59	60	60	58	52	41	39	27	25	22
Direction of change														
p-value	0.659	0.390	0.561	0.582	0.083	0.968	0.347	0.425	0.778	0.271	0.955	0.674	0.657	0.948
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	61	54	52	49	48	53	53	54	48	38	28	24	19	19
Direction of change										↑				
p-value	0.659	0.337	0.613	0.205	0.173	0.136	0.301	0.530	0.886	**0.031**	0.101	0.627	0.687	0.629

Table. A.3.a Statistical significance of **CCL5** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with primary graft function (n=69).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	10	9	8	7	5	10	10	10	10	9	5	8	7	6
Direction of change														
p-value	0.959	0.327	0.484	0.463	0.500	0.386	0.838	1.000	0.953	0.374	0.500	0.123	0.310	0.600
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	10	9	7	6	3	4	9	9	10	8	5	4	7	5
Direction of change														
p-value	0.959	0.889	0.063	0.345	1.000	1.000	0.678	0.374	0.646	0.484	0.686	0.854	0.091	0.225

Table. A.3.b Statistical significance of **CCL5** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with delayed graft function (n=11).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	65	61	61	56	57	63	64	60	57	44	39	32	29	24
Direction of change					↑									
p-value	**0.971**	0.641	0.766	0.870	**0.005**	0.691	0.529	0.944	0.741	0.347	0.322	0.132	0.619	0.875
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	65	58	54	50	45	50	56	57	53	41	29	25	23	21
Direction of change					↑									
p-value	**0.971**	**0.429**	0.256	0.153	**0.040**	0.261	0.194	0.378	0.668	0.260	0.256	0.609	0.107	0.590

Table. A.3.c Statistical significance of **CCL5** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with no episodes of rejection outside the study period (n=72).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	6	6	5	7	7	7	6	8	5	6	5	3	3	4
Direction of change		↓	↓	↓	↓	↓		↓			↓			
p-value	**0.116**	**0.043**	**0.043**	**0.028**	**0.028**	**0.043**	0.345	**0.025**	0.138	0.917	**0.043**	0.109	0.109	1.000
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	**6**	5	**5**	5	6	7	6	6	5	5	4	3	3	3
Direction of change														
p-value	**0.116**	0.416	0.686	0.500	0.173	0.237	0.116	0.917	0.225	0.138	0.144	1.000	0.593	0.285

Table. A.3.d Statistical significance of **CCL5** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with an episode of rejection outside the study period (n=8).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	37	35	40	41	34	36	35	35	35	28	23	13	11	9
Direction of change	↓	↓												
p-value	**0.002**	**0.041**	0.885	0.257	0.851	0.525	0.258	0.446	0.961	0.374	0.988	0.507	0.657	0.484
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	37	29	31	33	29	26	27	29	29	25	16	11	8	7
Direction of change	↓									↑	↓			
p-value	**0.002**	0.116	0.122	0.416	0.611	0.367	0.882	0.804	0.554	**0.032**	**0.034**	0.689	1.000	1.000

Table. A.4.a Statistical significance of **CXCL10** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with primary graft function (n=49).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	10	9	7	7	4	8	9	10	10	10	6	7	7	6
Direction of change	↓		↓			↓								
p-value	**0.013**	0.343	**0.028**	0.128	1.000	**0.043**	0.594	0.359	0.333	0.475	0.917	0.735	0.499	0.600
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	10	9	7	5	2	3	6	9	10	9	6	4	7	5
Direction of change	↓	↑												↑
p-value	**0.013**	**0.021**	0.063	0.080	0.655	0.109	0.116	0.484	0.053	0.066	0.917	0.465	0.237	**0.043**

Table. A.4.b Statistical significance of **CXCL10** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the non-**rejector group with delayed graft function (n=11).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	41	40	42	42	34	41	40	39	43	33	26	19	16	12
Direction of change	↓													
p-value	**0.001**	0.074	0.722	0.365	0.905	0.199	0.288	0.786	0.777	0.427	0.485	0.171	0.278	0.814
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	41	35	35	34	27	26	31	35	37	32	20	14	14	10
Direction of change	↓	↑												
p-value	**0.001**	**0.017**	0.481	0.980	0.866	0.128	0.854	0.675	0.621	0.224	0.126	0.660	0.221	0.074

