ENDOTHELIAL CELL ANTIBODIES AS BIOMARKERS FOR REJECTION IN KIDNEY TRANSPLANT

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Abstract

An immune response to foreign antigens from members of the same species is called alloimmunity. Alloimmune response results in graft rejection which finally can lead to complete loss of graft function. It is very important to develop a specific, sensitive, and especially a noninvasive read-out of the alloimmune risk in acute rejection (AR). Currently this is a need in transplantation which is still unaccomplished. With such an assay the number of invasive and expensive biopsies could be reduced and therefore a better and improved treatment for patients could be granted and following also a higher quality of life for the patients would be achieved. Additionally another consequence could be the decrease of financial strains on the insurance payer.

The initial contact point between a recipient's immune system and a transplanted organ is the vascular endothelium. By using high-density protein arrays, target antigens for Anti endothelial cell antibodies (AECA's) were isolated from sera of recipients of kidney transplants experiencing antibody- mediated rejection. The purpose and objective of this study was to investigate whether other AECAs activate the vascular endothelium, amplifying the alloimmune response and increasing microvascular damage and to find biomarkers to monitor acute rejection and identify what patients are at risk of AR even before its occurrence.

Antibodies CTLA4, CD7, TRIM21 and IFI6 were previously identified in discovery step using high-density protein arrays and are now further analyzed regarding their influence in early or late rejection and other clinical outcomes after transplantation.

ECL and common ELISAs for the endothelial proteins CTLA4, CD7, TRIM21 and IFI6 were established. Pre and post transplantation antibody levels against these proteins were determined and further statistically analyzed regarding their influence in early or late rejection and other clinical outcomes after transplantation. A predictive character was found for CTLA4, CD7 and IFI6 which should be evaluated further with additional samples.

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List of Abbreviations to be edited

Tx ..... Transplantation

TLR ..... toll-like receptors

Ab ..... Antibody

MHC ..... major-histocompatibility-complex

HLA ..... Human Leukocyte Antigen

ABO ..... Blood group system

APCs ..... Antigen Presenting Cells

Tregs ..... T regulatory cells.

IgG ..... Immunoglobulin G

PRA ..... panel reactive antibody

FDA ..... US Food and Drug Administration

TCMR ..... T-cell mediated Rejection

AR ..... Accute Rejection

AMR ..... Antibody Mediated Rejection

EMI ..... extramedullary infiltration

PCR ..... polymerase chain reaction

ELISPOT ..... Ezyme-linked immunosorbent spot

FSGS ..... focal segmental glomerulosclerosis

AECAs ..... Anti Endothelial Cell Antibody

DSA ..... Donor specific Antibody

FCXM ..... flow cytometric crossmatch

EPC ..... Endothelial precursor cells

dPBMCs ..... Donor peripheral blood mononuclear cells

GFR ..... Glomerular Filtration Rate

ECXM ..... Endothelial Cell Cross Match

IFI 6 ..... Interferon, alpha-inducible protein 6

PNPP ..... Para-Nitrophenylphosphate

PBS ..... Phosphate Buffered Saline

MSD ..... Meso Scae Discovery

ECL ..... Electrochemiluminescent

SrCr ..... Serum Creatinine

CV ..... Correlation Variation

Stdev ..... Standard Deviation

1 Introduction

1.1 Transplant History and Current Issues

A better understanding of the role of the immune system in allograft rejection lead to an intense progress in the science of kidney transplantation during this past half century. [1]

Emerich Ullmann was the first to perform experimental transplantations of a kidney. In 1902, in Vienna, he transplanted a kidney between dogs. Only four years later, professor of surgery Mathieu Jaboulay, connected the renal vessels of a sheep and a pig kidney, to the brachial vessels of two patients dying of renal failure in Lyon, France. Unfortunately, neither of the kidneys worked. However, the techniques that were used to join these vessels together, was the same technique developed by Alexis Carrel, who was a young surgeon in Jaboulay's lab, and is also just the same technique of vascular anastomosis still used in kidney transplantation nowadays.

It was in 1914 when Carrel said that the technical problems of transplantation were essentially solved, but until some method was developed to prevent the reaction of the organism against the foreign tissue, there would be no medical application of transplantation.

After the Second World War, a series of successful transplantations of deceased kidney donors were performed by David Hume at the Peter Bent Birgham Hospital in Boston. In these transplantations, although no intentional immunosuppression was used, some kidneys functioned for days or even for several months. It has to be taken into account that due to the profound uremia the recipients were suffering from, their immune system was weakened.

Soon after, the successful transplantation between identical twins in 1954 showed that kidney failure could be reversed completely while giving immunosuppression and total body irradiation. By 1960 however, it was clear that total-body irradiation, which lead to patient's' deaths, was not the perfect solution.

After Gertrude Elion and George Hitchings of Buroughs Wellcome had developed 6-mercaptopurine, an anticancer agent, Robert Schwartz and William Daeshek proved this drug to suppress immune response to a rabbit protein and also to prolong survival of skin allografts. Roy Calne, Charles Zukoski and David Hume showed that this agent could prolong renal graft survival in dogs. After the introduction of azathioprine, corticosteroids and anti-lymphocyte globulins more and more agents were presented to treat renal failure. [1] [2] With the introduction of cyclosporine, a calcineurin inhibitor, a significant reduction in graft loss was achieved. In the 1980s the first monoclonal antibody against T- lymphocytes was introduced.[2]. Also the better immunologic matching of graft and donor changed the outcome of transplantation significantly. [1] Lamentably, the rates of graft survival beyond 5 years have so far remained constant in most cases. [3]

Today, kidney transplant half-lives are around 8 to 11 years. [4]

1.2 Immunology

Transplantation of tissues or cells from a donor who differs genetically from the graft recipient will induce an immune response against alloantigens of the donor graft. If this immune response is not sufficiently controlled it will destroy the graft.

Rejection can be classified in many ways. By the time it takes for a graft to be rejected we differentiate between: hyperacute (occurring within minutes), acute (occurring within days to weeks), late acute (occurring after 3 months) and chronic (occurring months to years after transplantation).

It can also be classified looking into pathophysiological changes (cellular-interstitial, vascular, antibody-endothelial) or by the severity with help of the Banff Score which describes the extent of histologic inflammation and injury of the graft. Another differentiation can be achieved by whether adaptive or innate immune responses lead to graft injury. [1]

1.2.1 Innate Immune Response

Innate immunity reacts fast but with low specificity. It has no memory and expresses neutrophils, macrophages, natural killer cells and dendritic cells. Molecular components are toll-like receptors (TLRs), complement chemokines and cytokines.[5] Once a pathogen is recognized by the innate immune system, signals to activate the more specific adaptive immune response are provided. The innate immune system is necessary for tissue healing and pathogen removal, however, it can also contribute to transplant injury. Very often, delayed graft function follows. [6] Injured tissues express ligands of the toll-like receptor family. Toll-like receptors detect pathogens, but can also sense presence of foreign/tissue molecules and produce factors that cause maturation and activation of dendritic cells, which promote acute rejection.

By an increase in major-histocompatibility-complex (MHC) class I peptide–related sequence A (MICA) antigens found on endothelial surfaces, natural killer cells and CD8 T-cells can be activated. Additionally an association between poor graft outcomes and sensitization to the highly polymorphic MICA antigens in HLA-matched (Human leukocyte Antigen) transplants is associated with it. [7,8]

There are certain features a donor might have that lead to rejection. Older age, presence of hypertension or other persisting diseases like diabetes, as well as donation after cardiac death or prolonged ischemia of the graft himself, resulting from a delay in shipping can influence the decision of whether or not the donor is suitable. [9]

1.2.2 Adaptive Immune Response

Adaptive immune responses are less fast but more specific than innate immune responses. B cells have to be appropriately stimulated in order to differentiate into memory B cells and plasma cells that secrete antibodies. An antibody imagined as a Y shape has an FC region at the base of the Y, formed by two heavy chains. This region mediates diverse effector functions such as activation of the comple-

ment cascade. It also interacts with macrophages, natural killer cells and neutrophils through their FC receptors. [6]

Antibodies capable of mediating rejection comprise those against HLA molecules, endothelial-cell antigens, and ABO blood-group antigens expressed on endothelial cells and red cells. Unless they were sensitized by exposure to alloantigens through undergone blood transfusions, pregnancy or previous transplantation, usually recipients do not possess antibodies against HLA molecules before transplantation [1]

Before transplantation is conducted, Blood from recipient/donor pairs are tested to determine ABO compatiblity and kidneys are routinely assigned to recipients with a compatible blood group. Nevertheless ABO-incompatible kidneys have been successfully transplanted by means of plasmapheresis or immunoadsorption. This means the perioperative removal of antibodies from the recipient. After removal, anti–blood-group antibodies can increase back to pretreatment levels even after transplantation. There they can adhere to the microvasculature, and contribute to activation of complement. However, they generally do not injure the endothelium. This anomaly is now recognized as “accommodation” within the kidney. The mechanism responsible for this positive but incomplete response is yet unknown.[10] Unfortunately, injury to the graft by anti-HLA antibodies is very often insidious, and accommodation is unusual.

