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Genetic aspects of outcome in kidney transplantation: cytokine and thrombosis associated candidate genes and gene expression biomarkers

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ACADEMIC DISSERTATION

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TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS 6

ABBREVIATIONS 7

ABSTRACT 9

INTRODUCTION 11

REVIEW OF THE LITERATURE 12

1 KIDNEY TRANSPLANTATION 12
- 1.1 General aspects of kidney transplantation 12
- 1.2 Genetic aspects of end stage renal disease 13
- 1.3 Human leukocyte antigen 14
  - 1.3.1 Basic features of human leukocyte antigen biology 14
    - 1.3.1.1 Immunogenetics of the human leukocyte antigen system 14
    - 1.3.1.2 Antigen processing and presentation 17
    - 1.3.1.3 Human leukocyte antigen and T cell selection 18
    - 1.3.1.4 Interaction between specific human leukocyte antigen molecules and peptides 19
  - 1.3.2 Clinical histocompatibility and human leukocyte antigen matching 19
- 1.4 Complications in kidney transplantation 20
  - 1.4.1 Acute rejection 21
    - 1.4.1.1 Immune systems involved in rejection 22
    - 1.4.1.2 Allorecognition 23
    - 1.4.1.3 Tolerance 24
  - 1.4.2 Delayed graft function 24
  - 1.4.3 Cytomegalovirus infection 25
  - 1.4.4 Vascular complications 25
- 1.5 Genes associated with acute rejection, delayed graft function, cytomegalovirus and vascular complications 26
  - 1.5.1 Cytokine genes 27
    - 1.5.1.1 Tumor necrosis factor 29
    - 1.5.1.2 Transforming growth factor beta 1 29
    - 1.5.1.3 Interleukin 10 29
    - 1.5.1.4 Interleukin 6 30
    - 1.5.1.5 Interferon gamma 30
  - 1.5.2 Thrombosis-associated genes 31
    - 1.5.2.1 Coagulation factor V 31
    - 1.5.2.2 Coagulation factor II 31
    - 1.5.2.3 Methylenetetrahydrofolate reductase 32
    - 1.5.2.4 Protein C, tissue factor pathway inhibitor, coagulation factor XIII 32
  - 1.5.3 Other genes studied in renal transplantation 34
- 1.6 Methods and biomarkers studied by these methods for diagnosing acute rejection 34

1.6.1 Histology 35
1.6.2 Enzyme-linked immunoabsorbent assay 36
1.6.3 Fluorescence activated cell sorting 37
1.6.4 Mass spectrometry 37
1.6.5 TaqMan real-time quantitative polymerase chain reaction 38
1.6.6 Microarray 38

2 AIMS OF THE STUDY 42

3 PATIENTS 43

4 ETHICAL PERMITS 43

5 METHODS 44
5.1 Isolation of DNA (I, II, IV) 44
5.2 Human leukocyte antigen typing (I) 44
5.3 Determination of single nucleotide polymorphisms (I, II, IV) 44
5.4 Isolation, quantification and validation of RNA from whole blood (III, V) 45
5.5 Real-time quantitative polymerase chain reaction (III, V) 45
5.6 Low-density array (V) 47
5.7 Microarray (V) 47
5.8 Statistical analyses (I–V) 48

6 RESULTS AND DISCUSSION 49
6.1 Acute rejection (I, IV) 49
6.1.1 Only a few gene polymorphisms were associated with acute rejection (I) 50
6.1.2 Genetic variation in thrombosis-associated or cytokine genes did not associate with AR (IV) 52
6.2 Genetic variation in interleukin 10 but not in other cytokine genes predisposed to CMV infection (II) 53
6.3 Genetic variation in thrombosis-associated or cytokine gene polymorphism did not predispose to thrombosis (IV) 56
6.3.1 Thrombosis-associated or cytokine gene polymorphisms did not predispose to infarction (IV) 57
6.3.2 Thrombosis-associated or cytokine gene polymorphisms were not associated with graft survival 58
6.4 Gene expression markers showed similar time-related variance during hospital stay in control patients (III) 59
6.5 The expression of two genes was different in rejection patients than in control patients (III) 61
6.6 Rejection patients differed from non-rejection patients with regard to one gene expression marker (III) 63
6.7 Candidate gene expression markers did not differ between patients with subclinical rejection and control patients (V) 64
6.8 Gene expression markers of the low-density array were unable to identify patients with subclinical rejection (III) 65
6.9 A robust biomarker specific for subclinical rejection could not be identified in whole genome microarray analysis (III) 66

STRATEGIES FOR THE FUTURE 68
CONCLUSIONS 69
ACKNOWLEDGEMENTS 70
REFERENCES 71
ORIGINAL PUBLICATIONS 95

LIST OF ORIGINAL PUBLICATIONS

This thesis consists of the following original publications, referred to in the text by Roman numerals (I–V).

I Alakulppi NS, Kyllönen LE, Jäntti VT, Matinlauri IH, Partanen J, Salmela KT, Laine JT. Cytokine gene polymorphisms and risks of acute rejection and delayed graft function after kidney transplantation. Transplantation. 2004 Nov 27;78(10): 1422–8.

II Alakulppi NS, Kyllönen LE, Salo HM, Partanen J, Salmela KT, Laine JT. The impact of donor cytokine gene polymorphisms on the incidence of cytomegalovirus infection after kidney transplantation. Transpl Immunol. 2006 Nov;16(3-4):258–62.

III Alakulppi NS, Kyllönen LE, Partanen J, Salmela KT, Laine JT. Diagnosis of acute renal allograft rejection by analyzing whole blood mRNA expression of lymphocyte marker molecules. Transplantation. 2007 Mar 27;83(6):791–8.

IV Alakulppi NS, Kyllönen LE, Partanen J, Salmela KT, Laine JT. Lack of association between thrombosis associated and cytokine candidate gene polymorphisms and acute rejection or vascular complications after kidney transplantation. Nephrol Dial Transplant. 2007 Aug 17; [Epub ahead of print].

V Alakulppi NS, Seikku P, Jaatinen T, Holmberg C, Laine JT. Feasibility of diagnosing subclinical renal allograft rejection in children by whole blood gene expression analysis. Submitted.

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ABBREVIATIONS

ADCC	antibody-dependent cell cytotoxicity
APC	antigen-presenting cell
AR	acute rejection
CD3E	CD3e molecule, epsilon (CD3-TCR complex)
CD40LG	CD40 ligand (CD154)
CI	confidence interval
CIT	cold ischemia time
CMV	cytomegalovirus
CNF	congenital nephrotic syndrome of the Finnish type (NPHS1)
CTLA4	cytotoxic T-lymphocyte-associated protein 4
dbSNP	database of single nucleotide polymorphisms www.ncbi.nlm.nih.gov/SNP/
DC	dendritic cell
DGF	delayed graft function
DN	diabetic nephropathy
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
ESRD	end stage renal disease
F2	coagulation factor II
F5	coagulation factor V
F13A1	coagulation factor XIII
FASLG	fas ligand (FL)
GZMB	granzyme B (GB)
GNLY	granulysin (Gra)
GS	graft survival
GWA	genome wide association
HLA	human leukocyte antigen
HR	hazard ratio
ICOS	inducible T cell co-stimulator
IFNG	interferon gamma
Ig	immunoglobulin
IL6	interleukin 6
IL10	interleukin 10
LD	linkage disequilibrium
LTA	lymphotoxin alpha

MALAT1	metastasis associated lung adenocarcinoma transcript 1
MHC	major histocompatibility complex
MTHFR	methylenetetrahydrofolate reductase
NK	natural killer cell
OR	odds ratio
PBL	peripheral blood leukocyte
PDCD1	programmed cell death 1 (PD-1)
PRF1	perforin (Per)
PROC	protein C
RNA	ribonucleic acid
ROC	receiver operating characteristic
RT-QPCR	real-time quantitative polymerase chain reaction
SCR	subclinical rejection
T1D	type I diabetes
T2D	type II diabetes
TFPI	tissue factor pathway inhibitor
TGFB1	transforming growth factor beta 1
TNF	tumor necrosis factor
Tx	transplantation

ABSTRACT

Kidney transplantation (Tx) is the treatment of choice for end stage renal disease. Immunosuppressive medications are given to prevent immunological rejection of the transplant. However, immunosuppressive drugs increase e.g. the risk of infection, cancer or nephrotoxicity. A major genetic contributors to immunological acceptance of the graft are human leukocyte antigen (*HLA*) genes. Also other non-*HLA* gene polymorphisms may predict the future risk of complications before Tx, possibly enabling individualised immunotherapy.

Graft function after Tx is monitored using non-specific clinical symptoms e.g. fever and swelling of graft and laboratory markers e.g. creatine/cystatin-c measurement. Definitive diagnosis of graft rejection, however relies on an invasive core needle biopsy of the graft. In acute rejection (AR) diagnostics there is a need for an alternative to biopsy that would be an easily repeatable and simple method for regular use. Frequent surveillance of acute or subclinical rejection (SCR) may improve long-term function. This may be especially important in SCR, where by definition there are no clinical symptoms or indications from laboratory tests.

In this thesis associations between cytokine and thrombosis associated candidate genes and the outcome of kidney Tx were studied. Cytotoxic and co-stimulatory T lymphocyte molecule gene expression biomarkers that might be useful for the diagnosis of the clinical and subclinical forms of rejection were also investigated. We found that polymorphisms in the cytokine genes tumor necrosis factor (*TNF*) and interleukin 10 (*IL10*) of the recipients were associated with AR. However, the *TNF* association was not observed in a further study implying inflated odds ratios and p-values used in the power analysis of the further study based on results from first study. A *TNF* gene polymorphism of the donors was associated with delayed graft function. In addition, certain *IL10* gene polymorphisms of the donors were associated with the incidence of cytomegalovirus infection and occurrence of later infection in a subpopulation of recipients. Further, polymorphisms in genes related to the risk of thrombosis and those of certain cytokines were not associated with the occurrence of thrombosis, infarction, AR or graft survival.

In the study of biomarkers for AR, whole blood samples were prospectively collected from 50 adult kidney Tx patients. With real-time quantitative PCR (RT-QPCR) gene expression quantities of CD154 and

ICOS genes differentiated the patients with AR from those without, but not from the patients with other causes of graft dysfunction.

Biomarkers for SCR were studied in 31 paediatric kidney transplantation patients. We used RT-QPCR to quantify the gene expression of immunological candidate genes in a low-density array format. In addition, we used RT-QPCR to validate results of the microarray analysis. No gene marker differentiated patients with SCR from those without SCR. This research demonstrates the lack of robust markers among polymorphisms or biomarkers in investigated genes that could be included in routine analysis in a clinical laboratory. Even though weak associations were found, their predictive value was insufficient for use in clinical decision-making.

In genetic studies, kidney Tx can be regarded as a complex trait, i.e. several environmental and genetic factors may determine its outcome. A number of currently unknown genetic factors probably influence the results of Tx. Added complexity for assessing outcome is provided by the interplay of genetic with environmental factors.

INTRODUCTION

The human kidneys are two bean-shaped organs situated retroperitoneally one on each side of the backbone. Each kidney consists of one to two million nephrons. A nephron has a complex structure consisting of a glomerulus, a Bowman's capsule, a proximal convoluted tubule, a loop of Henle, a distal convoluted tubule and a collecting tubule. The nephron filtrates surplus or waste molecules and ions from the blood to urine and returns useful materials back to circulation. In this manner the kidneys maintain body homeostasis. The human kidney also secretes two hormones, erythropoietin and calcitriol, and the enzyme renin which contribute to regulation of red cell development, calcium metabolism and blood volume or blood pressure (http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/K/Kidney.html).

The deterioration of kidney function leads to disturbance of acid-base and water-solute balance and accumulation of end products of the nitrogen metabolism. Clinical symptoms of malfunction may be e.g. urinary retention, elevated blood pressure or fever. Laboratory markers may be e.g. high creatine quantities and protein in urine. Cardio-vascular diseases and diabetes among others expose to kidney diseases [1].

REVIEW OF THE LITERATURE

1 KIDNEY TRANSPLANTATION

1.1 General aspects of kidney transplantation

Kidney Tx is the treatment of choice for end stage renal disease (ESRD). Studies comparing the quality of patients' life before and after kidney Tx have showed a better quality of life after Tx [2]. Even though dialysis is a treatment for terminal kidney dysfunction, it results in inferior life expectancy. It is also more expensive to society than kidney Tx [3].

The first successful kidney Tx was performed in 1954 between English living identical twins [4]. In the 1960s, the invention of the first immunosuppressive medications, azathioprine and steroids, enabled allotransplantation between unrelated individuals. The first kidney Tx in Finland took place in 1964 [5,6]. Txs in Finland are performed at Helsinki University hospital with separate Tx units for adults (Kidney Transplant Unit, Department of Surgery) and children (Pediatric Nephrology and Transplantation, Hospital for Children and Adolescents). This has facilitated the adaptation of uniform treatment protocols and follow-ups of patients in a network of university clinics as well as regional and local hospitals [7]. Graft and patient survivals have improved with the introduction of new immunosuppressants, in particular cyclosporine at the end of the 1970s, and tacrolimus and mycophenolate mofetil in the 1990s [8]. In addition, various induction antibodies against lymphocyte subpopulations have augmented the treatment repertoire [9]. Further, novel immunosuppressants and antibodies are under development with several currently undergoing clinical trials [10,11].

Graft and patient survivals in kidney Tx worldwide vary considerably according to the treatment protocol, donor source and ethnicity. The American organisation collecting Tx statistics (ustransplant.org) gives 1-year graft and patient survival rates for adult patients with a first transplant between 2003 and 2004 of 89.5% and 94.7%, respectively. The European registry, the Collaborative Transplant Study (ctstransplant.org), in turn gives 91% and 95.5% 1-year graft and patient survival rates, respectively, for adult patients transplanted for the first time between 2003 and 2005. Graft half-life was 7.8 years in the USA for the era 1988 - 1996 and 14.2 years in Europe for the era 1985 to 2005 implying that half-life has increased during recent years [12] (ctstransplant.org).

The need for kidney Tx may arise from renal failure due to e.g. chronic or acute conditions like trauma, infection or drug toxicity. Chronic renal diseases include primary kidney diseases and certain autoimmune diseases, and may be secondary to the failure of another organ. Currently, the disease most likely to lead to kidney Tx in Finland is diabetes, with type 1 diabetes (T1D) being more often the problem than type 2 diabetes (T2D) as T2D patients are more often unfit for Tx. The second most common group of diseases are the glomerulonephropathies (excluding IgA nephropathy and focal segmental glomerulosclerosis), followed by polycystic kidneys. Other causes of uraemia include tubulointerstitial and dysplastic diseases [9]. A special feature of paediatric kidney Tx in Finland is the large number of patients transplanted because of congenital nephrotic syndrome of the Finnish type (CNF, NPHS1), a rare autosomal recessive disease presenting with massive proteinuria at birth [13,14]. The disease is responsible for approximately 50% of Tx in children.

1.2 Genetic aspects of end stage renal disease

Some of the kidney diseases leading to ESRD are Mendelian disorders, e.g. NPHS1, polycystic kidney disease 1 to 3, X-linked Alport syndrome, Wilm's tumor, and familial Mediterranean fever diseases (OMIM - Online Mendelian Inheritance in Man www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM). However, most of the diseases leading to ESRD are genetically complex and even heterogeneous, and hence their more detailed genetic dissection is still underway [15-18]. As diabetic nephropathy (DN) has attracted more studies than any other ESRD, DN genetics is briefly reviewed next.

DN accounts for 40% of ESRD in the Western world. Familial clustering of DN and ethnical variation have been observed, both in T1D and T2D, indicating genetic factors in the development of the disease. A large study on nearly 26000 incident US dialysis patients from more than 450 dialysis clinics has analyzed the family history of ESRD [19]. Independent risk factors for a family history of ESRD in first- or second degree relatives included earlier age at ESRD onset, female gender, black ethnicity, and diabetes-associated nephropathy.

Molecular genetic studies published on DN involve family-based linkage and association studies or association analysis in a case-control study design. Linkage studies on families have either analyzed a restricted chromosomal region using a few polymorphic microsatellite markers [20] or the whole genome using at least 900 microsatellite markers relatively

uniformly distributed throughout the genome [21]. SNPs are commonly used in association analysis nowadays.