Table. A.4.c Statistical significance of **CXCL10** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with no episodes of rejection outside the study period (n=53).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	6	4	5	6	4	3	4	6	2	5	3	1	2	3
Direction of change	↓		↓											
p-value	**0.046**	0.273	**0.043**	0.173	0.465	1.000	0.273	0.600	0.655	0.225	0.109	-	0.655	0.655
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	6	3	3	4	4	3	2	3	2	2	2	1	1	2
Direction of change	↓													
p-value	**0.046**	0.285	1.000	0.715	0.715	1.000	0.180	1.000	0.655	0.180	0.655	-	-	0.655

Table. A.4.d Statistical significance of **CXCL10** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with an episode of rejection outside the study period (n=7).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	53	51	49	49	52	50	52	53	46	35	31	21	20	17
Direction of change	↑		↑		↑									
p-value	**<0.001**	0.170	**<0.001**	0.276	**0.017**	0.118	0.249	0.547	0.134	0.116	0.096	0.135	0.411	0.795
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	53	47	44	41	41	44	44	48	44	32	22	19	14	14
Direction of change	↑		↑											
p-value	**<0.001**	0.397	**0.030**	0.079	0.211	0.339	0.327	0.482	0.357	0.350	0.685	0.277	0.363	0.826

Table. A.5.a Statistical significance of **CCR1** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with primary graft function (n=61).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	11	9	9	8	5	10	11	11	11	10	6	8	7	6
Direction of change		↑		↑				↑	↑					
p-value	0.182	**0.015**	0.086	**0.036**	0.225	0.333	0.286	**0.041**	**0.008**	0.114	0.249	0.362	0.237	0.600
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	11	9	7	7	3	4	9	10	11	9	6	4	7	5
Direction of change		↑											↓	↑
p-value	0.182	**0.015**	0.128	0.866	0.593	0.068	0.515	0.386	0.689	0.678	0.345	1.000	**0.018**	**0.043**

Table. A.5.b Statistical significance of **CCR1** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with delayed graft function (n=12).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	58	54	54	50	50	54	57	56	52	39	32	27	24	19
Direction of change	↑	↑	↑	↑	↑	↑	↑		↑	↑	↑	↑		
p-value	**<0.001**	**0.008**	**<0.001**	**0.020**	**0.002**	**0.023**	**0.043**	0.058	**0.005**	**0.043**	**0.007**	**0.008**	0.721	0.809
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	58	51	47	44	38	42	48	52	50	36	24	21	19	16
Direction of change	↑												↓	
p-value	**<0.001**	0.711	0.104	0.067	0.215	0.258	0.562	0.219	0.183	0.759	0.361	0.289	**0.008**	0.535

Table. A.5.c Statistical significance of **CCR1** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with no episodes of rejection outside the study period (n=65).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	6	6	4	7	7	6	6	8	5	6	5	2	3	4
Direction of change														
p-value	0.600	0.249	0.465	0.866	0.176	0.173	0.345	0.327	0.686	0.463	0.345	0.180	0.285	0.715
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	6	5	4	4	6	6	5	6	5	5	4	2	2	3
Direction of change														
p-value	0.600	0.138	0.465	0.715	0.600	0.463	0.893	0.600	0.345	0.225	0.068	0.655	0.180	1.000

Table. A.5.d Statistical significance of **CCR1** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with an episode of rejection outside the study period (n=8).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	31	28	28	33	30	26	24	25	25	25	19	8	9	8
Direction of change						↓								
p-value	0.199	0.110	0.716	0.372	0.271	**0.010**	0.057	0.288	0.628	0.893	0.809	0.236	0.214	1.000
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	31	25	22	25	27	22	18	18	19	21	14	7	6	6
Direction of change						↓								
p-value	0.199	0.459	0.745	0.757	0.542	**0.008**	0.089	0.486	0.658	0.218	0.778	0.499	0.345	0.345

Table. A.6.a Statistical significance of **CCR5** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with primary graft function (37).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	9	10	8	7	4	8	9	10	10	10	6	7	7	6
Direction of change			↓											
p-value	0.260	0.541	**0.035**	0.398	0.465	0.263	0.260	0.646	0.859	0.575	0.674	0.866	0.091	0.917
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	9	9	8	6	2	3	6	9	10	9	6	4	7	5
Direction of change			↓										↓	
p-value	0.260	0.086	**0.025**	0.249	0.180	0.285	0.463	0.859	0.646	0.484	0.753	0.715	**0.043**	0.225