1.2.3 Types of Rejection

1.2.3.1 Hyperacute Rejection

This type of rejection is known to occur immediately after opening of the vascular crossclamps. The kidney appears flaccid and mottled, reflecting antibodies against HLA Antigens expressed on the endothelium of the kidney’s glomerulus. As a result, a complementary cascade leads to final endothelial necrosis, platelet deposition and local coagulation and the graft has to be removed immediately.

1.2.3.2 Acute Rejection

In acute rejection one can differentiate between antibody mediated acute rejection and T-cell mediated acute rejection.

Antibody mediated acute rejection occurs within a few days after transplantation. Graft dysfunction is very often due to inflammation. Previous exposure to certain antigens rapidly generates high amounts of complement-activating antibodies which mainly attack MHC antigens exposed on the endothelium of the donor's glomerular or peritubular capillaries. [1]

Damaged endothelial cells then release several injury molecules such as von Willebrand factor or P-selectin which lead to platelet aggregation. Also cytokines and chemokines such as interleukin-1α, interleukin-8, and chemokine (C-C motif) ligand 2 (CCL2) are released and can cause leukocytes to attach to the glomeruli of the kidney. Chemoattractants such as C3a and C5a are also released. A marker of typical complement activation is C4d. This molecule is not only an important biomarker which will be explained further in detail later on, it also triggers the formation of membrane-attack complexes which lead to endothelial necrosis and apoptosis. Detachment of endothelial cells from the membrane comes with it. [11]

With the help of early diagnosis, treatments for rescuing grafts experiencing acute antibody mediated rejection can be approached. Removal of antibodies via the help of plasmapheresis or immunoadsorption as described before can decrease acute rejection. [1] High-dose pulses of glucocorticoids, intravenous immune globulin and antiproliferative agents are frequently given. These treatments are especially helpful when given as a prohpylaxis to ABO-mismatched recipients. [12] It is therefore very important to detect potentially harmful antibodies before transplantation and occasionally a donor or an aggressive post-transplantation management should be found and approached. [1]

Due to ongoing advances in understanding the immune system and human leukocyte antigen matching techniques, acute rejection rates have reduced to <10%. [6]

T-Cell mediated Acute Rejection is introduced when donor alloantigens are presented to the T-lymphocytes of the recipient by so called Antigen Presenting Cells (APCs). [1] There are immature dendritic cells within the graft that carry these donor antigens from the organ to the recipient's lymph nodes and spleen. On their way they differentiate into APCs. [13]

Once the APCs arrive to the lymphoid organs, they activate the recipient's T-cells which differentiate into subgroups and return to the graft to attack the transplanted organ. [1] Dendritic cells as well as macrophages present antigens to T-cells, B cells however can also function by capturing antigens with help of immunoglobulins and MHC class II molecules expressed on their surface. Also tubular epithelial cells and endothelial cells have the ability to present antigens to activated T-cells. [14]

1.2.3.3 Late Acute Rejection

This kind is rare but can develop in patients with high-grade immunity against the transplant itself or in patients who receive reduced amounts of immunosuppressive therapy due to cancer or other severe infections. Late acute allograft rejection is severe and often problematic because it can't be reversed that easily. The risk of graft loss is elevated.

1.2.3.4 Chronic Rejection

In chronic rejection an ongoing immune injury to the graft due to a failure to maintain sufficient immunosuppression is assumed. Indicators are a progressive waning in renal function. Sometimes smooth-muscle proliferation and hyperplasia in vessels, forming a neointima (scar tissue inside vessels); focal destruction of internal elastic lamina; and finally, vascular occlusion can be observed. [1] In chronic antibody-mediated rejection, undetected preexisting donor-specific antibodies or antibodies generated after transplantation deposit on the capillary endothelium. [11]

1.2.4 Major Histocompatibility Complex and HLA mismatch

MHC is responsible for encoding the HLA system. HLA genes encode glycoproteins that enable APCs (Antigen Presenting Cells) to display fragments of antigens to receptors on T-cells. These MHC molecules can be either class I or class II. Class I molecules present peptides derived from internal proteins to cytotoxic CD8 T-cells. Class II molecules present peptides derived from extracellular proteins such as bacterial proteins to CD4 T-cells.[1] Class I HLA molecules are HLA A, B or C and are expressed on all nuecleated cells. They are heterodimers and consist of a single transmembrane polypeptide chain and a macroglobulin. HLA DR, DP and DQ are molecules and belong to class II HLA molecules. They are expressed on professional APCs such as macrophages, dendritic cells and B cells.[6] Mismatches between recipient's and donor's HLA, even if only a few amino acids within the peptide-binding site of MHC, are sufficient to incite graft rejection.[1] Due to their polymorphic properties HLA provides a large number of potential antigens to be recognized by a recipient. [15]

Two pathways are described for T-lymphocytes recognizing alloantigens. The direct pathway is known for T-cells sensing alloantigens displayed on donor's APCs whereas in the indirect pathways recipient's APCs are detected.[1] The direct pathway is known to contribute to acute and chronic allograft injury.[16] After donor APC's have disappeared, the indirect pathway becomes more important.

In order to activate T-cells, more signals than MHC-complexes, called costimulatory signals, are needed.[17] When presented with an antigen in the absence of costimulatory signals, T-cells can become unresponsive (anergic), are delated or differentiate into protective regulatory cells (Tregs). Tregs are then capable of inhibiting cellular immune responses and are therefore essential for the prevention of autoimmune diseases. [1,6]