Genome-wide linkage studies on sibling pairs have identified several chromosomal regions that may harbour susceptibility genes/variants for DN. Chromosome 3q13-q25 has been indicated in DN in T2D African Americans [22] and in T1D DN in Caucasian families [20,21], and it has been reported that adiponectin gene on 3q is a likely susceptibility gene for DN in French and Danish families [23]. A large region on chromosome 7q has been indicated in DN, predominantly in T2D, in several populations of different ethnic origin. Chromosome 18q22-q23 region has also evolved in T2D DN in several reports on populations of different ethnic origin [22,24,25].

Some candidate genes studied code components of the renin-angiotensin system, e.g. angiotensin I converting enzyme 2 [26], lipid metabolism, e.g. apolipoprotein E [27], and membrane proteins such as xylosyltransferase I [28], nitric oxide synthase 3 [29], and superoxide dismutase 2 [30,31]. Currently, at least one genome wide association (GWA) study "Genetics of Kidneys in Diabetes (GoKinD)" (search at www.jdrf.org) is being conducted. New genetic factors in DN may open the path to new molecular targets for drug development and more accurate diagnosis of DN, ultimately perhaps reducing the incidence of DN leading to ESRD [32].

1.3 Human leukocyte antigen

1.3.1 Basic features of human leukocyte antigen biology

1.3.1.1 Immunogenetics of the human leukocyte antigen system

The major histocompatibility complex (MHC) in the human race is HLA. The *HLA* complex on chromosome 6p21.3 contains more than 200 genes, of which more than 40 encode the HLA antigens (Fig. 1). In fact, all the known *HLA* genes are located within the MHC. The remaining 150 genes or so in the MHC are not evolutionarily related to the *HLA* genes, although some are involved in HLA function, in particular to the processing of the peptide antigens, while some others have immunological functions, e.g. related to complement activation. Finally, many appear not to be related to the immune response at all [33].

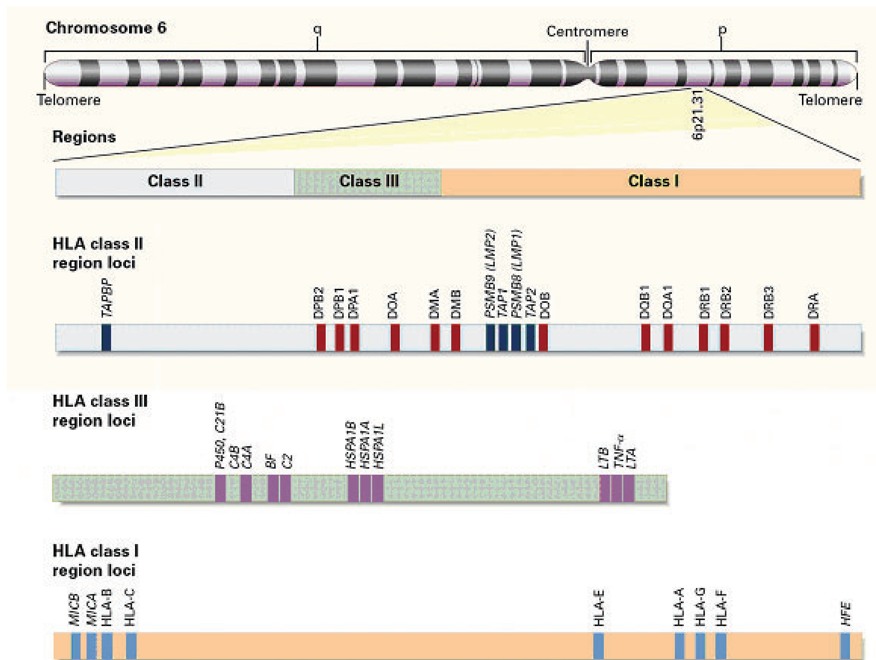

Figure 1. Location and Organization of the *HLA* Complex on Chromosome 6. BF denotes complement factor B; *C2* complement component 2; *C21B* cytochrome P-450, subfamily XXI; *C4A* and *C4B* are complement components 4A and 4B, respectively; *HFE* hemochromatosis; *HSP* heat-shock protein; *LMP* large multifunctional protease; *LTA* and *LTB* lymphotoxins A and B, respectively; *MICA* and *MICB* major-histocompatibility-complex class I chain genes A and B, respectively; *P450* cytochrome P-450; *PSMB8* and *PSMB9* proteasome ß 8 and 9, respectively; *TAP1* and *TAP2* transporter associated with antigen processing 1 and 2, respectively; *TAPBP* TAP-binding protein (tapasin); *TNF-α* tumor necrosis factor; and *HSPA1A*, *HSPA1B*, and *HSPA1L* heat-shock protein 1A A-type, heat-shock protein 1A B-type, and heat-shock protein 1A–like, respectively. Copyright © 2000 Massachusetts Medical Society. All rights reserved [34].

The *HLA* genes are often divided into two major classes. Class I includes in particular the classical Tx antigens HLA A, B and Cw, and class II the HLA DR, DQ and DP. These molecules are functionally and structurally similar (Fig. 2), although they do have certain important differences. Class III is a heterogeneous collection of genes where a few families dominate. These include genes of the immunoglobulin superfamily members as well as those involved in the activation cascades of the complement system, hormonal synthesis, inflammation and cell stress, and extracellular matrix organisation. The most of the remaining loci are mainly involved in more basic biological functions with no immediate connection in the immune system [35].

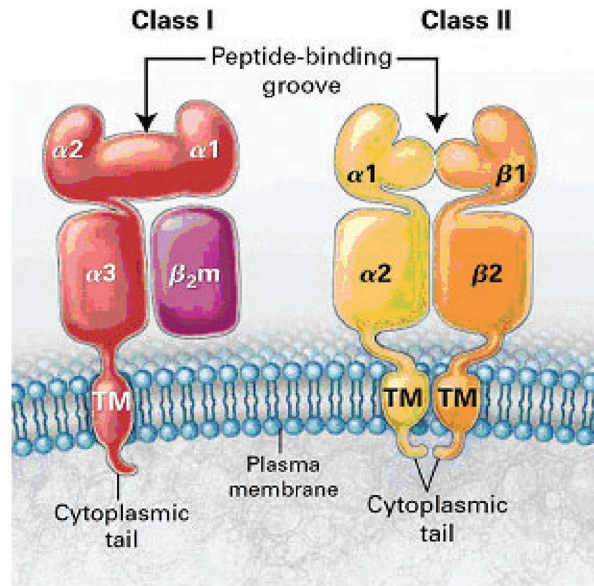

Figure 2. Structure of HLA Class I and II Molecules. β2m denotes beta2-microglobulin, TM transmembrane region. Copyright © 2000 Massachusetts Medical Society. All rights reserved [34].

The α chains of the HLA class I molecules are encoded by the *HLA* linked genes, whereas the β chain is encoded by the β2-microglobulin gene located on chromosome 15q21-q22. The α chains have five functional domains: two domains (α1 and α2) together form the functionally crucial peptide binding groove, an immunoglobulin-like domain (α3), the transmembrane region, and the cytoplasmic tail (Fig. 2).

The class II molecules are also formed of two chains, but here both chains (α and β) are encoded by genes located in the *HLA* class II region. Each of them has four domains, which are similar to those of class I, except that there is only one peptide-binding domain in each chain (α1 or β1); however, these two domains form a similar type of functionally important peptide binding groove (Fig. 2).

The *HLA* genes show the highest known quantity of classical, allelic genetic variation. The most polymorphic ones are *HLA-A* with over 500 alleles, *HLA-B* with over 800 alleles, *HLA-C* with over 300 alleles, and *HLA-DRB1* with over 500 alleles (www.ebi.ac.uk/imgt/hla/stats.html). There is also strong linkage disequilibrium (LD) between the *HLA* genes, in particular between the *B* and *Cw* alleles, and *DRB1* and *DQB1* alleles [36,37]. In other words, certain allelic combinations appear together more frequently than expected from their individual frequencies. Extended

haplotypes in the *HLA* region are conserved in many populations, e.g. haplotype *HLA-A1-B8-DR3-DQ2* is very common in many Northern European populations [38,39]. It is currently not established whether the LD results from some evolutionary advantage of certain allelic combination, recent population mixings, or missing recombination sites in some haplotypes.

Most human cells express the HLA A and B, and apparently HLA Cw, molecules, but their relative quantities vary between tissues. Only specific immune cells like B cells, activated T cells, macrophages, dendritic cells, and thymic epithelial cells express HLA class II molecules. However, interferon gamma (IFNG) can induce other types of cells to express the *HLA* class II genes. Basically, the HLA class I and II molecules share the same function: to present peptides to T lymphocytes. This presentation is an essential requirement for the activation of the adaptive immune response [34].

1.3.1.2 Antigen processing and presentation

A special molecule, ubiquitin, marks intracellular proteins that are no more needed for disassembly. Chaperones make marked proteins available to a proteasome degradation process where they are digested into peptides of variable length. Peptides are also produced in endocytic vesicles where extracellular proteins are digested by lysosomes and proteolytic enzymes [40].

In addition to foreign proteins, the proteasomes also digest self-proteins, the resulting peptides being picked up by transporters associated with antigen processing (TAPs). TAP1 and TAP2 genes encode TAP proteins. TAPs form channels through the endoplasmic reticulum (ER) and peptides move via these and bind to class I molecules. The class I molecules have been assembled from α chains and β2m in ER with the help of chaperones like TAP-binding protein (TAPBP) and others. After binding to the suitable peptide, the class I molecule moves to the cell surface where it is displayed [41].

Class II molecules are manufactured in the same way as class I molecules up until the class II molecule peptide binding in cytoplasm. Before peptide binding, the invariant chain binds to the peptide-binding groove. In the cytoplasm the class II molecules intersect with endosomes. Proteasomes, with the help of the HLA-DM molecule, cut the invariant chain and exogenous peptide binds to the groove. This complex is exported to the surface of the cell [42].

In general, the class I molecules present peptides from endogenous sources and class II molecules from exogenous sources. On an uninfected cell surface, the HLA molecules bind hundreds of thousands of self-peptides. Antigen-presenting cells (APCs) express HLA-peptide complexes, which T-cells bind and recognize as self or non-self in a process described in the following sections [34].

1.3.1.3 Human leukocyte antigen and T cell selection

T cells mature in thymus from lymphocyte progenitors. Immature double-positive T cells interact with their T cell receptor (TCR), as well as CD4 and CD8 coreceptors to HLA molecules. In the selection process if TCR binds to HLA class I, the T cell starts to down-regulate CD4 receptors and up-regulate CD8 receptors, and if TCR binds to HLA class II, the T cell starts to down-regulate CD8 and up-regulate CD4 [43,44] (Fig. 3).

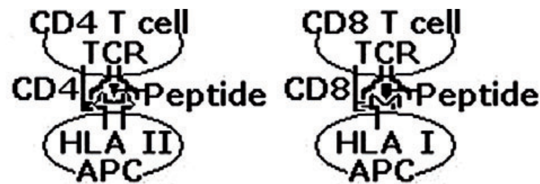

Figure 3. Mature CD4 and CD8 positive T cells make contact with TCR and CD4 or CD8 molecules to an APCs HLA class II and class I molecules representing peptide, respectively. TCR denotes T cell receptor; HLA human leukocyte antigen; APC antigen presenting cell.

During T cell maturation in the thymic cortex T cells encounter HLA molecules. T cells that are unable to recognize the self-HLA molecules will die by apoptosis. In the thymic medulla, T cells with strong binding to self-peptides presenting HLA molecules undergo apoptosis. On the periphery, T cell binding to HLA class I molecules with a recognized ligand leads to T cell maturation to a CD8 cytotoxic killer T cell while binding to HLA class II molecules results in maturation to a CD4 helper T cell. Maturation activates T cells to produce INFG and other cytokines, and these cytokines enhance immune response by increasing the expression of HLA class I and II molecules [45].

1.3.1.4 Interaction between specific human leukocyte antigen molecules and peptides

Only a few amino acids of each peptide bind directly to the peptide binding groove of HLA. These interactions have been described in detail by crystallographic studies [46,47]. One *HLA* allele has the ability to bind many different peptides if they all share certain critical amino acids at particular positions, that is, they fulfil the criteria for the peptide binding motif. The amino acids that form the critical pockets of the peptide binding groove and thus determine the peptides able to bind therein, show a high quantity of genetic variation between *HLA* alleles. Hence, different *HLA* alleles bind and present to the immune response different sets of peptides. Although the peptide binding motif gives the general outline of peptides bound by each of the *HLA* alleles, the requirements are not absolute. One *HLA* allele can therefore bind thousands of different peptides [48] (Fig. 4).

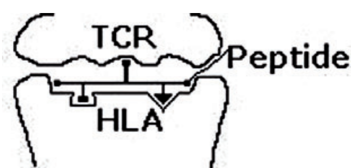

Figure 4. A TCR recognized HLA-peptide complex. TCR denotes T cell receptor, HLA human leukocyte antigen.

HLA is currently the most important known genetic factor influencing the outcome of kidney Tx. In the next sections, aspects of HLA biology and matching in kidney Tx are covered.

1.3.2 Clinical histocompatibility and human leukocyte antigen matching

Successful kidney Tx requires, in addition to special surgical skills, careful immunogenetic matching between the donor and recipients. In many centres worldwide, Tx is only considered if (i) the recipient and donor are blood group ABO matched, (ii) the recipient has not developed prior antibodies against the donors HLA antigens (a negative cross-match), and (iii) there is sufficient *HLA-A, -B* and *-DR* similarity between the recipient and the donor [49].

HLA matching for kidney Txs has two main aims. First, a good immunological histocompatibility between the donor and recipient diminishes immune activation against the graft. Second, emergence of antibodies against foreign HLA antigens of the graft is potentially harmful for the long-term graft survival (GS), and their emergence is certainly harmful if the recipient undergoes retransplantation. In other words, preformed antibodies, or a positive cross-match before Tx, may lead to acute or hyperacute rejection and deterioration of the graft. Long-term humoral immunity against the graft promotes chronic rejection and shortened GS [50]. The detection of anti-HLA antibodies is discussed in the section on methods for AR diagnosis.

Foreign HLA antigens promote cellular immunity. The cellular response can be divided into direct allorecognition, primarily by cytotoxic CD8 T cells, and indirect allorecognition by effector/helper CD4 T lymphocytes.

Worldwide there are several *HLA* matching protocols for kidney Tx. Centres may match for all HLA-A, -B, and -DR antigens, or some mismatching in HLA-A, -B, and -DR antigens, and either broad or split antigens can be tolerated. There are programs for acceptable and unacceptable mismatches for highly sensitized Tx candidates, DR-only matching, cross-reacting groups of antigen (CREG) matching, structurally based matching, amino acid residue mismatching, and HLA Matchmaker program matching [51]. In Finland, an acceptable *HLA* match involves the sharing of at least two 'broad' antigens in the *HLA-A*, and *HLA-B* genes, and one antigen in *HLA-DR* [6].

Other essential factors affecting the outcome of Tx include the quality of the donor kidney, donor age, cold ischemia time, and the experience of the Tx team [51]. The Collaborative Transplant Study (CTS) reports that *HLA* mismatch worsened GS independently of other known factors influencing GS (www.ctstransplant.org/). Good *HLA* matching also significantly improves GS in Finnish kidney Tx [52].

1.4 Complications in kidney transplantation

Complications experienced by transplant recipients include AR, delayed graft function, cytomegalovirus infection, and/or vascular complications, and these are reviewed in the following sections. Other significant adverse effects (first year incidences in spesific studies in parentheses) include BK-virus nephropathy (8% [53]) and other microbe infections, chronic rejection (4% [54]), post-transplant lymphoproliferative disease (1% [55]) and other cancers, and there are common minor problems, e.g.

cosmetic issues and mild gastrointestinal complications. These are not reviewed as they are beyond the focus of this thesis.

1.4.1 Acute rejection

Rejection is an immune response to an allogeneic graft. Immunosuppressive medication is given to prevent rejection in an alloTx. A kidney Tx patient experiencing an AR episode may present with fever, chills, night sweats, myalgias, and tenderness over the allograft, oliguria and hypertension. Laboratory tests may indicate increased creatinine or a decrease in glomerular filtration rate. An ultrasound scan may reveal a swollen kidney and delayed and decreased blood flow, among other functional parameters [56]. These, with or without biopsy findings, usually lead to suspicion of AR. Histology and other tests in use and in development are reviewed in the section 1.6. AR is treated with steroids, or in steroid-resistant rejection with antibodies to T cells, among others. Rejection episode length and response to therapy determine the resulting graft damage, which may only be partly reversible. Permanent damage may lead to long-term deterioration of graft function. It is important to recognise patients with high rejection risk and diagnose rejection early to limit damage to the graft and improve the Tx prognosis.