Table. A.6.b Statistical significance of **CCR5** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with delayed graft function (n=11).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	35	34	32	35	30	31	30	30	33	31	22	14	14	11
Direction of change						↓	↓							
p-value	0.096	0.416	0.556	0.408	0.465	**0.009**	**0.022**	0.544	0.674	0.799	0.897	0.064	0.096	0.959
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	35	31	27	28	25	22	22	25	27	28	18	10	12	9
Direction of change						↓							↓	
p-value	0.096	0.652	0.394	0.973	0.520	**0.003**	0.140	0.375	0.866	0.395	0.983	0.878	**0.005**	0.086

Table. A.6.c Statistical significance of **CCR5** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with no episodes of rejection outside the study period (n=42).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	5	4	4	5	4	3	3	5	2	4	3	1	2	3
Direction of change														
p-value	0.893	0.465	0.715	0.138	0.144	0.109	1.000	0.138	0.655	0.144	0.285	-	0.180	1.000
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	5	3	3	3	4	3	2	2	2	2	2	1	1	2
Direction of change														
p-value	0.893	0.285	1.000	0.285	0.144	0.593	0.180	0.157	0.655	0.655	0.655	-	-	0.655

Table. A.6.d Statistical significance of **CCR5** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with an episode of rejection outside the study period (n=6).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	39	40	41	44	39	37	33	37	34	29	23	14	13	11
Direction of change	↓	↓											↓	
p-value	**0.001**	**0.003**	0.795	0.227	0.548	0.338	0.427	0.308	0.745	0.863	0.891	0.730	**0.017**	0.328
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	39	33	35	36	34	29	27	30	28	24	17	12	10	8
Direction of change	↓		↑											
p-value	**0.001**	0.296	**0.005**	0.677	0.163	0.057	0.326	0.871	0.829	0.830	0.831	0.754	0.646	0.401

Table. A.7.a Statistical significance of **CXCR3** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with primary graft function (n=50).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	9	7	5	7	4	8	9	10	10	10	6	7	6	5
Direction of change														
p-value	0.139	0.128	0.500	0.176	0.715	0.069	0.139	0.386	0.202	0.241	0.600	0.735	0.116	0.500
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	9	7	5	4	2	3	6	9	10	9	6	4	6	4
Direction of change														
p-value	0.139	0.866	0.715	0.068	0.180	0.593	0.600	0.213	0.721	0.953	0.249	0.715	0.345	1.000

Table. A.7.b Statistical significance of **CXCR3** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with delayed graft function (n=11).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	43	41	42	44	37	41	39	40	41	34	25	20	17	13
Direction of change	↓	↓											↓	
p-value	**<0.001**	**0.010**	0.900	0.455	0.792	0.340	0.105	0.510	0.964	0.467	0.657	0.575	**0.006**	0.311
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	43	36	36	36	30	28	31	36	35	30	20	15	15	10
Direction of change	↓		↑											
p-value	**<0.001**	0.838	**0.016**	0.994	0.133	0.062	0.226	0.169	0.961	1.000	0.502	0.820	0.125	0.285

Table. A.7.c Statistical significance of **CXCR3** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with no episodes of rejection outside the study period (n=54).** The direction of any significant ($p<0.05$) change is shown.

Baseline	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11	PT-12	PT-13	PT-14
n	5	6	4	7	6	4	3	7	3	5	4	1	2	3
Direction of change		↓		↓										
p-value	0.225	**0.028**	0.715	**0.043**	0.075	0.068	0.109	0.063	0.593	0.893	0.273	-	0.655	0.593
Stepwise	PT-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
n	5	4	4	4	6	4	2	3	3	3	3	1	1	2
Direction of change														
p-value	0.225	0.068	0.144	0.715	0.345	0.465	0.180	0.109	1.000	0.593	1.000	-	-	0.180

Table. A.7.d Statistical significance of **CXCR3** gene expression changes for each day post-transplant compared to the pre-transplant (PT) sample (baseline) and between successive days (stepwise) in the **non-rejector group with an episode of rejection outside the study period (n=7).** The direction of any significant ($p<0.05$) change is shown.