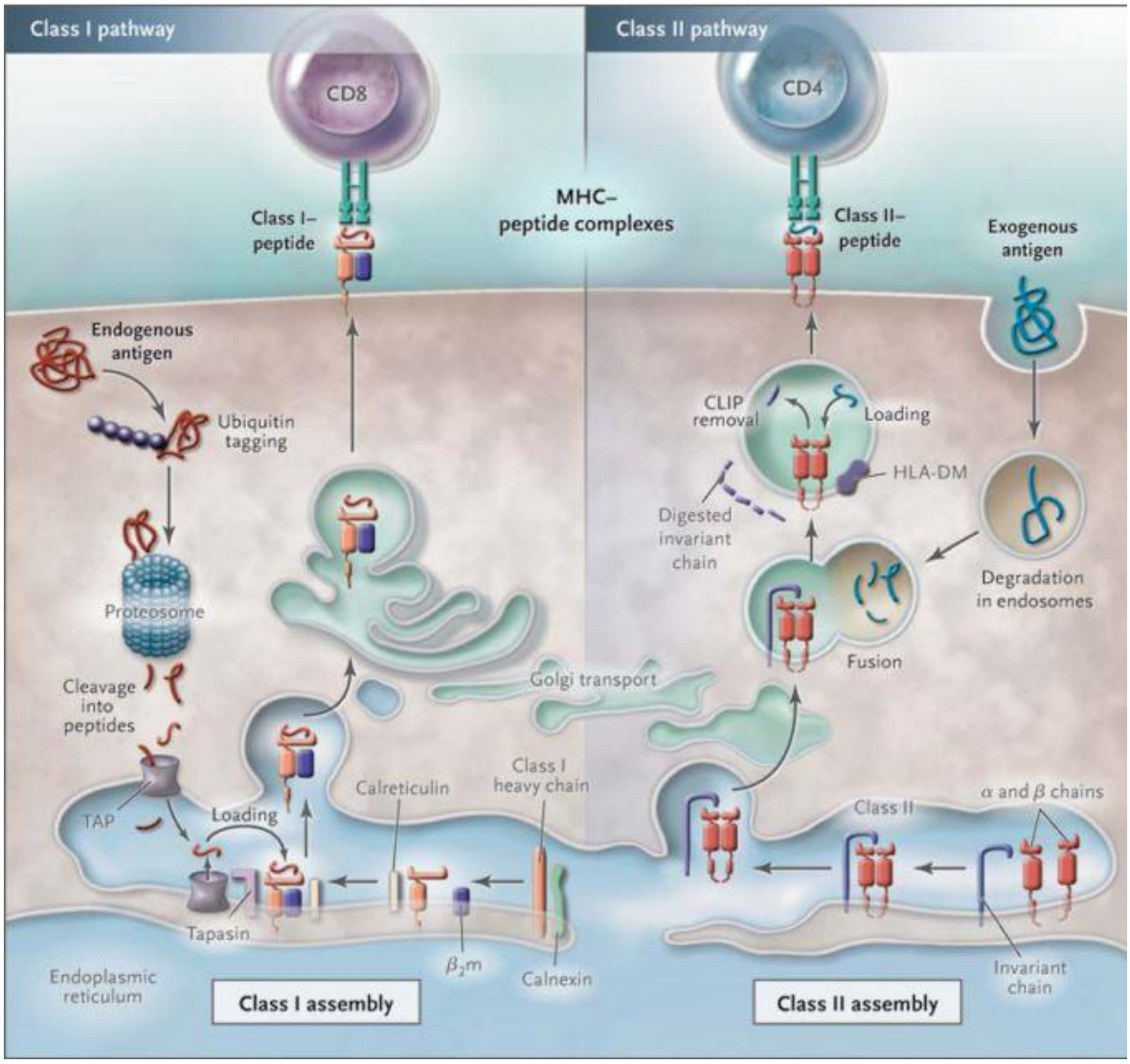

Figure 1 Major Histocompatibility Complex Class I and II Pathways. Endogenous antigens are digested into peptides by proteosomes and are loaded into class I MHC. Exogenous antigens are degraded in endosomes and are loaded into class II MHC. As it precedes its transport through the Golgi it is ultimately expressed on the APC surface where the MHC–peptide complex interacts with CD8+ or CD4+ T lymphocytes. Class I molecules present peptides derived from internal proteins to cytotoxic CD8 T-cells. Class II molecules present peptides derived from extracellular proteins such as bacterial proteins to CD4 T-cells. [1]

Among the costimulatory molecules displayed by APCs are CD80 (B7-1) and CD86 (B7-2); which are ligands for two T-cell–membrane receptors, CD28 and CTLA-4. Many therapeutics have been developed being able to block costimulatory signals in order to inhibit pathogenic T-cell immune responses.[6] Belatacept, a fusion protein containing CTLA-4 and the Fc fragment of IgG1 which will be ex-

plained further in detail later on, blocks T-cell stimulation engendered by the CD80–CD28 and CD86–CD28 pathways. [18–20]

Once activated, T-cells initiate a series of pathways resulting in the secretion of interleukin 2, T-cell proliferation or differentiation into effector cells which when primed is capable of migrating to sites of inflammation. Gotten to this point, they re-encounter specific antigens expressed on target cells and release proinflammatory cytokines such as interferon-γ and IL-17 to induce Fas-mediated cytolytic killing which results in a coordinated destruction of antigen-expressing cells. The production of peroforin from effector T-cells leads to pore formation which helps granzyme B to enter the cytosole where caspases activated by granzyme B results in cell death. [6] Interestingly, these two molecules found in urinary cells can serve as biomarkers for noninvasively diagnosed graft rejection. [21]

Whether a recipient disposes of antibodies reactive to donor HLA molecules can be tested in two ways. PRA (panel reactive antibody) tests are performed while the patient is still waiting for a transplant. Serum from a possible transplant candidate is tested for its ability to bind and lyse a range of donor cells that express various HLA molecules. A more recent method developed in order to define that reactivity against a cell is actually to an HLA molecule, and to exclude nonspecific binding, uses flow cytometry. HLA-coated beads are used instead of HLA typed cells. In case the patient's serum binds to a certain amount of HLA-coated beads, the serum is concluded to be positive to donor-reactive antibodies and a desensitizationmight have to be taken under consideration. Beads are also available with single HLA allele variants such as B7 or A2 to define more specifically reactivity of patient's sera. That way a surgeon might be able to decide to exclude any donor expressing A2 for patients reactive to HLA A2 right away.

Additionally, shortly before kidney transplantation a crossmatch test is performed to determine if the patient has reactive antibodies to his donor organ. If cells are lysed or antibody binding is positive, hospitals will not proceed with the surgery. [6]

1.2.5 Immunosuppresants and therapeutics

Belatacept, as mentioned before, is an effective immunosuppressant for kidney transplant patients. It was approved in 2011 by the FDA (US Food and Drug Administration) and has the potential to contribute against side effects of cyclosporine and tacrolimus. [18] It is a follow-up product of the earlier invented abatacept and will be explained more in detail later on.

Upon activation of T-cells, calcium flux activates calmodulin, an intracellular molecule that binds to calcineurin. A phosphatase is then activated and a number of downstream mechanisms finally lead to the binding of NFAT (transcription activating factor) to IL-2. Cyclosporine A and tacrolimus target calcineurin and block transcription activation.

IL-2 binds to IL-2R and is also one of the main targets of corticosteroids. These inhibit the expression and transcription of IL-2 as well as IL-1, IL-6 and IFN-y and tumor necrosis factor alpha, which leads to a blockage of T-cell proliferation and T-cell dependent immunity. IL-2R also leads to activation of another cascade involving mTOR (mammalian target of rapamycin) which leads to translation of new proteins that allow the cell to progress from G1 phase into S phase, which means proliferation. Azathioprine and Mycophenolic acid are two immunosuppressants used to inhibit DNA synthesis and therefore T-cell activation at this point.[6]

CD20 is a cell surface molecule expressed by mature B cells and memory B cells. The FDA-approved antibody Tiruximab is specific for CD20 and depletes B cells. Tiruximab is being tested for its preventive activities in alloantibody production in transplantation.[22] However, the predominant producers of antibodies, plasma cells, do not express CD20, are BAFF and APRIL independent and are therefore resistant to therapies against these molecules. Bortezomib as well as other proteasome inhibitor are being tested of their capability of depleting antibody-secreting plasma cells.[23]

Currently the most common immunosuppression regimens include triple therapy. Thereby a combination of Tacrolimus or Cyclosporin/MMF/steroids is given. Ex-

ception may apply for diabetics. Preferably Rapamycin instead of Tac or Cyclosporin is given.

Noninvasive biomarkers could serve as predictive tools and might help clinicians personalize immunosuppression and allow early prediction of transplant outcomes, ideally prior to established organ dysfunction.[24]

1.3 Biomarkers

NIH Biomarkers Definitions Working group states that a biomarker is “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”.[25]

Biomarkers can be differentiated in three groups. Prognostic, predictive and surrogate end points. [24] Prognostic biomarkers are linked with a clinical end point, regardless of whether a patient is receiving treatment or not. It is used to identify patients likely to get beneficial results out of interventions.[26] A high amount of donor-specific antibodies (DSAs) before transplantation is considered a prognostic biomarker of hyperacute rejection for example, and is regardless of any prophylaxis used consequently. [27] With predictive biomarkers, a change in biomarker concentration is associated with a change in the clinical endpoint. Surrogate endpoint markers can substitute any clinically relevant endpoint. It can therefore predict the clinical outcome and change in parallel with the true clinical end point responding to therapeutic interventions. [26]. To measure performance of a biomarker, specificity and receiver operator characteristic curves (ROC) are used. [24]

Currently, graft rejection requires histopathological evidence in a biopsy sample with the help of the Banff criteria [28,29]. The problem with kidney biopsy is that it is invasive and sampling errors can occur. Less invasive ways of detection on a molecular and cellular basis to predict or diagnose rejection might allow early treatment and prevention of allograft failure.