Most AR episodes occur in the first three months after Tx. AR occurring later is often suspected to be related to non-compliance. However, the incidence of AR has decreased with the evolution of medical care. This has made studies in cohorts difficult, because the incidence of AR is often as low as 10 to 15%. According to ustransplant.org, the incidence of AR has continually decreased from 1996 through 2004, with respective annual incidences of 51%, 29%, 21%, 19%, 17%, 17%, 15%, 13% and 11%. However, long-term GS for the patients with AR has not increased [57]. Future studies should probably use other endpoints to predict outcome, such as serum creatinine or glomerular filtration rates. In addition, the decreasing AR incidence has improved short-term, but not long-term, allograft survival. Factors associated with late allograft loss, such as calcineurin inhibitor toxicity, chronic rejection, and BK virus nephropathy, may provide better predictors of long-term allograft loss than AR [58].

1.4.1.1 Immune systems involved in rejection

AR results from the innate and acquired allogeneic responses to transplanted graft. These responses follow each other in sequence, in three processes: 1) naïve host T cells recognise alloantigens, 2) alloreactive T cells are activated and expand, 3) target cell destruction takes place in an effector phase (Fig. 5) [59]. Alloantigens can be divided into two

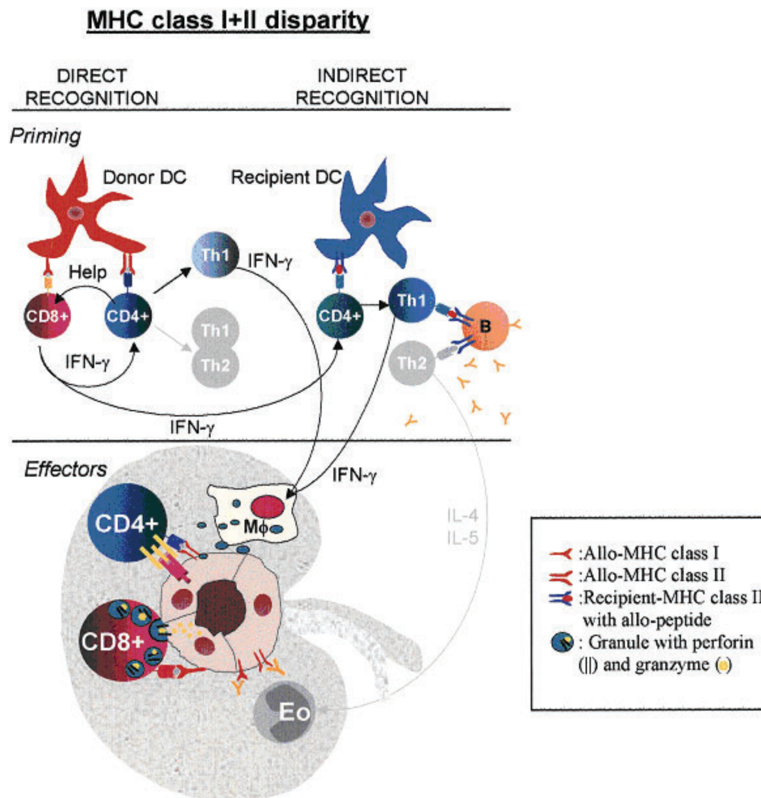

Figure 5. Effector mechanisms responsible for the rejection of combined class I and class II MHC-mismatched allografts. In the direct pathway, host CD4 T cells recognize intact MHC molecules on donor dendritic cell (DC) (stained red). In the indirect pathway, recipient DC (stained blue) engulf and process donor MHC molecules before donor MHC molecules present themselves as MHC-derived allopeptides to host CD4 T cells. CD8 T cells that recognize donor MHC class I molecules will receive help from activated alloreactive CD4 T cells. The effector phase of rejection (depicted around a tubule within a kidney graft) involves: (a) CD8 and CD4 T cell cytotoxicity with the perforin/granzyme and Fas/FasL pathway, respectively; (b) CD4 and CD8 derived IFNG production and delayed-type hypersensitivity, where macrophages release toxic molecules, such as NO, TNF, and oxygen species; (c) antibodies reactive to donor MHC molecules, which will mediate complement activation or antibody-dependent cell-mediated cytotoxicity. The cells and pathways not involved in rejection are shown in grey. Copyright © 2002 Lippincott Williams & Wilkins. All rights reserved [59].

classes: foreign MHC and minor alloantigens. The above discussed *HLA* matching attempts to lessen the MHC differences between the donor and recipient [60]. Even though full *HLA* matching were to be achieved e.g. in sibling Tx, minor alloantigen allopeptides would still result in a degree of immunoactivation as they are presented by shared donor and recipient MHC molecules [61]. Allopeptides derive from differences between donor and recipient proteins, e.g. male antigen in sex-mismatched Tx or proteins having amino-acid differences, or di-allelic polymorphisms in coding or controlling sequences [62,63].

1.4.1.2 Allorecognition

In direct allorecognition the TCR on CD8 T cells of the recipient recognize the donor's foreign HLA class I molecules directly on the cell surface. The recognition takes place in lymph nodes and spleen, where naïve alloreactive T cells contact donor APCs. Transplantation surgery is associated with ischemia/reperfusion injury which activates donor APCs to migrate from allograft to recipient lymphoid tissues [64]. In the indirect allorecognition recipient APC cells engulf donor HLA I and II molecules, digest them into peptides and present them via recipient HLA II molecules to the recipient's CD40 T lymphocytes. Recipient DCs circulate to the transplanted graft attracted by the chemokines and increased graft endothelial cell adhesion molecule expression. Recipient DCs circulate back to spleen and lymph nodes to prime naïve T cells [65]. In addition to the signal from the HLA–TCR complex, other co-signals are needed for the immune activation, in particular co-stimulatory molecules e.g. in DCs B7, CD40 and OX40 ligand and their receptors, and in T cells CD28, CTLA4, CD40LG (CD154) and OX40. Binding of these molecules triggers intracellular pathways involving IL2 secretion and activation of transcription factors such as NFKB, which activates genes expressing chemokines and cytokines [66].

CD4 T cells secrete cytokines according to their differentiation into Th1 and Th2 cells. The major cytokines of Th1 cells are IFNG and IL2, which activate CD8 T cells and themselves by the expression of Fas ligand (FASLG) to cytotoxicity. Th2 cells mainly secrete IL4, IL5, IL9, IL10 and IL13, which activate eosinophils [67].

Alloreactive T cell cytotoxicity is mainly mediated by perforin/granzyme and the Fas/FASLG system, leading to target cell apoptosis [68]. In the tight junction between cells, CTL makes contact with donor MHC molecule and cytotoxic granules fuse to the target membrane. Granule molecules trigger caspase production, which leads to apoptosis [69].

B cells produce alloantibodies, which internalize foreign HLA molecules bound by their immunoglobulin (Ig). Foreign HLA molecules are cleaved into peptides which are presented by B cells' own MHC class II molecules to CD4 T cells in order to obtain signals enhancing B cell alloantibody production [70]. Alloantibodies binding to cells of the graft may activate the complement system, and subsequently graft-infiltrating immune cells like macrophages, neutrophils and eosinophils [71]. In addition, natural killer (NK) cell binding to Ig molecules initiates perforin/granzyme-mediated NK cytotoxicity, otherwise specified as antibody-dependent cell cytotoxicity (ADCC) [72].

Th1 cell mediated delayed-type hypersensitivity (DTH) appears as tissue swelling and induration, where T cells, macrophages and neutrophils induce vascular permeability and inflammatory infiltrate. Antigen-specific Th1 cells release IFNG and TNF, which trigger the macrophage production of nitric oxide (NO) and oxygen intermediates. NO elicits cytotoxicity, vasodilation and edema. TNF induces apoptosis and necrosis through caspase activation. These activations lead to DTH mediated allograft rejection [73].

1.4.1.3 Tolerance

The ultimate aim in the prevention of AR with immunosuppression is to induce immunological tolerance between the recipient and graft. Tolerance induction is complex. It can be relatively easily induced in mouse, rat, pig and primate models of transplantation e.g. by blockade of immunological co-signals [74]. However, in clinical transplantation tolerance induction remains an elusive goal that seems impossible to reach with current immunosuppressive protocols. The use of cyclosporine may lead to either beneficial or adverse interaction depending on the balance between the efficacy of the co-stimulatory signal blockade and the stringency of the alloimmune response [75]. Tolerance induction is currently one of the main goals in transplantation research [76,77].

1.4.2 Delayed graft function

The recipient usually starts secreting urine within the first day after kidney Tx and will no longer need dialysis. If not, the situation is called DGF. DGF is not a well-defined entity but is usually a centre dependent definition. The definition for DGF in Finland is from Halloran et al. [78], i.e.

plasma creatinine concentration higher than 500 μmol/L throughout the first post-transplant week, or need for more than one dialysis session in the first week, or oliguria <1L/24hr lasting more than two days [79]. The incidence of DGF varies with different factors in the donor and recipient, and in the transplant procedure [80]. Major factors predisposing to DGF are long cold ischemia time (CIT), high donor age, female kidney to male recipient, and high creatinine quantities in the donor [81]. GS is reported to be approximately 10% lower with DGF [82]. This fact has led to efforts to shorten CIT and to find novel drugs for diminishing the ischemia-reperfusion injury [83,84].

1.4.3 Cytomegalovirus infection

Cytomegalovirus (CMV) is a betaherpesvirus with linear double-stranded DNA genome. CMV infection is usually mild and subclinical, and stays as latent after infection. However, immunosuppressive medication predisposes to infections and re-activation of microbes and viruses. CMV infection occurs in 25 - 60 % of kidney graft recipients, depending on the serological status of the recipient and donor, and on prophylactic treatment. CMV serologically negative recipients (R-) who receive a serologically positive donor (D+) organ have the highest risk. These recipients often receive prophylaxis 3 - 6 months post-Tx [85,86]. New antiviral prophylactic treatments have reduced the risk for CMV infection considerably. CMV infection is commonly diagnosed using antigenaemia test or PCR. CMV prophylaxis reduces the one-year post-Tx incidence of AR in R-/D+ groups from 32.4% to 26.3% [86]. CMV disease during the first 100 days after Tx is associated with 71% GS, compared to 86% GS without CMV [87].

1.4.4 Vascular complications

Kidney Tx involves suture and catheterization of veins and urinary bladder. These procedures increase the risk of thrombosis (the formation, development or presence of a blood clot) immediately after operation. Recipient factors such as age, immobility, and primary disease influence the long-term risk of thrombosis and infarction. Recipient death with a functioning graft is mainly due to vascular complications, e.g. myocardial and cerebral infarctions [88-91]. Vascular AR may also promote microvascular thromboses, which may lead to graft loss [92]. In addition,

renal artery- or vein thrombosis due to surgical complications or factor V Leiden mutation may lead to immediate graft loss [93,94].

1.5 Genes associated with acute rejection, delayed graft function, cytomegalovirus and vascular complications

Several gene polymorphisms have been studied for their potential to predict Tx outcome (reviewed in several articles [95-100]). So far, these studies have been candidate gene studies on solid organ Tx, although GWA studies are underway (reviewed in later section). After the completion of human genome sequence [101] the International HapMap consortium has made available for everyone studying the human genome over three million SNPs validated in several populations [102,103]. The HapMap internet database includes also the information of LD between SNPs in same chromosome (hapmap.org). This LD can be used to choose tag SNPs which represent all the SNPs in LD with tag SNP for genotyping entire genes. In the HapMap CEU samples represent adequately basis for tag SNP selection in Finnish individuals [104]. In addition, tag SNPs are used among others in the genotyping platforms for GWA studies.

A good genetic association study is designed to include a sufficient number of samples, implemented to collect reliable phenotypic data, perform genotyping rigorously, and interpret results correctly [105]. Solid organ Tx candidate gene studies are founded on the immunological basis of Tx. Cytokines, chemokines, the receptors of mononuclear blood cells and other related genes have been screened for possible genetic variations associated with the outcome. The results from these studies have been mostly contradictory or non-conclusive due to their small size. The small patient number in these genetic studies has led to various problems, including sensitivity to variability in the patient and donor characteristics, and analysis methods. In addition, the non-consistent nomenclature of gene polymorphisms makes the comparison of studies difficult. The refSNP (rs) numbers, the standard nomenclature for gene polymorphisms from the database of single nucleotide polymorphisms (dbSNP), or Human genome variation society (HGVS) mutation nomenclature recommendations are not commonly used. Currently there is not a single gene outside the *HLA* and *ABO* blood groups that is commonly accepted to be of importance in kidney Tx [106]. However, comprehensive prospective studies are yet to come.

Genetic epidemiology is a term that indicates a combination of the old formal epidemiology (variables changing during an individual person's

life-time) and new emerging genetic variation typing and analysis methods (variables not changing during an individual person's life-time). Traditional epidemiology follows strict guidelines when reporting associations between environmental factors and outcomes, e.g. diseases. However, even with extremely detailed studies not everything can be taken into account as a risk factor, or measured variables are confounding, intervening, suppressing or interacting without an acknowledgement of this. Adding this to the genetic diversity, which is not limited to SNPs, several factors can influence the outcome [107]. Thus, many published articles may report false-positive or false-negative results [108].

1.5.1 Cytokine genes

Cytokines can be divided into subgroups of interleukins, interferons and miscellaneous hematopoietins. Since the launch of the HapMap project, several thousands of gene polymorphisms have been found in genes encoding cytokines. Some of these may, or have been shown to, have an effect on gene function or its expression quantities where certain allele/genotype/haplotype is associated with decreased or increased gene expression compared to the other variant. The mechanisms of these mediation effects are not usually known [109]. The published studies still mainly include only a single or a few polymorphisms per cytokine gene, and haplotype analyses are rare. All the cytokine genes and polymorphisms studied in this thesis are listed in table 1 which also denotes the primary source and effect of each cytokine. Relevant studies to this thesis are discussed in more detail in Results and Discussion.

Table 1. Summary of findings in the current literature demonstrating association or lack of association for each described genetic variation on graft outcome (DGF, AR, GS or patient survival).

Gene polymorphism	dbSNP rs number	Allele function	Primary source	Effect	Citation: Association	Citation: No association
TNF G(-308)A	rs1800629	-308A: Transcription factor sensitivity, increases TNF transcription [110,111]	Macrophage, natural killer cell, T cell	Proinflammatory, local inflammation, endothelial activation	[96,112-123]	[98,100,124-133], donor: [112,119,131,134-136]
TGFB1 T(+869)C and C(+915)G	rs1982073 rs1800471	T(+869)C: Cellular TGFB1 transport, C(+915)G: Structure enzyme cleavage site [137]. Haplotype +869T +915C: Increased production of TGFB1 [138]	Chondrocyte, monocyte, T cell	Anti-inflammatory, immunosuppressive, profibrogenic, inhibits cell growth	[100,118,119,121,122,128,139], donor: [118,122,123,131,136]	[98,113,117,124,127,130-132], donor: [119,134,135]
IL10 G(-1082)A	rs1800896	-1082G: Transcription factor binding site, increased IL10 production [140]	T cell, macrophages	Prohumoral, anti-inflammatory, macrophage suppressant	[98,112,119,121-123,125,128,130,139]	[96,100,113,117,124,126,127,129,131-134,141-143], donor: [112,119,122,131,134-136,143]
IL10 C(-819)T and C(-592)A	rs1800871 rs1800872	Haplotype GCC: Increased IL10 production [144]			[119,121,122,128,131,139], donor: [119]	[117,124,126,127,129,130,132,142,143], donor: [122,131,135,136,143]
IL6 G(-174)C	rs1800795	-174C: Negative regulatory domain, decreased IL6 production [145]	T cell, macrophage, endothelial cell	Procellular and humoral, acute phase protein production, fever	[96,115,122,127], donor: [123,131,135]	[98,119,121,126,128,130-132,135,136,143], donor: [119,122,143]
IFNG T(+874)A	rs2430561	+874T: NFKB binding site, increased IFNG production [146]	T cell, natural killer cell	Proinflammatory, procellular	[121,128], donor: [123,136]	[96,98,119,127,130-133], donor: [119,131]

1.5.1.1 Tumor necrosis factor

The most studied cytokine is TNF, mainly known for its devastating effects on sepsis leading to massive inflammatory reactions. TNF causes pro-inflammatory responses in response to several immunological challenges. Systemic TNF causes e.g. fever, hypotension, and shock [147]. TNF is also involved in the AR cascade [148]. The *TNF* gene lies on 6p21.3 in the region of MHC class III and is in LD with classical *HLA* genes [149]. The *TNF* gene is 2778 bases long and encodes a protein of 233 amino acids. The most studied polymorphism in the *TNF* gene is the G(-308)A promoter polymorphism that influences the expression of TNF protein [111]. The -308A allele associated with high cytokine expression is found in the ancestral *HLA-A1-B8-DR3* haplotype which confers susceptibility to many autoimmune diseases [150]. The G(-308)A polymorphism has been found to associate with the outcome of kidney Tx, although results are contradictory (Table 1).