In order for a biomarker to be relieable, levels should accurately mirror the progression of a disease and not to be confused with other causes of inflammation or kidney damage. Another important condition is to find the right time to measure a certain biomarker and the right threshold concentration. In terms of acute rejection that means that nephrologists should set time points of how frequently a particular biomarkers should be measured. Also the type of biomarker should be assessed. If as a prognostic to increase surveillance or as a predictive tool to prompt further evaluation such as obtaining a biopsy sample, or as a surrogate end point to start empirical rescue therapy without a confirmatory biopsy sample needed. Ideally a biomarker provides the clinician with sufficient information to weigh up the potention risks and benefits of treatment.

Mathematically, the relationship between the predictive value of a biomarker, sensitivity, specificity and prevalence can be defined by Bayes Theorem, which can be reduced to the following equations:

$$PPV = \frac{(sensitivity * prevalance)}{(sensitivity * prevalance) + (1 - sensitivity) * (1 - \ prevelance)}$$

(1)

$$NPV = \frac{\big(specificity * (1 - prevalance)\big)}{(specificity) * (1 - prevalence) + (1 - sensitivity) * (prevelance)}$$

(2)

[24,30]

Not only the sensitivity and specificity of a biomarker are important. positive predictive value as seen in Equation 1, number of true positives, negative predictive value as seen in Equation 2 and the number of true negatives are just as significant.

In a more practical sense that means that for a disease assumed to have a low prevalence it is very difficult for a biomarker to demonstrate a high PPV. In that case its specificity and sensitivity values must be extra high. Likewise in order to

avoid a low NPV, a disease appearing to have a high prevalence also calls for a biomarker with particularly high sensitivity and specificity values. [24]

1.3.1 Importance of Biomarkers in Transplant research

Several methods are currently in use for the exploration and finding of biomarkers. Microarray is one of the most broadly used methods. With the help of standardized gene arrays, simultaneous evaluation of thousands of gene expressions from one single sample are possible.[31] A more sensitive method to changes in expression of genes is quantitative real-time PCR (qPCR). It can also be used for validation purposes of microarray data. Today, also micro RNAs (miRNA) and small non coding nucleotide sequences can be analysed.[32] Additionally advances in mass spectrometry have been achieved which enable large-scale profiling and quantification of proteins in different kinds of biological samples [33] In the past twelve years also multiparameter flow cytometry has grown to enable intracellular and very detailed cell-surface phenotyping, Even rare and complex cell populations can be studied this way. [34] Another way to assess cellular function includes the ELISPOT technique. Similar to ELISA, enzyme-linked immunosorbent spot, results in a color reaction, spotting the cells of interest. Also, today multiplex platforms are a lot simpler and are sufficiently cost effective to be able to use routinely for biomarker assesment in nontransplant fields. [35]

Although there seems to be an endless variety of possible biomarkers, in nephrology only three substances can be used: urine, blood and through invasive biopsy, tissue. Samples taken from blood and urine are inexpensive and easy to obtain. Moreover, point-of-care testing could be developed for both substrates and might enable monitoring biomarker levels from home. (As for glucose control in diabetes). [24]

Recently 9000 antigens in pretransplant sera and selected 10 antibodies targeting glomerular antigens for enzyme-linked immunosorbent assay (ELISA) validation were screened. In the end seven antibodies (CD40, PTPRO, CGB5, FAS, P2RY11, SNRPB2, and APOL2) were able to predict posttransplant focal segmen-

tal glomerulosclerosis (FSGS) recurrence with a 92% accuracy. These seven antibodies enable to identify FSGS patients at risk to suffer of recurrence even before transplantation. [36]

Gene array data from Sis et al [37] showed that increased endothelial cell transcripts are a more sensitive biomarker for AMR than C4d levels in acute renal biopsies. Other studies have shown hyperacute rejections occurring in patients positive for AECAs (Anti Endothelial Cell Antibody) without HLA-DSA present.[38] Therefore growing evidence exists, proving antibody induced endothelial cell activation may be sufficient for immune cell recruitment and graft injury. [39]

1.3.2 Serum Creatinine

The concentration of creatinine found in serum is widely used as a measure for glomerular filtration rate (GFR) in the kidney. In clinics it is seen as an indicator of renal functionality. Unfortunately, filtration of creatinine via the glomeruli is only one of many ways that lead to different concentrations in serum. Therefore, proper interpretation of the obtained values is critical in order to conclude physiological and pathological outcomes from glomerular filtration rates.

Each of the human kidneys contains around 106 glomeruli. These capillary units filter plasma via pressure driven filtrations across the semipermeable membrane of the glomerular capillary. If urine and plasma concentrations of a substance are known and the urine flow rate is measured, clearance can be calculated. This is seen as the theoretical volume of plasma that has been filtered of this substance during a specific amount of time.

Insulin is seen widely as the perfect filtration efficacy marker. Unfortunately these measurements require intravenous infusions and a complicate chemical assay so that clinical practice remains inconvenient and it is only used as a research tool.

In order to use serum creatinine as a marker of GFR, one has to assume that two criteria are fulfilled. Creatinine has to be a perfect filtration marker. The metabolism among individuals has to be constant over time and the creatinine production

rate must be equal to the renal excretion rate. It follows that if the serum creatinine is inversely proportional to GFR, halving of the GFR must lead to a doubling in serum creatinine.[40]

1.3.3 C4d staining

In AMR donor-specific antibodies bind complement factors and activate the complementary pathway. During the activation cascade, C4d, is formed as a complement split product. C4d is capable of covalently binding to target molecules expressed on the endothelium of peritubular capillaries (PTCs) and is therefore seen as a footprint of AMR [41] Sensitivity (95%) and specificity (96%) of C4d staining in PTCs for the presence of DSAs is considered high[42]. Other studies have also shown that there is a solid relation between diffuse C4d staining of PTCs and the presence of DSAs [43] [44] [45] Since 2003, PTC staining also served as criteria of pathological diagnosis for AMR in Banff classification. In 2007, the C4d PTC staining was one of the diagnostic trio for chronic AMR with the morphological features and the presence DSAs. However, the specificity and sensitivity of C4d staining have both been questioned [46], after C4d-negative AMR has been found in renal allograft pathology. [37]

1.4 Endothelial Crossmatch Tests

Endothelial cells of donor organs are the immediate targets for the host's immune system. It has been proven that anti-endothelial cell antibodies (AECAs) play a important role in some kidney [47] heart and liver [48] transplant rejections.

In history, donor-specific antibodies were detected in complement-dependent cytotoxicity assays after patients sera had been incubated with donor T- and/or B-lymphocytes in the presence of rabbit complement. Many centers in the US and more in Europe still use CDC-crossmatch as the final test to determine transplant eligibility. Until now, flow cytometric crossmatch (FCXM) techniques added sensi-

tivity to the detection of DSAs.[49] A more recently used method includes ELISA [50], flow cytometry, and Luminex protocols which use soluble HLA antigens coated onto microtiter wells or microbeads, allowing a more specific determination of HLA-DSA. [38] [51]

In Breimer's multicentre evaluation the XM-ONE kit was used and Endothelial precursor cells (EPC) expressing the angiopoietin receptor, Tie-2, were isolated from donor peripheral blood mononuclear cells. According to the instructions of the manufacturer, (AbSorber AB, Stockholm, Sweden) peripheral blood was collected in Vacutainer CPT tubes with heparin (BD, Franklin Lakes, NJ) and centrifuged for 15 min at 1500 to 1800 g in a swing-out rotor. Donor peripheral blood mononuclear cells (dPBMCs) were then collected and EPCs could be isolated by immunomagnetic separation with the help of nanobeads coated with anti-Tie-2 antibody. For crossmatch testing, washed Tie-2 positive cells were incubated with patient serum. EPC- reactive IgG and IgM was then detected by flow cytometry using fluorochrome-conjugated secondary antibodies. Patients with a positive ECXM test performed pretransplant run a higher risk of graft rejection. The occurrence of rejection is significantly higher among patients presenting AECAs compared with patients without. Serum creatinine levels were significantly higher in patients with donor-reactive AECA than without at 3 months and 6 months post transplant. The highest creatinine levels administered at 3 and 6 months were found among kidney transplant patients having HLA-specific antibodies and a positive ECXM test before transplantation.[51]

1.5 Banff Criteria

To describe histologic lesions for renal allograft rejection in a standardized classification system, the Banff process was introduced. This new method permitted classification of lesions and end point biopsy proven rejection.

The aim of this uniform method was to equalize the recording of allograft pathology worldwide. With its help, criteria for classifying several types of rejection as well as pathologies and drug toxicity were established.

In order to achieve this goal of a standardized renal biopsy, a small group of pathologists, nephrologists, surgeons and tissue typing physicians met in August 1991 to build a classification system for allograft injury. Initially this was called "Workshop on International standardization of the Nomenclature and Criteria for the Histologic Diagnosis of Kidney Transplant Rejection", however rapidly it was simply called "Banff" after the city in Canada where this meeting was held. Since then, this meeting takes place every two years in a different country in order to keep the internationality.

The Banff score defines graft injuries in different compartments of the kidney and outlines how to semi-quantitatively note these lesions. [52]

The Banff Score describes the main lesions in the four different compartments of kidney. To diagnose TCMR, (T-cell mediated Rejection) the Banff classification outlines how to semi-quantitatively record these injuries. The lesion scores (g-, i-, t-, v-score) are then summarized to become the final Banff TCMR grade. [28] Defined thresholds of scores and grades then show whether a case is considered TCMR. The existing lesions of TCMR are: interstitial inflammation, (i-score); tubulitis (t-score = invasion of tubular epithelium by lymphocytes) and intimal arteritis (v-score = invasion of inflammatory cells beneath the endothelium), whereas the more lesion present the more severe considered is the rejection.

Because concerns of over-diagnosing and over-treating TCMR arose, the founders later introduced minimum thresholds for the i- and t-score. [28] Whenever well-conducted studies point out that the current criteria should be improved, or that the new technology can help in already established procedures, a process follows that might lead to inclusion of this new knowledge. The biggest concern is always reproducibility. Two types of reproducibility have to be considered: intra- and interobserver reproducibility. With the mathematical help of Kappa statistics, the per cent agreement between two votes can be measured. However studies on Banff scoring [53] [54] showed reproducibility only to be 'moderate' (Kappa: 0.41–0.60) and

more often ‘fair’ (Kappa: 0.21–0.40). because the Transcriptome assays can be internally controlled and normalized, those measurements can be highly reproducible. In 2005, the development of a genomic approach was already considered as an important objective at the Banff meeting.[55]

The drive of the Banff classification to adapt constantly, led to the implementation of six working groups on the following areas: isolated v-lesion, fibrosis scoring, molecular pathology, glomerular lesions, polyomavirus nephropathy and quality assurance. Banff working groups conduct multicenter studies to evaluate the clinical relevance, practical feasibility and reproducibility of potential changes to the Banff score. [52]

Today the multiple mechanisms of alloantibodies employed in allograft rejection are still under investigation. Reports of allograft rejection in HLA identical siblings let suggest a role for non-HLA antibodies. [56,57] Today we know that complement activation is the mechanism of action for alloantibodies in many rejections. Additional mechanisms are still being investigated.

In this presented study, an effort was made to find non-HLA specific AECAs able to predict allograft rejection before transplantation. The vascular endothelium is the very first contact point between a recipient's immune system and the transplanted organ. We looked deeper and asked us whether certain AECAs are linked to rejection of the kidney. Through identification of AECAs' targets expressed on endothelial cell precursors (ECPs) a potential test for AECAs in solid phase immunoassays could provide an opportunity for therapeutic intervention.

In order to find target antigens, a very sophisticated approach using high-density protein arrays on patient's sera, suffering from antibody-mediated rejection, was conducted. All rejections took place in the absence of donor-specific HLA antibodies. Among the findings were CTLA4, CD7, MATN2, IFI6 and TRIM21. [58]

2 Materials and Methods

2.1 Identification of Endothelial Cell Targets via Protein Arrays

In anticipation of this study, Jackson et. al. isolated AECAs from a panel of 10 kidney transplant recipients. All were tested positive for AECAs in pretransplant endothelial cell crossmatch tests. In order to focus on AECA targets, antibody eluates were conducted using ECPs derived from blood. With the help of high-density protein platforms, 9500 human proteins were eluted, Fiftyfive showed to exist in a significant concentration. Four were expressed on vascular endothelium in all eluates and already tested in Jackson's study [39], four more were tested in this study.

2.2 Patient's Sera

Sera from 150 sequential recipients of renal transplants for whom there were adequate pre- and post-transplant sera were tested using common ELISA and ECL ELISAs specific for CTLA4, CD7, MATN2, IFI6 and TRIM21. This retrospective study cohort were recipients sensitized to HLA, with 91% (137 of 150) of recipients testing positive for HLA-specific class I and/or II antibodies. Here we analysed the most strongly reacting sera in each ELISA.

While conducting experiments our samples were blinded and any clinical data was not known.

Table 1 Patient Demographics ELISA Test Cohort (n=150)

Recipient Age (mean,SD)	49 ± 15
Race (% non-white)	44%
Male Gender (%)	41%
Previous transplantation (%)	40%
HLA Sensitization1 (%)	91%
Mean % CRPA2 (CDC-XM, FCXM)	29, 39
Original ABO or HLA barrier3	
ABOi	6%
CDC-XM+	2%
FCXM+	19%
FCXM-, DSA+	45%
NO DSA	34%
Live Donor (%)	55%
Deceased Donor (%)	45%
HLA-A;B;DR;DQ mismatch (mean)	4.7
Plasmapheresis Treatments:	
No Pre- or Post- Treatments (%)	45%
Pre-Transplant (Mean, Median)	1.0, 0.0
Post-Transplant (Mean, Median)	4.0, 2.0
Anti-CD25 Induction (%)	17%
Thymoglobulin induction (%)	83%
Rituximab induction (%)	36%

[1] HLA- specific antibody detected on Luminex® platform

[2] Calculated panel reactive antibody (CPRA) was determined for HLA-antibodies of suffiecient strength to yield a positive CDC crossmatch (CDC-XM) or flow cytometric crossmatch (FCXM)

[3] Original donor HLA-specific antibody (DSA) strength prior to desensitization treatments

2.3 Antigens

2.3.1 CTLA4

Ab69787 purchased through Abcam

The soluble recombinant immunoglobulin fusion protein, CTLA4-Ig (abatacept), was developed to competitively bind to and therefore inhibit the interactions of both B7 molecules with their receptors. [20] CTLA4-Ig can be a potent inhibitor of immune responses in vitro and in vivo [59,60] However, soon researchers found that a more potent CTLA4-Ig protein could be developed. They engineered a molecule with higher avidity to B7 ligands and especially to CD86. This was achieved by designing a mutagenesis screening strategy to identify high-avidity mutants of CTLA4-Ig that doesn't dissociate from the B7 molecules as fast. As a result a daughter compound which was found to bind with a ~4-fold higher avidity to CD86 and ~2-fold more avidly to CD80 than the parent CTLA4-Ig. This molecule is LEA29Y (belatacept) and resulted in approximately 10-fold more potent inhibition of T-cell activation in vitro.

In synergy with conventional immunosuppression, LEA29Y has proven to have the ability to prolong renal graft survival. Also, no adverse side effects are related to the usage of LEA29Y. However, in a study conducted by Larsen et al, after cessation of LEA29Y treatment on day 70, all recipients ultimately rejected their allografts between 116 and 145 days after transplantation.

Importantly, animals that were treated with LEA29Y did not develop anti-donor antibodies while receiving this treatment, however in the end rejection occurred even during LEA29Y therapy. After withdrawal of LEA29Y, animals did start to develop anti-donor antibodies at time of rejection. [20]

2.3.2 CD7

Human CD7 full length protein ab114926 purchased through Abcam.[61]

CD7 plays an important role in autoimmune diseases as well as in leukemia. A recent study showed that CD7 induces integrin β2 and enhances cell adhesive-

ness and invasiveness in Tanoue cells. The study highlights the role of the CD7/integrin β2 axis as a critical pathway in the process of EMI (extramedullary infiltration) of human B-cell acute lymphoblastic leukemia. [62]

Furthermore, another recent study tried to identify a common rejection module (CRM) A suggestion that there is a common rejection mechanism in all transplanted solid organs led the group to search for such a common rejection mechanism. The lab hypothesized that the minimal set of genes that are significantly overexpressed in organ rejection, irrespective of the set of organs analyzed, would be common in solid organ transplant rejection. Twelve genes that were overexpressed were identified as such, among them was CD7. [63]

2.3.3 IFI6

interferon, alpha-inducible protein 6 ab158482.