1.5.1.2 Transforming growth factor beta 1

Transforming growth factor beta 1 (TGFB1) regulates proliferation and differentiation of many cell types. In the immune system TGFB1 affects many cell types, e.g. T cell survival, proliferation, Th differentiation and effector functions [151]. The *TGFB1* gene lies in 19q13.2, is 23402 bases long and encodes a protein of 390 amino acids. In kidney Tx TGFB1 is known as fibrogenetic factor, whose increased expression has been associated with chronic rejection [152,153]. In addition, a significant association between *TGFB1* gene and graft and patient survival has been reported [154]. Polymorphisms in the exon 1 of the *TGFB1* gene codons 10T/C and 25C/G have been associated with the outcome of kidney Tx in many studies. Again, however, there are also many studies that have not found the association (Table 1).

1.5.1.3 Interleukin 10

IL10 promotes the Th2-type immune response leading to antibody production. The effects of IL10 are generally thought to be anti-inflammatory, and to suppress the Th1-type immune response. The *IL10* gene lies in 1q32.2, is 4892 bases long and encodes a protein of 178 amino acids. There are several polymorphisms in the promoter region of *IL10*. However, the effects of these on IL10 production remain

unclear. The -1082G allele has been associated with a higher expression quantity *in vitro* and *in vivo* [140]. In another study, the -1082A allele was reported to confer a two-fold increase in the transcriptional activity of the *IL10* promoter compared to the G allele [155].

The conflicting results of the *in vitro* and *in vivo* studies of the *IL10* polymorphisms are reflected in the clinical studies of the role of the cytokine in the development of AR [140]. While *IL10* polymorphisms have been reported to affect the outcome of kidney Tx in several studies, there are many reports that have been unable to detect the influence of *IL10* (Table 1).

1.5.1.4 Interleukin 6

IL6 regulates many aspects of the immune response, e.g. T cell differentiation [156]. The IL6 quantities increase in the incidence of inflammation, tissue damage, hypoxia, or infection [157]. The *IL6* gene lies in 7p15.3, is 6113 bases long and encodes a protein of 212 amino acids. The *IL6* promoter haplotype regulates the IL6 mRNA and protein quantities [158]. The promoter polymorphism G(-174)C, together with nearby polymorphisms, have been the subject of several kidney Tx studies (Table 1).

1.5.1.5 Interferon gamma

Activated T cells produce IFNG. Mitogens for T cells may mimic this induction. IFNG has several properties: it modulates immune activity by e.g. activating macrophages; it mediates the lytic effect; it potentiates the actions of other interferons; and it inhibits intracellular microorganisms other than viruses [159]. The *IFNG* gene lies in 12q15, is 4974 bases long and encodes a protein of 166 amino acids long.

IFNG acts both as an anti-rejection and pro-rejection cytokine, e.g. by induction of microvascularisation in the grafted organ and increasing the expression of MHC. Whether the main effect will be anti- or pro-rejection depends mainly on the secretion time after kidney Tx, being protective early and then later antagonistic [160]. The most studied *IFNG* polymorphisms in kidney Tx are intron 1 SNP T(+874)A and CA short tandem repeat microsatellite (rs3138557). These have been associated with AR, and donor polymorphism to chronic allograft nephropathy (Table 1).

1.5.2 Thrombosis-associated genes

The blood coagulation cascade involves several molecules which lead to thrombosis and infarction [161]. In addition, in the epithelium of rejecting graft blood vessels the coagulation cascade activates forming infiltrates in the inflammation area. Infiltrates consist of e.g. platelets, neutrophils and monocytes [162]. All the thrombosis associated genes and polymorphisms studied in this thesis are listed in table 2 where the effect of each molecule is also denoted. The relevant studies to this thesis are discussed in more detail in Results and Discussion.

1.5.2.1 Coagulation factor V

The *F5* gene lies in 1q24.2, is 72422 bases long and encodes a protein of 2224 amino acids long. In the context of kidney Tx the factor V Leiden mutation (FVL, *F5* R506Q, G1691A) is currently the most thoroughly investigated polymorphism of the genes of the coagulation cascade. The FVL mutation associates with thrombotic events, rejection episodes, GS and infarction. Patients with the FVL mutation are often given anti-thrombotic prophylaxis. However, in some studies investigators have been unable to demonstrate any association between the FVL mutation and outcome of renal Tx (Table 2). A second *F5* mutation (F5R2, H1299R, A[R1]4070G[R2]) occurs in the B-domain of the protein that is released upon the activation of F5. The H1299R mutation is postulated to lower F5 concentrations either by reducing F5 biosynthesis or increasing F5 molecule turnover [163]. The effects of the *F5* H1299R mutation on the outcome of kidney Tx have not been previously investigated. In the general population, the *F5* H1299R mutation has been associated with increased risks of infarction and thrombosis [164].

1.5.2.2 Coagulation factor II

The coagulation factor II (*F2*) gene lies in 11p11.2, is 20300 bases long and encodes a protein of 622 amino acids long. The *F2* mutation G20210A changes the F2 pre-mRNA processing. More F2 mRNA is translated to the F2 protein, which leads to increased F2 concentrations and elevated risk of thromboembolism [165]. The G20210A mutation associates with decreased GS after kidney Tx (Table 2). However, in other studies the results have been conflicting (Table 2).

1.5.2.3 Methylenetetrahydrofolate reductase

The methylenetetrahydrofolate reductase (*MTHFR*) gene lies in 1p36.22, is 20335 bases long and encodes a protein of 656 amino acids long. An *MTHFR* mutation (A223V, C677T) results in elevated plasma homocysteine quantities and higher risk of thrombosis secondary to cytotoxic effects on endothelial cells. In kidney Tx, the mutation has been associated with AR and the development of chronic allograft nephropathy, but not with decreased patient or GS (Table 2).

1.5.2.4 Protein C, tissue factor pathway inhibitor, coagulation factor XIII

Protein C (*PROC*), tissue factor pathway inhibitor (*TFPI*) and coagulation factor XIII (*F13A1*) are also candidate genes for thrombosis but these have not been studied in kidney Tx patients. The *PROC* gene lies in 2q14.3, is 10802 bases long and encodes a protein of 461 amino acids long. PROC inactivates e.g. factor Va, thereby inhibiting clot formation. The *PROC* W380G, T8853G loss-of-function mutation, which may be regarded as a member of the 'Finnish disease heritage' [166], results in a deficiency of PROC, and an increased risk of thrombosis. The *TFPI* gene lies in 2q32.1., is 87880 bases long and encodes a protein of 304 amino acids long. The pro-thrombotic *TFPI* mutation (P151L, C536T) is thought to increase prothrombinase complex activity and thrombin production [167]. The *F13A1* gene lies in 6p25.1, is 176584 bases long and encodes a protein of 732 amino acids long. *F13A1* mutation (V34L, G103A) is associated with protection from thrombosis. The mutated protein has a lower ability to catalyze fibrin polymer formation than the wild type protein. In a recent meta-analysis of diagnosed thrombosis cases and controls in the general population, the V34L mutation had a significant protective effect against thrombosis (homozygote odds ratio (OR) 0.63, 95% confidence interval (CI) 0.46 - 0.86) [168].

Table 2. A summary of findings in the current literature demonstrating association or lack of association for each described genetic variation on graft outcome (AR, cardiovascular complications and markers, GS or patient survival).

Gene polymorphism, aminoacid and nucleotide change	Allele function	Effect	dbSNP rs number	Citation	
				Association	No association
F5, R506Q, G1691A	1691A allele: Inactivation site mutation, increased coagulation [169]	Factor Xa activation	rs6025	[170-175]	[89,176-180]
F2, -, G20210A	20210A allele: Pre-mRNA cleavage site mutation, increased F2 production [165,181]	Factor XIII and fibrinogen activation	rs1799963	[175,182]	[89,178-180]
MTHFR, A223V, C677T	677T allele: Decreased enzyme activity, increased homocysteine production [183]	Procoagulation	rs1801133	[175,184-192]	[89,180,193-196], donor: [184,193]

1.5.3 Other genes studied in renal transplantation

Polymorphisms in other genes such as cytokines *LTA*, interleukin 1, interleukin 2, interleukin 4, chemokines like chemokine ligand 5, chemokine ligand 12, chemokine receptor 2, chemokine receptor 5, melanin concentrating hormone receptor 1, monocyte chemotactic protein-1, adhesion molecules like intercellular adhesion molecule 1, platelet/endothelial cell adhesion molecule, selectins, costimulatory molecules like cytotoxic T-lymphocyte-associated protein 4, CD28 molecule, renin-angiotensin system molecules like angiotensin I converting enzyme 1, angiotensinogen, angiotensin type 1 receptor, angiotensin type 2 receptor, cytochrome P450 family 11 subfamily B polypeptide 2, and other molecules like growth factors and G proteins have also been studied in the kidney Tx setting [99]. However, these individual reports await confirmation from replication studies. Candidate gene studies may become fewer as they are replaced by future GWA studies. The only so far published GWA study from Tx patients was a case-control association study using 50,947 gene-based SNPs to identify genetic variations that might be associated with cardiovascular risk factors in 72 renal transplant recipients with cyclosporine therapy. The results were used to analyse predictive performance of cardiovascular risk factors associated with SNPs [197]. The Transplant Genomics Collaborative Group is conducting a GWA study on kidney Tx recipients and donors (www.genetics.ucla.edu/transplant-genomics/research/programs/project3.php).

1.6 METHODS AND BIOMARKERS STUDIED BY THESE METHODS FOR DIAGNOSING ACUTE REJECTION

Biomarkers are variables or predictors of a specified clinical condition measured by various laboratory methods. These variables may change over time according to the progression of the disease. Biomarkers have conventionally been protein molecules measured from bodily fluids or tissue biopsies. Gene expression and cell surface structure profiling using microarray and flow cytometry or mass spectrometry are the latest methods used [198]. In the next sections biomarkers are reviewed according to the method used.

1.6.1 Histology

A radiologist or nephrologist takes the kidney biopsy and a pathologist interprets the histologically stained sample and in some cases the sample is further stained with specific antibodies to molecules specific for AR subtypes. Histopathological evaluation may allow the identification of the cause of acute allograft dysfunction e.g. acute tubular injury, calcineurin inhibitor toxicity, obstruction or various inflammatory infiltrate processes. Standardization of renal allograft biopsy interpretation is necessary to guide therapy and to establish an objective end point for clinical trials. The first meeting for this purpose was in Banff, Canada in 1991, to develop an international classification for the histological grading of solid organ transplants. The biennial congresses have reported their proceedings in peer reviewed journals since the international consensus discussion began at Banff, and the process continues via the internet (cybernephrology.ualberta.ca/Banff/). In the Banff schema, kidney biopsy findings are rated and categorized to assist diagnosis and therapy. Banff 97 classification "defines types of acute/active rejection. Type I is tubulointerstitial rejection without arteritis. Type II is vascular rejection with intimal arteritis, and type III is severe rejection with transmural arterial changes. Biopsies with only mild inflammation are graded as borderline/suspicious for rejection. Chronic/sclerosing allograft changes are graded based on severity of tubular atrophy and interstitial fibrosis. Antibody-mediated rejection, hyperacute or accelerated acute in presentation, is also categorized, as are other significant allograft findings" [199]. Antibody mediated rejection in particular is diagnosed using immunostaining of capillaries or arteries for C4d [200]. Other molecules associating with AR include B7-1, B7-2, CD14, CTLA4, allograft inflammatory factor-1, TNF, IL18 and growth arrest-specific gene 6 [201-205]. Moreover, AR does not associate with molecules such as CD20, CD40, CD40L, CD25, perforin, TNF and TGFB1 [201-203,206,207]. In the future, biopsy sections may be stained with a wider repertoire of antibodies to explore the molecular pathway involved in the rejection process [208].

The latest Banff classification from 2005 has abandoned the non-specific term chronic allograft nephropathy and recognizes the entity of chronic antibody-mediated rejection. Renal biopsies are classified to six classes: 1. normal, 2. antibody-mediated rejection, 3. borderline changes, 4. T-cell-mediated rejection, 5. interstitial fibrosis and tubular atrophy, 6. other changes not considered to be due to acute and/or chronic rejection [209].

In addition, a clinician may take a fine needle aspiration biopsy (FNAB) [210]. The FNAB samples are scored according to a total corrected increment (TCI) value which is based on counting immunoblasts, activated lymphocytes, plasmablasts and activated monoblasts in the cytological samples [211]. FNAB can be used as a frequent monitoring of AR in kidney graft [212]. However, FNAB cannot identify vasculitis and tubulitis, which are important components of the Banff grading systems for rejection [213].

1.6.2 Enzyme-linked immunoabsorbent assay

Enzyme-linked immunoabsorbent assay (ELISA) is an immunoassay utilizing an antibody labelled with an enzyme marker. The enzyme or the antibody is bound to an immunosorbent substrate, and the change in enzyme activity from the enzyme-antibody-antigen reaction is proportional to the concentration of the antigen and can be measured e.g. spectrophotometrically [214]. The risk of AR can to some degree be estimated by determination of pretransplant anti-HLA antibodies. One of the methods used is the ELISA, where solubilized class I or class II HLA antigens are fixed onto microtiter plates. Antibodies against both HLA class I and class II increase rejection episodes in *HLA* mismatched first grafts. ELISA assay is perhaps more sensitive for detecting additional antibodies with clinical relevance. However, it cannot detect other Tx-relevant non-HLA antigens or isotype antibodies other than IgG [215].

Another pre-transplant test for predicting rejection is measurement of soluble CD30 (sCD30), most often using ELISA. CD30 is preferentially expressed on Th2-type T cells with alloimmune responsiveness. Elevated quantities of sCD30 are associated with increased risk of AR, independently of anti-HLA antibodies or Tx sequence [215]. However, in the Finnish Txs, the quantities of sCD30 did not predict rejection incidence [216]; but on day 21 posttransplant the rejecting patients had significantly higher relative sCD30 quantities than the non-rejecting patients [216]. A pilot study implied that high sCD30 quantities at post-Tx days 3 to 5 predicted AR well [217]. In addition, pre- and post-transplant CD40 quantity predicts AR. CD40 is expressed in injured kidney and is involved in the extravasation of lymphocytes at sites of inflammation [218,219]. From other molecules, the upregulation of IL10, IL12, IL18, macrophage-colony stimulating factor (M-CSF), IFNG, and the downregulation of IL4, associate with AR [204,220,221]. In addition in other studies, molecules IFNG, sIL1RA, IL2, sIL2R, IL3, IL4, IL6, sIL6R, IL10, TGFB2, TNF have not associated with AR when studied with ELISA [222,223]. However, internationally transplantation centres do not use any of these routinely.

1.6.3 Fluorescence activated cell sorting

In fluorescence-activated cell sorting (FACS) a monocellular stream of cells, platelets or other microscopic particulate elements is passed through a beam of laser light of a computerised instrument which categorises them by size and form. Cells can be labelled with fluorescent dye and a laser based detector system used to excite fluorescence and give fluorescent cell droplets an electric charge. Cells can subsequently be separated and collected and/or counted [224]. Anti-HLA antibodies can be detected using FACS analysis. In one application, patient serum reacts with beads coated with specific HLA antigens and anti-HLA antibodies are detected by fluorescence-conjugated anti IgG or IgM antibodies [225]. If antibodies are found these can be further specified to certain HLA antigens with another FACS analysis using beads coated with single HLA antigens [226]. High titer post-Tx HLA antibodies monitored by FACS bead analysis are associated with increased rejection incidence [227].

AR diagnostics with FACS analysis is based on specific antibodies, which detect their antigens at the surface or inside recipient cells. The T lymphocyte surface molecule CD69, a marker for T cell activation, associates with AR [228]. Other associations with AR are downregulation of CD45 and upregulation of CD61, CD63 and PAC-1 [229,230].