This gene is also known as G1P3 and was first identified as one of the many genes induced by interferon. The encoded protein could play a critical role in the regulation of apoptosis. Alternatively spliced transcript variants that encode different isoforms by using the two downstream repeat units as splice donor sites have also been described. [65]

Hormonally regulated survival factors can have an important role in breast cancer. G1P3 is known to be a target of estrogen signaling and a contributor to unfortunate outcomes in estrogen receptor-positive breast cancer. Compared to healthy breast tissue, G1P3 was elevated in the malignant epithelium (50× higher) and was prompted by estrogen ex vivo. [66]

2.3.4 TRIM21

Human TRIM (tripartite motif-containing)[68] 21 full length protein ab136590 purchased through Abcam[67]

The encoded protein is part of the RoSSA ribonucleoprotein, which includes a single polypeptide and one of four small RNA molecules. The RoSSA particle localiz-

es to both the cytoplasm and the nucleus. In patients with Sjogren syndrome and systemic lupus erythematosus, RoSSA interacts with autoantigens. For this gene alternatively spliced transcript variants have been described but the full-length nature of only one has been determined. [69]

TRIM2, also known as Ro52 is often target of circulating autoantibodies in autoimmune diseases. Studies showed that anti-Ro52 antibodies are associated with different clinical outcomes. As it belongs to the TRIM proteins, Ro52 disposes of E3 ligase activity and functions upon ubiquitination. Since Ro52 is expressed in the immune system as a predominantly cytoplasmic protein it can be upregulated and in a proinflammatory environment, transported to the nucleus. Substrates for Ro52-mediated ubiquitination are the transcription factors IRF3, IRF5, IRF7 and IRF8, and activate Ro52 which then regulates type 1 interferon and cytokine production. Ro52 is altered at sites of autoimmune inflammation, like cutaneous lupus lesions. This suggests that Ro52 may play an important role in the pathogenesis of autoimmunity and might also be interesting in transplant research. [70]

2.4 Incidence of AECAs using Antigen-Specific ELISA

2.4.1 Plates and Reagents

Coating Buffer

15nM Na_2CO_3, 30mM $NaHCO_3$

Reagent Diluent Buffer and Blocking Solution

2% Nonfat dry milk in PBS-T (0.05% Tween 20)

Wash Buffer

PBS-T (0.05% Tween-20)

Secondary Antibody

Anti-IgG-AP

Color developing reagent

PNPP 1 Step Solution

Stop Solution

2N NaOH

2.4.2 ELISA Protocol

To achieve the desired concentration of protein in coating buffer, first optimization was conducted. From 125ng/ul CTLA-4 a 2,5ng/ul stock was achieved by dilution with coating buffer. Eight plates were coated with 15ng CTLA4 and incubated o/n at 4deg C. The next day, in order to prevent unspecific protein binding, plates were blocked for 1 hour with 2% Milk in PBS-T. The following 5 washing steps with PBS-T and blotting plates against paper towels should remove all blocking solution left in the wells. 100ul Patient's Sera diluted 1:50 in 2% Milk in PBS-T was added to each well. In order to enhance statistical validation, all samples were added in duplicates. After sample addition, plates were sealed and incubated for 1 hour at RT. Another five washing steps, follows the addition of secondary antibody. Anti-IgG-AP diluted 1:1000 in 2% milk in PBS-T is added to each well and incubated for another hour at room temperature. After the last five washing steps, PBS-T buffer is left in the wells for 20 minutes. 75ul of 1 step PNPP is added to the wells after removing any remaining PBS-T buffer. Plates are incubated for 30 minutes containing PNPP in order to achieve color development. With 50ul of 2N NaOH, the reaction is stopped and the plate was read at 405nm. Absorbance was measured and the mean values of samples plated in duplicate were calculated with Microsoft Excel. Samples with a CV higher than 10% were excluded of the analysis.

2.5 Incidence of AECAs using Antigen-Specific Electrochemiluminescence ELISA

In MSD ECL multiple excitation cycles lead to an increase in signal and sensitivity, a high signal to noise ratio can be achieved. Due to the fact that only labels near the electrodes are excited, washing steps can be spared, a lower background is achieved and a lot of time is saved. Samples are precious and not always available in a great amount. ECL has a larger dynamic range. Experiments conducted comparing ELISA and MSD's ECL showed that MSD assays give a dynamic range at least one log greater than traditional ELISA. [71]

2.5.1 Plates and Reagents

QUICKPLEX plates

Bare uncoated plates were used for custom and user-developed assays. Plates were coated with specific protein manually.

MSD Blocker A Kit, 250mL

Catalog Number: R93AA-2

Containing MSD Phosphate Buffer (5x), Catalog Number: R93SA-2 and MSD Blocker A, Catalog Number: R93BA-2.

MSD Phosphate Buffer (5x) was stored at room temperature. Reconstituted MSD Blocker A Solution was kept at 4deg C. MSD Blocker A solution is bovine serum albumin in a PBS-based buffer that blocks non-specific binding of proteins to the plate surface and enhances sensitivity and reduces background.

MSD Sample Dilution Buffer

MSD 5% Blocker A diluted in 1x PBS to get 1% MSD Blocker A.

MSD 4x Read Buffer T

Catalog Number: R92TC-1 is a Tris-based buffer containing tripropylamine (TPA) as a co-reactant for light generation in electrochemiluminescence immunoassays.

Stored at Room Temperature, diluted in ddH_2O to 1x Read Buffer.

Wash Buffer

PBS-T (0.05% Tween-20)

2.5.2 Protocol for MSD ECL ELISA

Spotting Coating

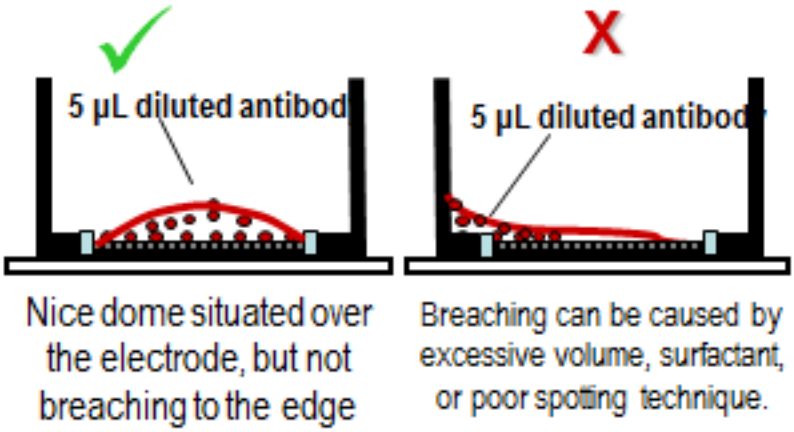

Figure 2 Spotting scheme as provided by MSD Technician

Because of very precious proteins and high value of samples, spotting technique was introduced for TRIM21, CD7 and IFI6. PBS containing 0.03% Triton X-100 used as buffer. 5ul of antigen was added directly to the center of the electrode. After coating, plates were left unsealed at RT to dry overnight.

In order to increase specificity and decreasing unspecific protein binding the next day, plates were blocked with 150ul 5% Blocker A solution. During coating, bare plates were not put on shaker. Plates were sealed and stored at RT.

After blocking, plates were washed thoroughly three times by adding 150ul of PBS (0.05% Tween) and blotting plates against paper towels.

After optimization of sample dilution, all samples were diluted accordingly in 1% Blocker A reagent diluent. With a multichannel pipette, 25ul were distributed equal-

ly in every well. Attention had to be taken by adding the sera into one corner of the wells in order to achieve full submersion of the electrodes. Plates were sealed with adhesive films and incubated in RT on a 450rpm shaker. After the sample addition and two hour incubation, five washing steps followed.