1.6.4 Mass spectrometry

Mass spectrometry measures the mass-to-charge ratio of ions. The main components of a mass spectrometer are an ionization source, a mass analyzer, and a detector system [231]. All methods described above have been applied to the candidate molecules derived from studies where the known pathways of AR or alloimmunity were investigated. A recent research approach that has become feasible for well-funded research centres is global protein analysis based on mass spectrometry with or without two-dimensional gel electrophoresis. Global urine protein profiling has been proposed as a potential non-invasive method for AR monitoring in kidney Tx, and several studies have been published [232-239]. However, large consortiums have yet to publish their results (www.allomark.ubc.ca/ and www.genetics.ucla.edu/transplant-genomics/research/programs/project2.php). They may discover novel molecules associated with AR that can be monitored with routinely used methods like ELISA AR and other outcomes.

1.6.5 TaqMan real-time quantitative polymerase chain reaction

TaqMan RT-QPCR (from now on referred to as RT-QPCR) is based on the two sequence specific primers and a probe, fluorescently labelled with a reporter and quencher dye. In the PCR extension phase Taq polymerase cleaves the hybridized probe and releases the reporter dye for fluorescence detection [240]. Real-time quantification can be done with various instruments that vary in excitation and emission wavelengths available, speed, number of reactions that can be run in parallel, and the reaction container. However, the software and performance of these instruments are basically identical [241]. The statistical analysis of RT-QPCR has not reached a consensus, with standard curve and deltadeltaCt relative expression methods being most commonly used [242]. In the standard curve method the efficiency of PCR reactions is taken into account by running known dilutions of the studied gene and a reference gene from one sample in every RT-QPCR run. In the deltadeltaCt method the efficiency of PCR reactions is validated to be the same in the studied gene and the reference gene and dilutions are not run after validation run [243].

RT-QPCR studies in the kidney Tx setting for AR markers are listed in Table 3. Results may reflect different sources of sample material, like kidney biopsy, urine or different blood components, and different methods for RT-QPCR. Studies relevant to this thesis are discussed in more detail in Results and Discussion.

1.6.6 Microarray

Microarray is quite young as a method , the results of the first self-made cDNA arrays being published in 1995 [244]. Commercially manufactured oligoarrays, in particular those by Affymetrix, together with centralised core facilities have made the technique available to all users. Even though the first kidney Tx microarray studies were all performed with self-made cDNA arrays [245-256], studies have also been done with commercially available chips [257-259]. While gene expression can be studied from any cellular source, kidney Tx has obvious sources that may indicate what is going on in the graft and recipient, i.e. the blood, urine and biopsy from the graft. From these sources RNA is isolated and purified. Often there is too little RNA for direct hybridization to the arrays. RNA is therefore amplified, followed by labelling and hybridization onto the array chips. A scanning apparatus reads the results. The raw data must be normalized

using an appropriate technique and the samples are compared by various statistical methods depending on the hypothesis. All this processing makes the comparison of studies very difficult, but not impossible. Based on microarray analysis only, there are no commercial laboratory tests available that would utilize novel biomarkers. However, there are two consortiums developing new biomarkers based on the combination of proteomics and genomics (www.allomark.ubc.ca/ and www.genetics.ucla.edu/transplant-genomics/research/programs/project2.php), and if clinically relevant, their results are to be made available as a commercial test package.

Many candidate gene markers are expressed in such low quantities that not all microarrays can detect the expression of the studied genes. These include many cytokines which are major contributors to the rejection process. However, RT-QPCR can be used to quantify the low expressing genes [260].

Until now (November 2007), four studies have investigated clinical rejection but not subclinical rejection in human kidney Tx using human samples in microarrays [248,252,261,257]. All have been done with different microarray platforms and only one [257] has reported a comparison of biopsy to peripheral blood leukocyte (PBL) samples. The comparison of immune response genes in the microarray studies shows that different genes are significantly upregulated or downregulated (Table 4). The biomarkers in Transplantation and Transplant Genomics Collaborative Groups are conducting a whole genome expression study from kidney Tx recipients and donors (www.allomark.ubc.ca/ and www.genetics.ucla.edu/transplant-genomics/research/programs/project1.php). Rapid advances in technology may bring expression analysis into clinical use in the next few decades [263].

Table 3. Summary of the current literature on RT-QPCR studies demonstrating association or lack of association for each described gene for predicting AR.

Sample source	Gene	Function	Citation	
			Association	No association
Kidney biopsy	perforin 1 (PRF1, Per)	Cytotoxic molecule, membrane-perturbing protein	264-270	271,272
	fas ligand (FASLG, FL)	Cytotoxic molecule, T cell effector protein	265,266,268-273	264
	granzyme B (GZMB, GB)	Cytotoxic molecule, granule serine protease	264-266,268,269,272-274	270,271
	granulysin (GNLY, Gra)	Cytotoxic molecule, membrane-perturbing protein	268	
	CD40 ligand (CD40LG, CD154)	Costimulatory molecule, positive effect on T cell signalling	268	
	inducible T cell co-stimulator (ICOS)	Costimulatory molecule, positive effect on T cell signalling	268	
	cytotoxic T-lymphocyte-associated protein 4 (CTLA4)	Costimulatory molecule, inhibitory effect on T cell signalling	264,268	265
	programmed cell death 1 (PDCD1, PD-1)	Costimulatory molecule, inhibitory effect on T cell signalling	268	
Urine	perforin 1 (PRF1, Per)		275-279	280
	fas ligand (FASLG, FL)		278,279	280
	granzyme B (GZMB, GB)		275,276,278,279,281	280
Blood	perforin 1 (PRF1, Per)		266,282-286	251,270,287-289
	fas ligand (FASLG, FL)		266	270,282,283,285,287,289
	granzyme B (GZMB, GB)		251,266,282,283,286,288	270,285,287,289
	granulysin (GNLY, Gra)		251,290	289
	CD40 ligand (CD40LG, CD154)		287,289,291	
	inducible T cell co-stimulator (ICOS)		289	
	cytotoxic T-lymphocyte-associated protein 4 (CTLA4)			289
	programmed cell death 1 (PDCD1, PD-1)			289

Table 4. Microarray studies in kidney Tx with AR as outcome [255].

Reference	Platform	Tissue	Sample number	Selected key genes	Gene category
[257]	Oligonucleotide (Affymetrix HG-U95Av2)	Kidney biopsy, PBL	32 (7 AR)	AIF, CD14, CD163, CD2, CD3D, CD48, CD53, chemokines, interleukins, C1q, immunoglobulins, INFG, TCR, TNF, and HLA	Immune response, inflammation, cell cycling, and DNA metabolism
[252]	Custom cDNA (number of transcripts not reported)	Kidney biopsy	26 (AR subtypes)	HLA Class I and II, CD20, MIG, MIP-1, CCR5, CX3CR1, DARC, SCYB10, SCYA5, SCYA3, SCYA13, SCYA2, interleukins, DEFA1, DEFB1, SCYA2, SCYA5, MST1, STAT1, STAT6, CD69, MAL, NFATC3, annexins, CASP10, PECAM1 and VCAM1	T- and B-cell activation, immune response, apoptosis, cell adhesion, cell cycling, HLA genes, and innate immunity
[248]	Custom cDNA (28032 transcripts)	Kidney biopsy	67 (25 AR)	TCR, HLA class I and class II, immunoglobulins, lactotransferrin, chemokines, CD20, CD34, IGF1R, TNFR, MST1, NK4, DARC, STAT1, TGFR1, GZMA, PRF1, IL2R, CD53, LTA, LTBR, NFKB1, CD59, IFNGR1, and annexins	Immune response (T and B cell), innate immunity, apoptosis, transcriptional regulation, and cell cycling
[261]	Oligonucleotide (Affymetrix Hu6800)	Kidney biopsy	10 (7 AR)	CXCL9, TCR, RING4, ISGF-3, CD18	Immune response

2 AIMS OF THE STUDY

The present thesis investigates the role of genetic factors in the clinical outcome of kidney Tx. In addition to the currently performed histocompatibility testing several other genetic factors may influence the outcome of kidney Tx and these factors could potentially be analysed and the results used to improve patient care.

We hypothesized that cytokine and/or thrombosis associated gene polymorphisms may predict the outcome of kidney Tx in adults, because cytokines are major mediators of immunological responses in Tx, in CMV infection and cytokines and coagulation cascade molecules are mediators of immunological responses in the vessels of transplanted organs.

In addition, we hypothesised that by studying gene expression quantities on blood samples using microarrays and/or RT-QPCR, we could determine the clinical applicability of a non-invasive method to diagnose AR or SCR in kidney Tx.

Spesific aims:

1) To study the association between spesific SNPs in genes *TNF, TGFB1, IL10, IL6, IFNG, F5, F2, F13A1, PROC, TFPI*, and *MTHFR* and outcomes in kidney Tx.

2) To study the association between AR and gene expression of Per, FL, GB, Gra, CD154, ICOS, CTLA4, and PD-1.

3) To study the association between SCR and gene expression of CD154, ICOS, immunologically related genes, and whole genome gene expression.

3 PATIENTS

Study I. The retrospective study comprised 291 adult cadaver kidney recipients and their 206 donors transplanted between 1999 and 2002 at the Renal Transplant Unit of Helsinki University Hospital, Finland.

Study II. The retrospective study comprised 71 CMV seronegative recipients of kidney grafts from 62 CMV seropositive donors transplanted between 1999 and 2002 at the Renal Transplant Unit of Helsinki University Hospital, Finland.

Study III. The prospective study comprised 50 adult renal graft recipients transplanted between 2001 and 2005 at the Renal Transplant Unit of Helsinki University Hospital, Finland.

Study IV. The retrospective study comprised 772 adult cadaver kidney Txs for 764 recipients (eight recipients had 2 Txs during the study period) and their 462 donors transplanted between 1999 and 2003 at the Renal Transplant Unit of Helsinki University Hospital, Finland.

Study V. The prospective study comprised 31 consecutive paediatric kidney allograft recipients transplanted between 2003 and 2006 at the Hospital for Children and Adolescents of Helsinki University Hospital, Finland.

4 ETHICAL PERMITS

Ethical permits for the studies were obtained from the relevant Ethical Committees of Helsinki University Hospital.

5 METHODS

5.1 Isolation of DNA (I, II, IV)

Whole blood samples were originally taken before transplantation for histocompatibility testing. The extracted DNA or buffy coats from citrate anticoagulated peripheral blood were stored at -20°C. DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN Inc, CA) or the FlexiGene DNA Kit (QIAGEN Inc, CA, USA).

5.2 Human leukocyte antigen typing (I)

For *HLA-A* and *HLA-B* typing serological lymphocytotoxicity method and for *HLA-DRB1* typing INNO-LiPA *HLA-DRB1* kit were used (Immuno Diagnostic Oy, Hämeenlinna, Finland).

5.3 Determination of single nucleotide polymorphisms (I, II, IV)

The SNPs for this study were chosen based on literature review of most promising candidate gene polymorphisms predicting the outcome of kidney Tx. Genetic variations in *TNF* G(-308)A, *TGFB1* T(+869)C and C(+915)G, *IL10* G(-1082)A, *IL10* C(-819)T, and C(-592)A, *IL6* G(-174)C, and *IFNG* T(+874)A genes of recipients and donors of studies I and II were determined using the Cytokine Genotyping Kit (One Lambda Inc. CA, USA). Study IV recipients and donors were genotyped for *F5* G1691A rs6025, *F5* A4070G rs1800595, *F2* G20210A rs1799963, *F13A1* G204T rs5985, *PROC* T8853G, *TFPI* C536T, and *MTHFR* C677T rs1801133, and those not included in study I also for *TNF* G(-308)A rs1800629, *IL10* A(-1082)G rs1800896, *IL10* C(-592)A rs1800872, and *IL6* C(-174)G rs1800795 at the Finnish Genome Centre, University of Helsinki, Finland, using the Sequenom MassARRAY system, based on MALDI-TOF technology and primer extension chemistry [292]. In addition, a few samples were only or also tested for *F5* G1691A rs6025 and *F2* G20210A rs1799963 at the Thrombosis Laboratory of the Finnish Red Cross Blood Service using a Factor V Leiden Kit and Factor II (Prothrombin) G20210A Kit (LightCycler® Instrument, Roche Diagnostics GmbH, Mannheim, Germany).

5.4 Isolation, quantification and validation of RNA from whole blood (III, V)

In study III 119 and in study IV 52 whole blood samples were drawn into Paxgene Blood RNA tubes (PreAnalytiX, Qiagen Nordic) and stored within 24 hours at -70°C until RNA purification. RNA was purified using the Paxgene Blood RNA kit (PreAnalytiX, Qiagen Nordic) according to the kit protocol and with an on-column DNase I treatment. The RNA was quantified with a GeneQuant™ pro RNA/DNA Calculator (GE Healthcare, Finland). The integrity of the isolated RNA was verified by electrophoresis using ssRNA Ladder Sample Buffer and ssRNA Ladder (New England Biolabs, USA).

5.5 Real-time quantitative polymerase chain reaction (III, V)

Synthesis of cDNA from isolated total RNA was done with a High Capacity cDNA Archive Kit (Applied Biosystems, Finland) according to the manufacturer's instructions. The relative gene expression was quantified using TaqMan Gene Expression Assays (Applied Biosystems, Finland), TaqMan Universal PCR Master Mix with no AmpErase UNG (Applied Biosystems, Finland) and ABI PRISM 7000. The probe and primer assays used in the studies are listed in Table 5. The endogenous control gene *PGK1* was chosen from a TaqMan Human Endogenous Control Plate (Applied Biosystems, Finland) as the stably expressed gene among three recipients with a concurrent rejection episode and their post-treatment samples. The relative expression was calculated using the standard curve method (User Bulletin 2, P/N 4303859B, Applied Biosystems) using one non-patient volunteer as a calibrator sample.

Table 5. Quantified genes in studies III and V.

Gene	Abbreviations	Assay identification code
perforin 1	PRF1, Per	Hs00169473_m1
fas ligand, tumor necrosis factor (ligand) superfamily, member 6	FASLG, FL	Hs00181225_m1
granzyme B	GZMB, GB	Hs00188051_m1
Granulysin	GNLY, Gra	Hs00246266_m1
tumor necrosis factor (ligand) superfamily, member 5	CD40LG, CD154	Hs00163934_m1
inducible T cell co-stimulator	ICOS	Hs00359999_m1
cytotoxic T-lymphocyte-associated protein 4	CTLA4	Hs00175480_m1
programmed cell death 1	PDCD1, PD-1	Hs00169472_m1
phosphoglycerate kinase 1	PGK1	Hs99999906_m1
transformer-2 alpha	TRA2A	Hs00203263_m1
interferon regulatory factor 2 binding protein 2	IRF2BP2	Hs00766250_g1
myristoylated alanine-rich protein kinase C substrate	MARCKS	Hs00158993_m1
transportin 1	TNPO1	Hs01007848_g1
solute carrier family 8 member 1	SLC8A1	Hs01062251_m1
homeodomain interacting protein kinase 1	HIPK1	Hs00380232_m1
Calreticulin	CALR	Hs00944361_g1
IQ motif containing GTPase activating protein 1	IQGAP1	Hs00896582_g1
Dcr-1 homolog (Drosophila)	DICER1	Hs00998583_g1
lysophospholipase I	LYPLA1	Hs00377829_g1
PRO1073 protein/metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	PRO1073/ MALAT1	Hs00273907_s1
CD3e molecule, epsilon (CD3-TCR complex)	CD3E	Hs00167894_m1
B-cell CLL/lymphoma 2-like 1	BCL2L1	Hs00169141_m1
polycomb group ring finger 5	PCGF5	Hs00260713_m1
CGI-69 protein	CGI-69	Hs00924962_g1
BCL2-associated athanogene	BAG1	Hs01105460_g1
N-ethylmaleimide-sensitive factor attachment protein alpha	NAPA	Hs00943303_m1
FK506 binding protein 8 38kDa	FKBP8	Hs01014666_g1

5.6 Low-density array (V)

RT-QPCR from 90 immune-related and six housekeeping genes 18S ribosomal RNA, beta actin, glyceraldehyde-3-phosphate dehydrogenase, *PGK1*, beta glucuronidase and transferrin receptor was performed in a low-density array format at the Section for Medical Genetics and Molecular Medicine, University of Bergen, Norway, according to the manufacturer’s protocol (TaqMan® Low Density Human Immune Panel card, 4370573, Applied Biosystems). The amount of cDNA counted from RNA concentration was 1 μg per card.