25ul of Sulfo Tag Goat Anti-Human R32AJ-5 diluted 1:1000 in 1% Blocker A diluent reagent were added to each well and incubated for 1 hour on a 450rpm shaker. During the incubation the plates were sealed from light. This followed another three washing steps. Ultimately 150ul of provided read buffer is immediately added to each well and the plates are evaluated via MSD MESO QuickPlex SQ 120 Plate Reader. RLU (relative light units) were measured and the mean of samples plated in duplicate was calculated with Microsoft Excel. Samples that exceeded a CV of 20% were excluded for further analysis.

2.6 Optimization

Coating optimization was not only the most important but also the most work-intensive part of this study. Since none of the used antigens had been examined in this certain group of people before and actual levels of antigen-antibody interactions were not known beforehand, it had to be assured that the highest binding capacity possible is achieved. Therefore, every protein was tested in different amounts until an expected saturation plateau was achieved. This plateau can be observed in Figure 3 where the optimal coating for CD7 was tested. Additionally, different sample dilutions were tested in order to get the best and most reliable signal.

2.6.1 CTLA4

Due to saturation at around 15ng CTLA coating, it was decided that the optimal coating was achieved at that point.

2.6.2 CD7

For CD7 coating, first no plateau could be observed. A second optimization including different dilutions of patient's sera was necessary, When samples were diluted 1:500 instead of 1:100, a saturation (Fig3) could be observed at 15ng of CD7 human protein coated.

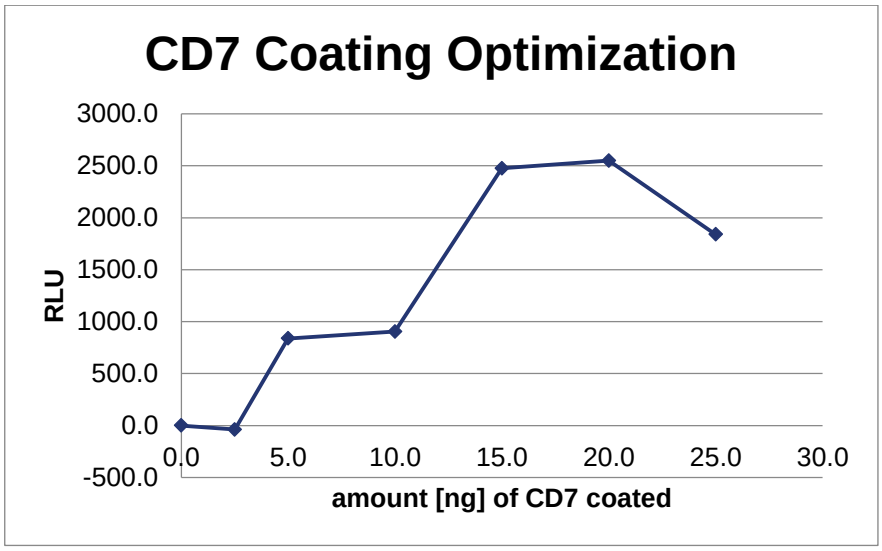

Figure 3 CD7 Coating and Sample dilution optimization finding that 15ng is the most adequate amount. Signal intensity plateaus between 15 and 20ng CD7 antigen coated.

2.6.3 IFI6

When looking at IFI6 10 or 15ng might be adequate. In the second try however, more duplicates were available and so we decided to take 15ng/well.

2.6.4 TRIM21

Finding the right amount of coating for TRIM21 was especially troublefull. When comparing TRIM21 data from the first and second optimization we found that up to 10ng both results were stable so we chose 10ng to be our amount of coating in the further experiments.

3 Results

Protein array data were analyzed by Prospector Analyzer (LifeTechnologies) using robust linear model normalization. A minimum relative fluorescent unit 500 and a Z factor of 0.4 were required for positive detection. [39] Summary statistics, including mean, trimmed mean, median, confidence norm distribution, correlation coefficient, coefficient of difference, and SD, were calculated using Microsoft Excel and Graphpad Prism5. Statistical significance was determined using t tests (two tailed), and P values 0.05 were considered significant. Samples on ELISA were excluded if their CV exceeded 10% and for ECL MSD ELISA Samples with a CV up to 20% were accepted.

3.1 CTLA4

Serum samples were analysed with the help of direct ELISA by coating the plates with CTLA4 antigen in order to detect Anti-CTLA4 antibody levels in 306 human sera. The used samples split into 153 samples taken prior to transplantation and 153 samples collected up to 3 months post transplantation. One sample had to be excluded from the analysis and so 152 patient's sera were analysed.

All samples were run in duplicates and the mean of the two results was taken. For a better comparison between data with different means, we used the CV (coefficient of variation). For CTLA4, the overall CV came to 4% and Stdev was 0.038954.

As a first analysis we asked whether the signals, and therefore the antibody levels for CTLA4 correlate between samples taken prior to transplantation and those taken after surgery. Antibody signal for CTLA4 for pre- and post- transplant samples strongly correlate. This was expected and can be observed in Figure 4.

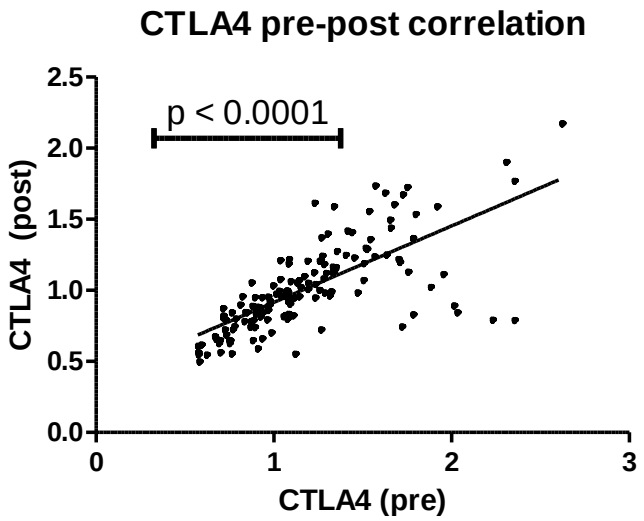

Figure 4 Correlation of Signals from Samples taken Pre- and Post transplantation. All 152 pre-samples were plotted against their corresponding post-transplant sample and expressed in this correlation graph.

Table 2 Statistics to Correlation of Pre- and Post- samples

Number of XY Pairs	152
Pearson r	0.7127
95% confidence interval	0.6244 to 0.7831
P value (two-tailed)	< 0.0001
P value summary	***
Is the correlation significant? (alpha=0.05)	Yes
R square	0.5080

Next we asked whether there was a change in antibody concentration between blood taken prior to transplantation or after. A slight decrease in signal was expected in post-transplant sera. Due to the sample date being after the transplantation and therefore after patients were treated with various immunosuppressants, the antibody levels in the blood fell significantly.

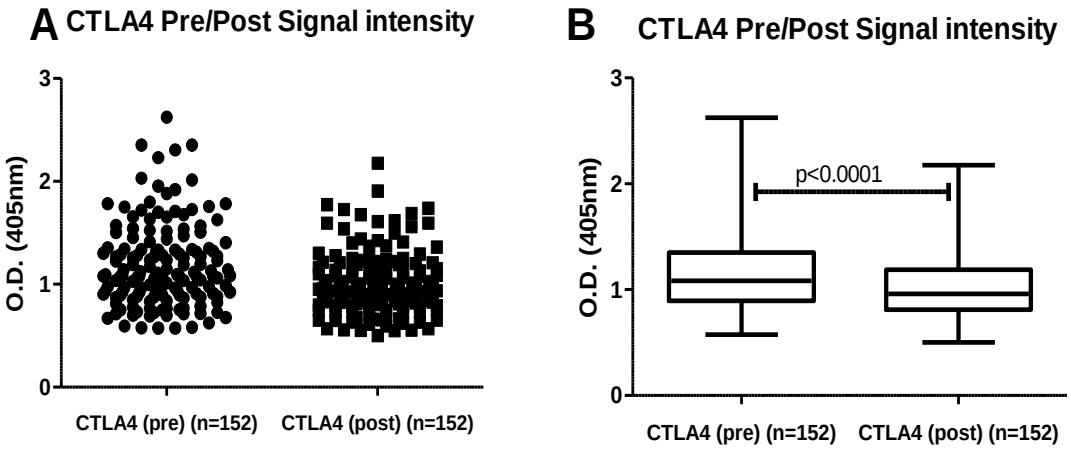

*Figure 5: **A)** Scatter Plot showing different Signal intensities Pre and Post Transplantation. Signal decreased significantly. **B)** Block diagram with whiskers showing Min to Max values and 25% of Data in the Box*

Table 3 Statistical Data to compared pre- and post- transplant antibody levels

Mean ± SEM of column A	1.183 ± 0.03282 N=152
Mean ± SEM of column B	1.014 ± 0.02475 N=152
Stdev Pre Transplant	0.402587
Stdev Post Transplant	0.290011
Difference between means	0.1695 ± 0.04111
95% confidence interval	0.08896 to 0.2501
R square	0.05331
Median Pre Transplant	1.082783
Median Post Transplant	0.958645

The next question we wanted to answer was if a trend could be seen in high antibody levels pre transplant and the time of rejection post transplant. To evaluate this, two approaches were made. First 48 samples were split into two groups according to their signal intensity. Patients with higher CTLA4 antibody levels in their sera showed to develop rejection earlier than patients with a lower concentration. This can be observed in Figure 6.