5.7 Microarray (V)

After isolation, 5 μg of RNA was treated using the Globin Reduction Protocol according to the manufacturer's directions (Globin Reduction Protocol: A Method for Processing Whole Blood RNA Samples for Improved Array Results Part No. 701497 Rev. 2, www.affymetrix.com). All experiments were performed using Affymetrix Human Genome U133 Plus 2.0 oligonucleotide arrays according to the manufacturer's directions. Sample labelling and hybridisation were carried out at the Finnish DNA Microarray Centre at Turku Centre for Biotechnology, Turku, Finland.

The Affymetrix GeneChip Operating Software (GCOS) detection algorithm was used to determine the presence or absence of expression for each transcript. A transcript with either a present or a marginal detection call was considered expressed. The complete gene expression data are available at http://qp01.novogroup.com/vpu. The GCOS change algorithm was used to compare the data of patients with rejection at three months post-Tx against the same patient’s six-month post-Tx sample data, or against the data of patients without rejection at three months, to detect and quantify changes in gene expression. The transcripts assigned with increased, decreased, marginally increased or marginally decreased change calls were considered differentially expressed. Only those genes that were increased or decreased by at least a two-fold change in all rejection patients compared with normal patients at three or six months were studied further.

5.8 Statistical analyses (I - V)

Preliminary power analysis was done with the Genetic Power Calculator [293] based on known baseline characteristics (odds ratio 3 - 10 according to outcome and SNP) and results published by others on the incidence of the studied factors (IV) [170,175,182]. The Hardy-Weinberg equilibrium of genotypes was tested using Fisher's Exact Test (I, II, IV). Descriptive statistics were used to describe the baseline characteristics and duration of follow-up. In bivariate analysis, Fisher's exact test was used to compare the occurrence of AR, thrombosis, infarction or composite endpoint from the above with respect to categorical baseline characteristics. Corrections for multiple testing were not done and a P-value <0.05 was considered statistically significant in studies I - III and V. The correction for multiple testing was done using the Bonferroni correction according to 11 DNA polymorphisms studied in 9 candidate genes without taking into consideration the number of studied phenotypes that give statistically significant P-values less than 0.0045 (IV). In addition, the distributions of continuous baseline characteristics with respect to studied complications and available laboratory results after Tx were compared using a Mann-Whitney U test and a Kruskall-Wallis test. Bivariate survival analysis was done with a Kaplan-Meier analysis and log rank test (II - IV). To adjust for confounding factors, multivariate logistic regression (I, II, IV) and Cox proportional hazards models (II, IV) were used to analyse the risk factors associated with AR, thrombosis, infarction, composite endpoint or 1st year GS after Tx. Statistical analyses were done using SPSS 13.0.1 (SPSS, Finland), StatsDirect 1.8.10 (StatsDirect, UK) or MedCalc 8.1 (MedCalc Software, Belgium).

6 RESULTS AND DISCUSSION

6.1 Acute rejection (I, IV)

Studies I and IV aimed to find associations between SNPs and AR, thrombosis, infarction, or GS. Such findings would justify further study of the associations prospectively in a larger study population. Eventually it might be possible to determine whether asymptomatic patients would benefit from individualized immunosuppression, antithrombotic prophylaxis or other medication according to their genetic status.

The overall incidence of AR in the study I was 17.2 %, i.e. 50 episodes. Two pre-transplantation factors were found to increase the risk of AR: presence of 3 - 4 *HLA-A, -B* or *-DR* mismatches (MM) (OR 1.8, 95 % CI 1.1 – 3.1 and P = 0.027), and a graft from CMV-seronegative donor to CMV-seronegative recipient increased the risk of AR (OR 2.2, 95 % CI 1.2 - 4.1, P = 0.038).

The cytokine gene polymorphisms studied in study I were *TNF* G(-308)A, *TGFB1* T(+869)C and C(+915)G, *IL10* G(-1082)A, *IL10* C(-819)T, and C(-592)A, *IL6* G(-174)C, and *IFNG* T(+874)A. *TNF* and *IL10* are the cytokine genes previously reported as most likely to have relevance for allograft outcome [154]. In addition, *IFNG, IL6* and *TGFB1* have in some studies been found to have an impact on the development of AR [96,128,135,294]. All of these studies were relatively small of under 200 recipient or donor participants, the obtained p-values were rarely clearly less than 0.05 or corrected for multiple testing and usually derived from only a few SNPs. However, these were the SNPs most studied and associated positively to kidney Tx outcomes so we limited the scope of the study to these five genes.

Among 772 transplant recipients in study IV, an AR episode was diagnosed in 139 (18%) subjects. We genotyped *F5* G1691A, *F5* A4070G, *F2* G20210A, *F13A1* G204T, *PROC* T8853G, *TFPI* C536T, *MTHFR* C677T, *TNF* G(-308)A, *IL10* A(-1082)G, *IL10* C(-592)A, and *IL6* C(-174)G, giving a total of 11 SNPs in nine genes in the study IV. This resulted in a Bonferroni corrected significant P-value of 0.005. None of the SNPs were significantly associated with AR. The level of *HLA* mismatch (Hazard ratio (HR) 2.3, 95% CI 1.6 - 3.3), DGF (HR 1.8, 95% CI 1.2 - 2.6) and the age of recipient (HR 0.97, 95% CI 0.96 - 0.99) showed association with AR by Cox proportional hazard model. The last mentioned two known risk factors were also statistically significant in bivariate analysis and in logistic regression analysis.

6.1.1 Only a few gene polymorphisms were associated with acute rejection (I)

The distribution of the frequencies of the cytokine genotypes was found to be in the Hardy-Weinberg equilibrium. There were no differences in frequencies between the recipients and donors. The cytokine polymorphisms at genotype level in the recipient and respective donor were compared by univariate analysis with respect to AR status (I). The recipient *TNF* -308AA genotype was statistically significantly associated with AR (Rejection rate 80%, OR = 5.0, P = 0.0034).

Chronic rejection triggers TNF production in stimulated monocyte cultures of affected patients [295]. In addition, TNF is more often detected in plasma and urine in renal allograft recipients with AR [296]. The dbSNP lists more than 37 polymorphisms at the 5′ end of the *TNF* gene, of which the G(−308)A (rs1800629) SNP is probably the most frequently studied. After HapMap project and other extensive resequencing projects, it is now known that only 3 SNPs (among them -308) are needed to capture 80 % of the common genetic variation in *TNF* overall among European–Americans [297]. More extensive approach by studying all the *TNF* SNPs in a small number of samples and defining the LD between them was out of reach of this study, even though this would have given more information about the definitive SNP affecting the association between *TNF* and AR. *TNF* -308A allele is considered a six- to seven-fold stronger activator of transcription than the more common G allele [110]. However, Bayley et al. reported that the -308 SNP had no functional relevance for *TNF* transcription [298,299]. Probably due to conflicting results in expression studies, the reported effects of *TNF* A(-308)G SNP on Tx outcome have been variable. The -308 SNP was found to be associated with AR in heart [300] and renal allograft recipients and the A allele predisposed to AR in these studies [96,112-114]. A combination of *TNF* -308 AA or GA genotypes with *IL10* -1082AA genotype in the same heart Tx patient significantly increased AR risk [301]. However, several studies of kidney and heart Tx patients have failed to find an association of -308 SNP with AR [98,124,126,127].

Some of the studies have included small groups (100 - 200 participants) of uniform genetic subjects, e.g. Caucasians and African-Americans, having genetic backgrounds with different risk factors [98]. Further, multivariate analysis has not always been performed due to missing clinical factors (known or hypothesized confounding factors) or insufficient numbers of subjects (not enough power perform subgroup analyses) [96,114,127]. The multivariate analysis in the present study was done with both a prior hypothesis and hypothesis-free approaches.

However, the impact of genetic factors in predicting AR was small and did not exceed the impact of known clinical factors.

In published reports the genotype frequencies that may suggest that the Hardy-Weinberg equilibrium is not fulfilled are usually not listed, probably due to lack of space or because of being perceived as uninteresting to a journal audience. In addition, the studied outcome incidences vary to a large degree, probably reflecting major variances in the amount of immunosuppression administered to patients [124,126]. This may mask or inflate the reported importance of the studied SNPs by altering the observed association of outcome with gene polymorphisms.

TNF and *HLA* genes are in strong LD [302]. Only some studies take this into account as a confounding effect when considering *HLA* mismatches between recipients and donors and *TNF* gene polymorphisms associating with outcome [96,112,127,301,303]. The *TNF* -308A-allele lies within the *HLA-A1-B8-DR3-DQ2* extended haplotype which is associated with various autoimmune diseases e.g. IgA deficiency and systemic lupus erythematosus [304]. In our study, the *HLA-A1-B8-DR3* haplotype was not associated with AR despite the recipient *TNF* -308AA genotype being associated with AR.

In addition, *IL10* polymorphisms have been reported to associate with AR in several reports [98,112,123,134], but not in others [96,113,124,126,127,135]. Contradictory results may in part be result of insufficiently powered studies. Gene polymorphisms in the IL10 gene have been found to constitute conserved haplotypes in many populations (only certain haplotypes are found), combined (low and intermediate or high and intermediate are combined) or separate genotypes associating with low, intermediate or high IL10 protein production *in vitro* [305]. We did not find association between *IL10* -1082AA (low producer) or AG/GG (high producers) genotypes and AR, whether or not they occurred together with the *TNF* -308 AA/AG (high producers) or GG (low producer) genotypes. The *IL10* low/*TNF* high profile associates with AR in heart Tx [301] and the *IL10* high/*TNF* high profile associates with AR in kidney Tx [112]. The association with AR of *IL10* gene polymorphism may be influenced by the allograft, the immunosuppressants used, the criteria of AR diagnosis, and other unknown or known factors between hospitals and studies.

6.1.2 Genetic variation in thrombosis-associated or cytokine genes did not associate with AR (IV)

Heidenreich et al. reported a medium sized prospective study showing an association between AR and *F5* G1691A, *F2* G20210A or *MTHFR* C677T in 165 kidney Tx recipients [175]. Their study had an exceptionally high incidence of AR, 44% as compared to 18% in our study, which may indicate under-immunosuppression in their study. However, our results were similar to the findings of Pherwani et al. who found no association in recipients or donors between AR and *F5* G1691A, *F2* G20210A or *MTHFR* C677T. They reported a retrospective study on paediatric and adult patients who were given heparin for postoperative thrombosis prophylaxis. They did not report overall AR incidence, but their first-year graft failure rate due to AR was 4.1% compared to only 1.4% in our study, suggesting that different immunosuppression and *HLA* matching policy was used [178].

An advantage of our study was that the Finnish population is genetically relatively homogeneous, which probably helps identification of weaker associations between genetic factors and disease outcome [306]. One might postulate that in the absence of statistically significant genetic associations of the marker polymorphism in the Finnish population, these cannot be found in genetically more mixed populations either, assuming equal prevalences of the marker polymorphism in the populations. The converse may also be true, i.e. that an association of the marker polymorphism significant in the Finnish population may not be found in a more mixed population.

A more ambitious approach than analysis of candidate genes would be a genome-wide analysis of gene polymorphisms, as other genes involved in the thrombotic cascade may associate with AR. However, we studied 11 DNA polymorphisms in nine candidate genes, including eight SNPs not previously studied in a kidney Tx setting, making our investigation the most extensive study of thrombosis-associated polymorphisms in adult kidney Tx so far.

Despite almost 800 Txs in the study IV, the AR incidence was only 18% and several SNPs were found to be quite rare (four rare allele incidences <2%). A larger study material is therefore needed for definite conclusions. We could not replicate the association found in our study I between *TNF* SNP and rejection. This may possibly be due to the first finding being a false positive result. Other possible reasons for non-replication are not correcting for multiple testing in the first study and the inflated OR in the small sample size of *TNF* -308AA genotype carriers.

In conclusion, we were unable to find associations between SNPs and AR in the retrospective adult kidney Tx setting. We could not provide data to support the routine determination of gene polymorphisms before Tx for AR prediction.

6.2 Genetic variation in interleukin 10 but not in other cytokine genes predisposed to CMV infection (II)

We aimed to predict the incidence and timing of CMV infection in adult deceased kidney Tx by analyzing cytokine gene polymorphisms, because cytokines are major mediators of immunological responses to CMV infection and CMV infection may impair kidney graft function after Tx. CMV diagnosis was defined as clinical CMV suspicion (fever and/or elevated serum creatinine value without rejection) and positive pp65 antigenaemia test. This study targeted the high-risk group of CMV serology negative recipients (R-), with CMV serology positive (D+) donors. We genotyped 71 recipients and 62 donors for *IL10* G(-1082)A, *IL10* C(-819)T, and C(-592)A, *IL6* G(-174)C, and *IFNG* T(+874)A cytokine gene SNPs. There were 24 diagnosed CMV episodes. Of the recipients, 22 received three-month CMV prophylaxis of gancyclovir or valgancyclovir and five a short prophylaxis (6-21 days) during a concurrent AR episode. Three-month prophylaxis, immunosuppressive treatment without antibody induction, and normal onset of graft function reduced the incidence of CMV, but not significantly (P = 0.26, 0.15, and 0.16, respectively) (Fig. 6a-b).

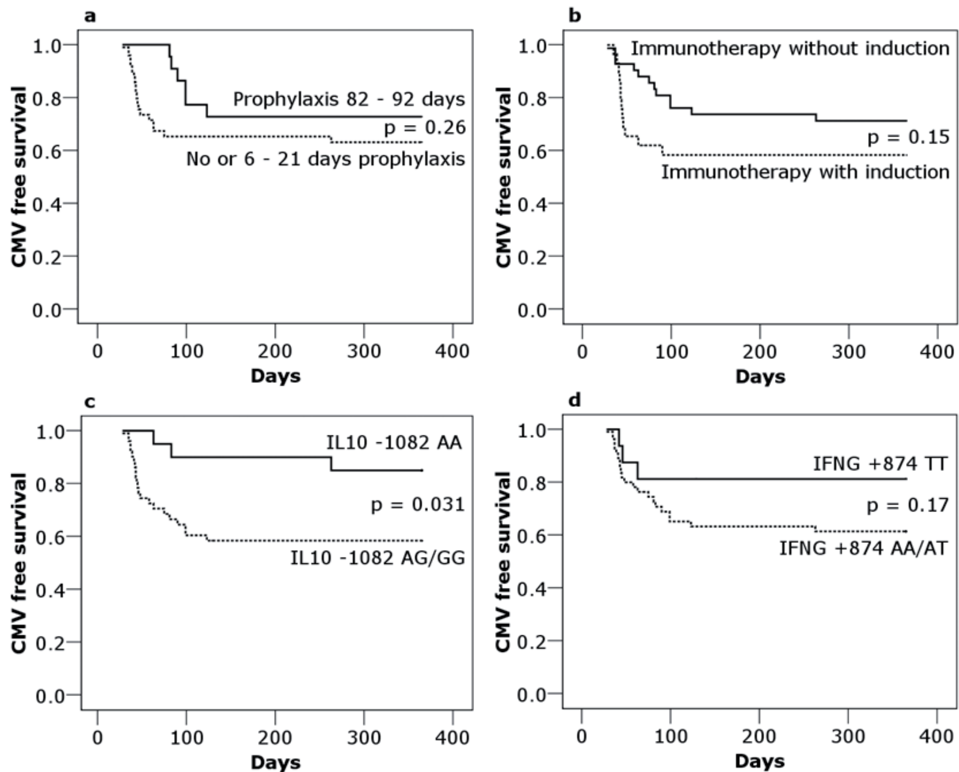

Figure 6. Kaplan–Meier survival curves of recipient clinical features and donor cytokine polymorphisms affecting CMV incidence. a) Long prophylaxis compared to no or short prophylaxis, b) immunotherapy (black) and delayed graft function (DGF, grey), c) donor *IL10* −1082 genotype, and d) donor *IFNG* +874 genotype. Copyright © 2006 Elsevier B.V. All rights reserved [307].

Recipient and donor cytokine polymorphisms were compared by Kaplan-Meier analysis. The analysis was done between patients with and without CMV diagnosis, at genotype and/or phenotype level. The donor *IL10* -1082AA genotype decreased the incidence of CMV (P = 0.031) (Fig. 6c). The *IL10* -1082AA genotype has been associated with low IL10 cytokine production [308]. CMV incidences were three out of 20 (15%) in AA and 21 out of 51 (41%) in AG/GG genotypes. The median time to CMV diagnosis was 83 days for AA and 45 for AG or GG genotypes (Mann-Whitney p for trend = 0.072). Further, none of the recipients receiving a graft from a donor with *IL10* -819TT/-592AA genotype (which is also associated with a phenotype of low IL10 cytokine expression) developed CMV. However, since there were only five such patients the effect did not reach statistical significance (P for trend = 0.051) and would not have been clinically relevant.

Donor *IL6* -174CG/GG and donor *IFNG* +874TT genotypes (both associated with high cytokine expression phenotype) [308] were both slightly less common, but not significantly, in cases developing CMV (P = 0.33, 0.17, respectively) (Fig. 6d). The 66 available recipient genotypes did not associate with CMV incidence.

Multivariate analysis with the Cox proportional hazards model gave no other variable except donor *IL10* -1082AA genotype, which was alone significant with respect to reduced incidence of CMV (HR 0.3, 95% CI 0.09 – 0.96, P = 0.043).

Human IL10 is an immunosuppressive cytokine which suppresses cytokine and chemokine production by macrophages, T-helper cells, NK cells and peripheral blood mononuclear cells [309]. CMV encodes a homologue of human IL10, which the virus uses to evade immune surveillance [310]. The homologue probably allows the virus to survive in infected cells, replicate and develop a full infection [311]. Accordingly, a genetically CMV susceptible mouse strain produced higher quantities of IL10 leading to the Th2 type of immunoresponse compared to a mouse strain genetically resistant to CMV [312]. Thus, in the viral microenvironment, high quantities of IL10 probably augment the spread of infection. In our study, the IL10 low production genotype in donors was found to protect against CMV reactivation. It seems possible that CMV infected cells in donor organs produce extra IL10 with an effect similar to the viral homologue.

On the other hand, Conti et al. reported that systemic production of IL10 by recipient cells stimulated B cell proliferation, antibody secretion and development of cytotoxic T cells [309]. However, these late occurring systemic responses to infection probably limit the extent of the infection and damage, rather than inhibiting the early stages of CMV reactivation. The genetic background of the recipient can be assumed to influence more the systemic response than that occurring locally within the graft with donor background.

The known predisposing factors in kidney allograft recipients for CMV disease are high-dose immunosuppressive protocol, co-morbidity, viral load, and DGF [313,314]. In a large meta-analysis, anti-viral prophylaxis significantly reduced the risk of CMV disease (19 trials, 1981 patients; relative risk 0.42 [95% CI 0.34–0.52]) and infection (17 trials, 1786 patients; 0.61 [0.48–0.77]) [315]. We found only a trend-level association between prophylaxis and reduced CMV incidence, probably due to the relatively small patient (71) population.

In conclusion, we found that donor *IL10* -1082AA genotype was associated with a reduced incidence of CMV in CMV-seronegative recipients of allografts from seropositive donors. Further and more extensive investigations may confirm the clinical relevance of this risk

factor. Moreover, the genetic background of the donor may help to assess post-Tx risks in kidney allograft recipients.

6.3 Genetic variation in thrombosis-associated or cytokine gene polymorphism did not predispose to thrombosis (IV)

In this study there were 38 (4.9%) recipients diagnosed with venous thrombosis and/or pulmonary embolism. We genotyped *F5* G1691A, *F5* A4070G, *F2* G20210A, *F13A1* G204T, *PROC* T8853G, *TFPI* C536T, *MTHFR* C677T, *TNF* G(-308)A, *IL10* A(-1082)G, *IL10* C(-592)A, and *IL6* C(-174)G, totalling 11 SNPs in nine genes. Of the non-genetic factors, patient age over 50 years increased the risk of suffering a thrombotic complication (OR 2.8, 95% CI 1.3 - 5.8). The SNPs studied in the recipients or donors were not significantly associated with any of the studied outcomes after correcting for multiple testing.

Increased risk for thrombosis in renal transplant recipients and association with *F5* G1691A has been found in two reports [170,173]. These studies had 300 and 202 recipients, and 26% and 11% incidence of thrombosis, respectively. Sample size was moderate in both, but by current standards the thrombosis incidence was exceptionally high, which may augment the risk associated with *F5* G1691A. A negative association was found in three reported studies involving 109, 394, and 509 recipients (and also 457 donors in the last study) with 27%, 6%, and 8% incidences of thrombosis, respectively [172,174,178]. The first two negative studies probably did not have enough statistical power to detect positive association and in the last study all the recipients had heparin prophylaxis after surgery, which probably prevented all renal vein thromboses and immobility-based thromboses.

The effect of *F5* G1691A mutation (FVL) has been well established in thrombosis patients and their relatives to increase the risk of thrombosis by seven- to 80-fold, whether heterozygous or homozygous for the mutation [316]. ESRD patients with increased susceptibility to thrombosis are commonly identified during haemodialysis with problems of blood clotting in dialysis catheters. However, whether anticoagulation medication is continued after Tx depends on the centre, in the absence of uniform international consensus [88].

Pherwani et al. reported negative *F2* G20210A mutation association with thrombosis in 562 renal transplant recipients and 457 donors [178]. In their study all recipients had heparin prophylaxis for thrombosis, which may modify the negative genetic association. Even though *F2* G20210A

mutation may not specifically increase the risk of thrombosis in renal Tx recipients, it may decrease GS by association with other complications like vascular rejection [88]. However, *F2* G20210A mutation heterozygosity is associated with a 2.8-fold increased risk of thrombosis compared with non-mutation carriers [316].

Apart from the present report (IV), there are no kidney Tx studies regarding the association between *MTHFR* C677T polymorphism and thrombosis. However, a recent meta-analysis of published epidemiological studies of 8364 first-time or recurrent thrombosis cases and 12 468 controls found that the 677TT genotype was associated with a 20% higher risk of thrombosis compared with the 677CC genotype. This may be modified by the nutritional intake of folate and riboflavin, which can cause contradictory association results between populations with different diets [317]. Finnish kidney Tx patients often take multivitamins including low doses of folate and riboflavin, which may in part explain our negative results if low doses are considered to be effective.

6.3.1 Thrombosis-associated or cytokine gene polymorphisms did not predispose to infarction (IV)

In this study there were 48 recipients with cerebral infarcts (35.4%), with myocardial infarcts (62.5%), and with kidney infarcts (2.1%). Non-genetic or genetic factors of the recipients and donors did not associate with infarcts.

These results are in accordance with studies that did not find association between infarction and *IL6* G(-174)C [318,319], or *F5* A4070G [320], or *F5* G1691A [319], or *F13A1* G204T, *IL10* A(-1082)G, *IL10* C(-592)A, *MTHFR* C677T and *F2* G20210A [319]. There is only one published study on kidney Tx patients and it did not report association between *F5* G1691A and myocardial infarction [170]. Studies finding association between infarction and *F5* G1691A [321], or *MTHFR* C677T [183] did not study kidney Tx outcomes but myocardial infarction among other cardiovascular diseases. In one genome-wide association study LTA associated with infarction in a Japanese population [322] but not in a replication study also among Japanese [323]. This non-replication may be due to different diagnostic criteria and lack of statistical power [324].

6.3.2 Thrombosis-associated or cytokine gene polymorphisms were not associated with graft survival

Graft loss within the first year after Tx was due to AR, thrombosis or infarct in 28 (62%) out of 45 losses (other graft losses were due to nine patient deaths and eight other causes, e.g. primary disease relapse). We genotyped *F5* G1691A, *F5* A4070G, *F2* G20210A, *F13A1* G204T, *PROC* T8853G, *TFPI* C536T, *MTHFR* C677T, *TNF* G(-308)A, *IL10* A(-1082)G, *IL10* C(-592)A, and *IL6* C(-174)G, totalling 11 SNPs from nine genes. The one-year GS was not significantly influenced by the studied genetic or non-genetic factors of recipients or donors analysed by the bivariate analyses.

Recently, one large multicentre study compared the association between the GS of 676 cadaver kidney first or 651 retransplants and *F5* G1691A, *F2* G20210A or *MTHFR* C677T polymorphisms. In this study *F2* G20210A associated with the three-year survival of first transplants [180]. We analysed our data separately for the first and retransplant subgroups but did not find an association with GS. This may have been due to our small study size.

In addition, FVL was reported to associate with GS in a study of 109 kidney recipients with a high incidence (27%) of vascular rejection [172]. In our study the vascular rejection incidence was 2%, which probably affected our negative finding. Our results are similar to three other studies which were also unable to find association between GS and FVL [175,177,178].

Two further studies reported association between *F2* G20210A and GS [175,182]. The first had a 13% retransplant rate compared to our rate of 1%. The latter study was a prospective investigation with high AR (44%, ours 18%) and acute vascular rejection (28%, ours 2%) incidences, suggesting under-immunosuppression or over-selection of patients with additional risk factors. A study reporting negative association between *F2* and GS [178] involved the use of heparin post-Tx thrombosis prophylaxis which may have reduced the incidence of fatal complications post-Tx. In our study most patients were on acetylsalicylic acid, which may have reduced graft thrombosis and cardiac complication incidences leading to graft loss.

Although several studies have reported an association between *MTHFR* C677T and homocysteine or lipid levels [184-192], none has reported a positive association between *MTHFR* C677T and GS [175,193,194,196]. Most study patients take multivitamins including low doses of folic acid and riboflavin, which may reduce homocysteine levels irrespective

of *MTHFR* C677T genotype status. The *MTHFR* C677T genotype may have an influence on GS through increased (OR = 1.2) susceptibility to cardiovascular complications [325], probably warranting large prospective studies including diet and multivitamin use information.

Cytokine gene polymorphisms were not associated with GS in our study. Similarly, several studies have reported negative associations between *TNF* G(-308)A, *IL10* G(-1082)A, C(-592)A, *IL6* G(-174)C and GS [96,100,116,117,141,326]. Mytilineos et al. reported an association between 59 retransplanted *TNF* -308AA carriers and GS [117], which we were not able to study as we did not have retransplanted *TNF* -308AA carriers in the study population.

6.4 Gene expression markers showed similar time-related variance during hospital stay in control patients (III)

The aim of this study was to determine whether analysis of whole blood gene expression of lymphocyte marker molecules would offer a robust means to diagnose AR after kidney Tx. If clinically useful, such a method would provide a non-invasive, easy, and rapidly performed test that could be routinely applied to detect rejection at an early phase before permanent injury has occurred. In particular, collection of whole blood into tubes with RNA stabilising solution that can be stored at room temperature would allow easy adaptation to routine hospital sample collection.

We decided to test the relevance of this method by investigating eight candidate molecular markers: perforin 1 (Per), fas ligand (FL), granzyme B (GB), granulysin (Gra), CD40 ligand (CD154), inducible T cell co-stimulator (ICOS), cytotoxic T-lymphocyte-associated protein 4 (CTLA4), and programmed cell death 1 (PD-1). Per, FL, GB and Gra are lymphocyte cytotoxic molecules that have a clear role in the molecular process of allograft rejection and that have previously been suggested to have diagnostic value [327]. In addition, we hypothesized that the gene expression quantities of co-stimulatory molecules CD154, ICOS, CTLA4, and PD-1 associated with T-cell activation would be significantly altered at a time of an AR episode and therefore be able to distinguish rejection from non-rejection samples. These were chosen based on a literature search.

Control patients were studied in order to determine the changes in gene expression associated with pre- and post-Tx course without rejection. The relative quantity of four costimulatory (ICOS, CD154,

CTLA4 and PD-1) and four cytotoxic (FL, Gra, GB and Per) genes followed the same general pattern after Tx. There was a rapid decrease of gene expression by approximately 50% between day 0 and day 1, followed by a slow increase so that by day 21 the levels were again close to those on day 0 (Fig. 7).

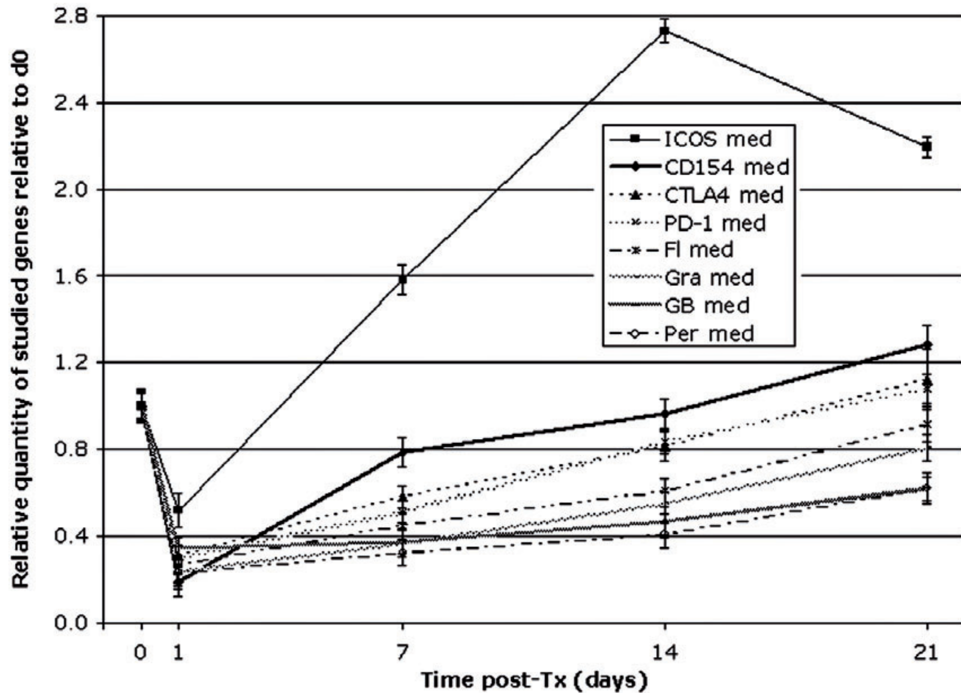

Figure 7. The relative gene expression quantities of studied genes relative to the d0 sample of control patients. Error bars represent the standard deviation of replicate wells normalised to endogenous control gene and calibrator. Med denotes median. Copyright © 2007 Lippincott Williams & Wilkins. All rights reserved [289].

The two studies of serial analysis of whole blood gene expression quantities after Tx are in accordance with ours [251,290]. One study reported lower quantities of granulysin on post-Tx day 1 than before Tx and higher levels on day 7 than on day 1 [290], and the other noted up-regulation of Per and GB in eight paediatric renal allograft recipients between day 1 and day 30 in peripheral blood leukocyte samples [251]. It seems likely that the early decrease in whole blood gene expression quantities reflects the massive infiltration of T-cells expressing the studied markers to the transplanted kidney at the time of Tx [328], which is gradually reversed as time passes and immunosuppressive medication stabilises the situation.

6.5 The expression of two genes was different in rejection patients than in control patients (III)

In order to determine whether AR episodes were associated with changes in gene expression, we compared 25 rejection and 15 control patients. The gene expression quantities of cytotoxic molecules were lower in the rejection patients than in the controls, although the differences were not statistically significant (Fig. 8). The gene expression quantities of the costimulatory molecules ICOS and CD154 were significantly lower

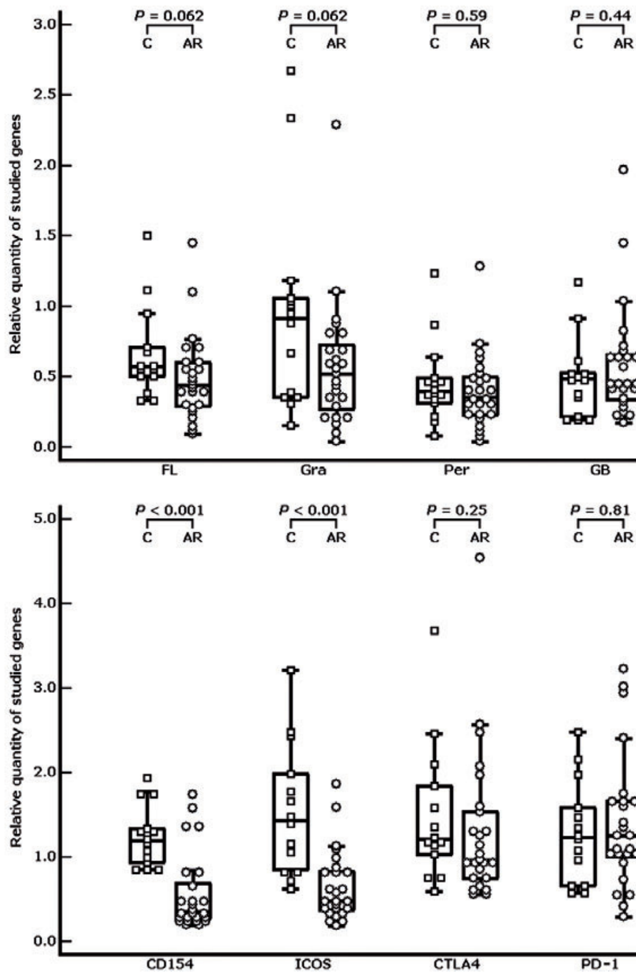

Figure 8. The relative gene expression quantity of eight studied genes compared between individual control patient medians and rejection patient samples from post-Tx day 8. The combined box/whisker and dot plots show 10%, 25%, 50% (median), 75%, and 90% percentiles, all the data points of individual outliers are between 90% and 100% percentiles. C, control patients; AR, rejection patients. Copyright © 2007 Lippincott Williams & Wilkins. All rights reserved [289].

in rejection patients ($P<0.001$, both). The gene expression quantity of the costimulatory molecules CTLA4 and PD-1 showed no statistically significant differences between rejection patients and controls (Fig. 8).

Our study design did not allow us to serially analyse samples taken from the same patient until rejection occurred. In the protocol, samples of individual patients are calibrated with their own pre-Tx sample and in the constellation Per and GB gene expression has been found to increase before the onset of an AR episode [283,284]. In a similar investigation, CD154 and ICOS might be better to differentiate rejection from non-rejection patients.

Mechanistically, it seems likely that the decreases in CD154 and ICOS gene expression in whole blood we observed during rejection and/or other complications are due to lymphocytes expressing the molecules migrating to the allograft during injury. CD154 and ICOS gene expression increases in renal allograft biopsies of recipients with subclinical or acute cellular rejection [268], and FL, Gra, GB, Per, CD154, ICOS, CTLA4 and PD-1 gene expression increases in SCR and AR biopsy material compared to stable allograft biopsies [267,268]. Similarly, in kidney biopsies CD154 expression increases more during AR episodes than during other types of renal dysfunction (cyclosporine toxicity, acute tubular necrosis, recurrence of primary disease, vascular complication or chronic rejection) [329].

The comparison of our results with data reported by other investigators is challenging since there are differences in patient population, immunosuppression, sampling schedule, sample type, sample handling and the analysis itself [198,330-332]. Possibly the single most important technical difference between our study and previous investigations is that we used a tube pre-filled with RNA-stabilising solution to draw samples into. To our knowledge there are no previously published results utilising this particular method in kidney transplant recipients, making direct comparison of results impossible. The whole blood sample drawn into the tubes did not give the same transcriptional profile as kidney biopsy, urine cells, isolated blood cells or whole blood in studies utilising different technology, probably due to the different sample processing method. In particular we were unable to detect the significant increase in the expression of cytotoxic gene markers that is usually reported to occur during an AR episode [333].

6.6 Rejection patients differed from non-rejection patients with regard to one gene expression marker (III)

The 25 rejection patients were compared to the 10 kidney biopsied non-rejection patients (RS). The gene expression quantities of all cytotoxic molecules (except FL, which did not differ) were lower in rejection patients; however the differences did not reach statistical significance (Fig. 9). The gene expression quantities of costimulatory molecules

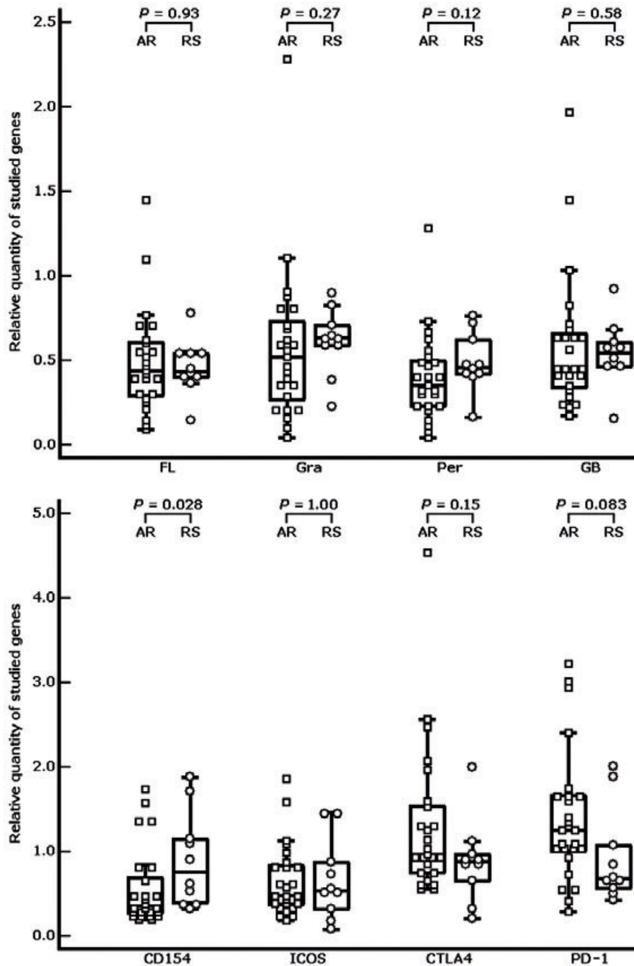

Figure 9. The relative gene expression of the studied genes compared between rejection and non-rejection patients. The combined box/whisker and dot plots show 10%, 25%, 50% (median), 75%, and 90% percentiles; all the data points of individual outliers are between the 0% and 10% or 90% and 100% percentiles. AR, rejection patients; RS, non-rejection patients. Copyright © 2007 Lippincott Williams & Wilkins. All rights reserved [289].

CD154 and ICOS were also lower in rejection patients, but only CD154 reached statistical significance (P = 0.028). The gene expression quantities of CTLA4 or PD-1 did not differ significantly (Fig. 9).

In conclusion, we were unable to determine that the use of whole blood gene expression of lymphocyte molecules would be feasible for the clinical diagnostics of acute kidney allograft rejection. We discovered that the whole blood gene expression of costimulatory molecules CD154 and ICOS reasonably robustly differentiated rejection patients from control patients. However, the clinical use of the analysis is limited by poor capability to differentiate rejection patients from those with other complications causing a suspicion of clinical rejection.

6.7 Candidate gene expression markers did not differ between patients with subclinical rejection and control patients (V)

The aim of this study was to analyse gene expression differences between SCR and non-rejection patients by microarray and RT-QPCR in whole blood samples, in order to determine whether the approach could be clinically useful as a non-invasive method to diagnose SCR in kidney Tx. We measured the relative gene quantities of CD154 and ICOS from whole blood samples taken three and/or six months after Tx. We analysed 31 samples taken at three months. Thirteen of these were from patients whose kidney biopsy was classified as SCR. Eighteen of the samples were from patients with biopsies showing no rejection signs.

At three months, the whole blood CD154 and ICOS expressions in patients with or without SCR were not significantly different (medians 1.16 vs 1.25 and 1.61 vs 1.95 for CD154 and ICOS, respectively) (Fig. 10). In addition, the three- and six-month samples showed no significant expression change in either CD154 or ICOS regardless of whether the patient had SCR at three months or not (medians 1.25 vs 1.67 and 1.50 vs 1.87 for CD154 and ICOS, respectively).

We collected blood samples into tubes pre-filled with a RNA-stabilising solution. These tubes may circumvent the problem of false gene expression results caused by the degradation of RNA or activation of blood cells during collection, transportation, storage and batch processing of samples [334-337]. However, whole blood sample results may be different to kidney biopsy or urine sample results as the different source samples may not carry the same qualitative and/or quantitative biomarker information [331].

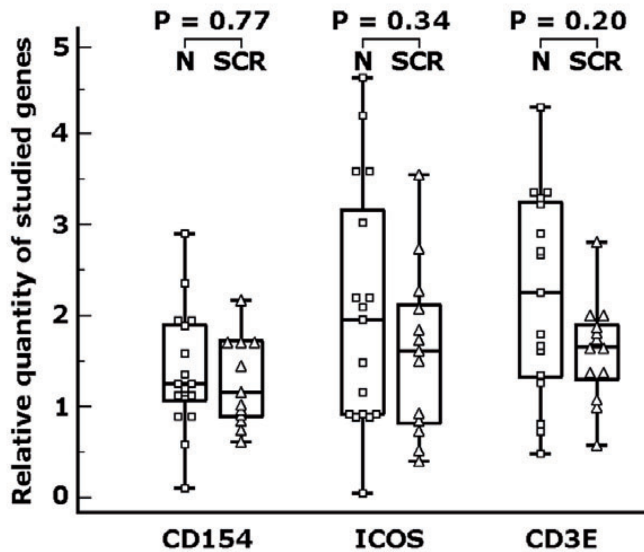

Figure 10. The relative gene expression quantities of CD154, ICOS, and CD3E in 13 subclinical rejection and 17 normal samples taken at the three-month clinical visit. N, normal patients; SCR, subclinical rejection patients.

We were unable to separate patients with or without SCR with CD154 and ICOS expression in whole blood samples, although they separated adult kidney Tx with or without clinical rejection in our study III. Subtle gene expression changes and/or different molecules involved in subclinical compared to clinical rejection episodes may explain the difference in results.

6.8 Gene expression markers of the low-density array were unable to identify patients with subclinical rejection (III)

We performed the LDA of immune genes from five kidney biopsy-proven normal, five SCR samples, and one calibrator sample from a healthy untransplanted volunteer. Of the 96 genes in the array, at least three normal, three SCR samples and the calibrator sample expressed 57 genes. The only significantly differently expressed (P = 0.04) gene was CD3E (CD3e molecule, epsilon [CD3-TCR complex]), which was slightly decreased in samples with SCR compared to samples without SCR (medians 1.12 vs. 2.65, respectively). For array finding validation, we measured with single RT-QPCR reactions the relative expression of CD3E

from the above samples and a further 12 normal and seven SCR three-month samples. The CD3E expression was not statistically significantly decreased in samples with SCR compared to samples without SCR (medians 1.66 to 2.25, respectively) (Fig. 10).

Since we did not find association between gene expression of CD154 or ICOS and SCR, we examined in LDA format the expression of immunological genes, including several of the candidate markers for rejection proposed by other investigators [268]. Although CD3E expression was significantly different between patients with and without SCR in LDA, we were unable to observe this difference in a larger number of samples, probably due to heterogeneous expression. A decreased CD3E molecule amount in peripheral blood lymphocytes associates in kidney Tx with long-term surviving kidneys [338], while increased CD3E gene expression associates with AR [277].

6.9 A robust biomarker specific for subclinical rejection could not be identified in whole genome microarray analysis (III)

We performed two separate hybridisations. The first hybridisation consisted of eight paired whole blood samples of four patients. The pairwise samples came from patients with SCR in the three-month protocol biopsy (Banff grade 1A) and six-month protocol biopsy with normal Banff grade. After GCOS analysis, we further analysed all the genes with at least two-fold average change. There were 26 probe sets representing 20 genes. From these, we quantified with RT-QPCR six genes (BCL2L, BAG, CGI69, NAPA, FKBP8, PCGF5) with the largest fold change. However, none of the genes expressed significantly differently in RT-QPCR.

In the second hybridisation, we processed four whole blood samples from patients with a normal three-month protocol biopsy. With GCOS analysis we compared these samples to those of four patients with a SCR in their three-month biopsy from first hybridisation. From these comparisons, we listed all the genes that were common to all the samples with significant change between SCR and normal samples. We found 29 shared probe sets representing 27 genes. From these, we further analysed 11 genes (MALAT1, TRA2A, IRF2BP2, MARCKS, TNPO1, SLC8A1, HIPK1, CALR, IQGAP1, DICER1 and LYPLA1) with over two-fold change in most patients or the largest average fold change. We performed RT-QPCR analysis from the samples that were in the microarray analysis and an additional 19 three-month samples (11 from

patients without and eight from patients with SCR). However, we could not find differences between patient groups with RT-QPCR as expression was heterogeneous and overlapping between normal and SCR patients.

As we recognised that many of the human genes involved in SCR were not present in the limited, immunologically oriented LDA, we performed a whole genome array to determine whether, with this approach, we could identify surrogate SCR marker genes. Since RNA isolated from the whole blood contains excessive amounts of globin mRNA, which may interfere with the microarray hybridisation [339], we performed globin reduction before hybridisation.

We found several differently expressed genes when we compared SCR with normal samples or samples taken after the clearance of SCR. However, the average expression fold changes were low and differences between samples large, suggesting that the genetic pathways contributing to SCR are heterogeneous. Further, because in the clinical setting SCR manifests itself solely in the graft, analysing whole blood samples drawn from peripheral veins may not fully reflect the graft processes. After validation with RT-QPCR from additional samples, none of the genes separated SCR and normal samples due to overlapping expression between groups.

In one Tx study, Horwitz et al. reported using the tubes by same manufacturer as us in their investigation of AR-induced gene expression in heart Tx [340]. In their study, minor fold changes of expression associate with AR. From their data, only one gene (Ubiquinol-cytochrome C reductase binding protein (UQCRB) associated with AR with over two-fold change, which was our criterion. None of our kidney Tx patient samples expressed the UQCRB, and it may be distinctive to heart Tx.

In conclusion, we discovered only subtle changes in peripheral whole blood gene expression profiles in paediatric renal Tx patients with or without SCR. Although the genes of the immune system associate with clinically suspected rejection episodes, these were not significant in our investigation of patients with SCR. Our relatively small series suggests that whole blood analysis is not able to find robust gene expression biomarkers for SCR. Further studies in larger populations may reveal whether any of the genes with small changes in expression in our study might provide a supporting diagnostic method.

STRATEGIES FOR THE FUTURE

As renal Tx treatment protocols have evolved to overcome the frequent rejection episodes of the early days of Tx, the aim of improving therapies currently emphasizes individualised immunotherapy to lengthen the graft and patient survival as well as to improve the recipient's quality of life. Excessively potent immunosuppression increases the risk of infections, cancer and nephrotoxicity, while insufficient immunosuppression results in rejection.

One approach towards optimal immunosuppression is to regularly monitor biomarkers reflecting processes leading to rejection. However, as every patient-graft pair is individual in immunological responses, no single biomarker is relevant for all. The current clinical monitoring of graft function is largely based on measuring serum creatinine/cystatin-c. This lacks sensitivity and specificity and may result in too late recognition of rejection or nephrotoxicity. The frequent use of core needle biopsy is limited by its cost and invasive nature. Immunosuppression is monitored also by pharmacokinetics measuring concentration of drugs in blood [341]. In addition functional assays can be performed - a new pharmacodynamic method detects cell-mediated immunity by measuring the concentration of ATP from whole blood CD4 cells following stimulation [342]. Constant search and development continues to find other noninvasive tools needed.

Currently tested monitoring sources include cells, mRNA and proteins in urine or peripheral blood. Two utilized strategies are to monitor alloimmune responses in the graft or global changes in immune responses of the host. Alloimmune monitoring takes place before and after Tx by measuring the reactivity of recipient HLA antibodies to donor cells or HLA antigens in beads. These antibody-based tools have been more successful than T cell-based tests for predicting rejection. However, the two strategies are successful only when an alloimmune response takes place also in circulation and not only in the graft. The second strategy, to monitor global changes in immune response, faces the problem of separating rejection and infection mediated immune responses from each other. Routinely used creatinine measurement detects mainly glomerular injury but less tubulointerstitial injury. Tubular markers have been found from urine that may separate ischemia from AR, chronic rejection, infection and nephrotoxicity, although not separating the latter from each other. This leads to the conclusion that whereas a single

biomarker for diagnosing any of the entities may never be found, a combination of many biomarkers may distinguish compartments and reveal the reason for injury.

A new emerging field is global analysis of different biomolecule classes that can be studied by proteomics, glycomics, lipidomics or metabolomics. However, as these scientific disciplines are much more complex than genomics, a large amount of work remains to be done simply to characterise the different molecules before even studying their performance as biomarkers. It is expected that a significant number of potential novel biomarkers will be found. However, only a few can be expected to be validated in prospective studies and enter clinical practice. [343].

CONCLUSIONS

This research demonstrates the lack of robust polymorphisms among studied SNPs or biomarkers in investigated genes that could be included in routine analysis in a clinical laboratory. Even though weak associations were found, their predictive value was insufficient for use in clinical decision-making. However, as future technology advances are likely to make large-scale genetic tests affordable, data on several hundreds of genetic markers may be combined and used in personalized patient treatment [344].

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ORIGINAL PUBLICATIONS

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