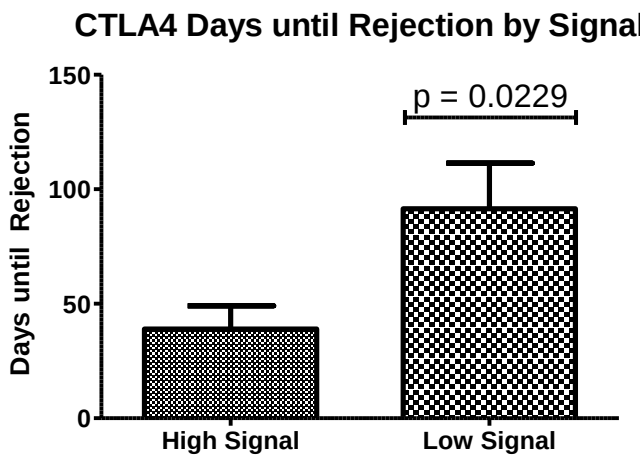

Figure 6: Signal strength devided into high signal and low signal groups were plotted against days until rejection. High CTLA4 antibody levels indicate a faster rejection of the transplanted organ.

As a second approach, 48 samples were separated according to their time of rejection post transplant. Occurences of acute rejection (AR) during the first 30 days after surgery, as observable in Figure 7, also showed to have a higher level of Anti-CTLA4 antibody pre- transplant. These two findings indicate a predictive character for high CTLA4 antibody levels found prior to transplantation.

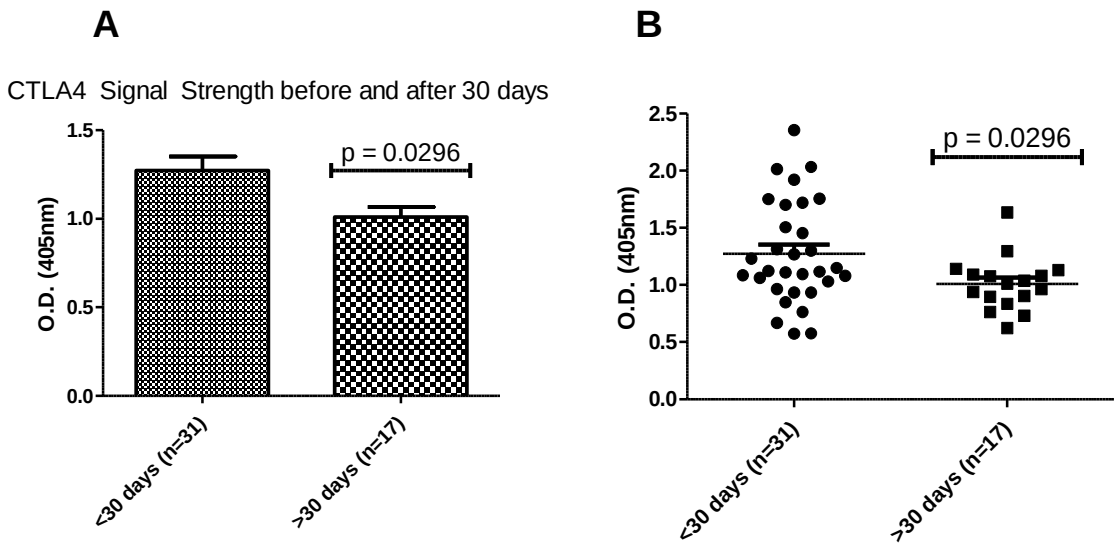

Figure 7 Rejection occured within the first 30 days after transplantation plotted against rejection that occured after 1 month post surgery. ***A)*** *Showing a box diagram with whiskers and* ***B)*** *showing the same data in a scatter diagram in order to visualize specific data points.*

Considering that different patients might generally have different amounts of antibody present, we also wanted to see whether a higher decrease or increase of antibody signal in post- transplant samples lead to rejection of the graft or if the organ was stable. Our findings for all antibodies did not show a significant correlation between samples with a high delta signal and rejection. The obtained p-value for this question was 0.5405 for CTLA4.

Additionally, we analyzed whether a higher or lower decrease in antibody levels post transplant would lead to earlier rejection. Therefore we took the delta signal of pre and post transplant samples and plotted samples with a high decrease in antibody levels against samples that had a more or less stable amount of antibodies before and after surgery. This happened for all four antibodies but no trend could be observed so the idea was rejected. The results showing the days until rejection for CTLA4 can be seen in Figure 8

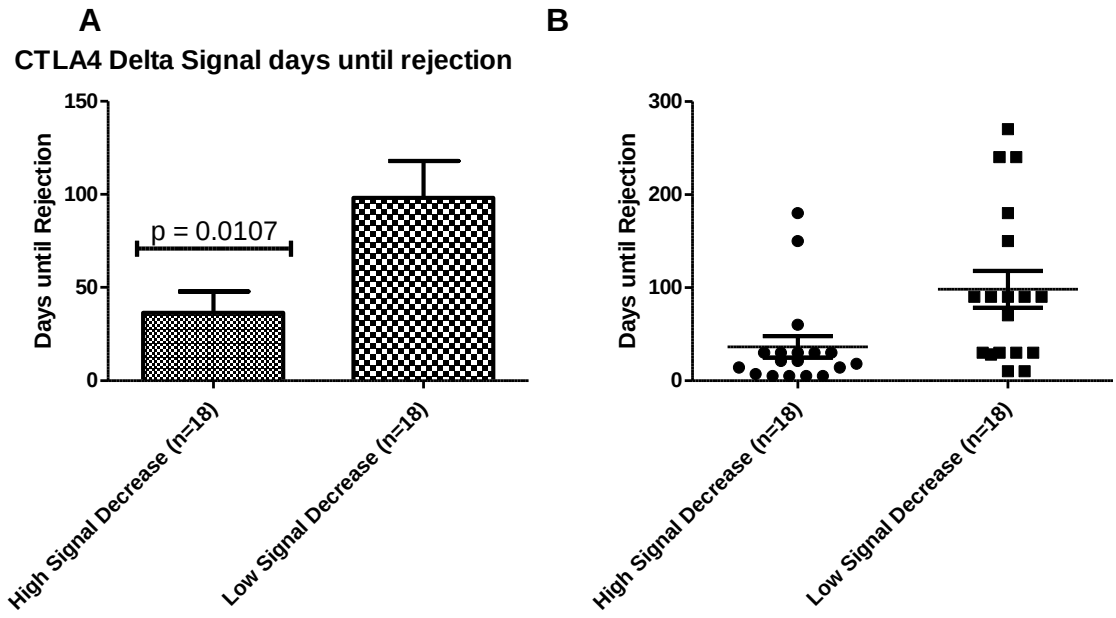

Figure 8: Presenting days until rejection according to high/low decrease in antibody levels post-transplant. ***A)*** *Shows a block diagram with whiskers.* ***B)*** *Scatter diagram to indicate specific datapoints.*

Furthermore we wanted to know whether there was a difference between samples that had an increase and samples that had a decrease. Unfortunately for all antibodies only little samples actually had an increase in antibodies post-transplant and so the findings would have turned out as no significant.

3.2 CD7

For CD7 298 serum samples from transplant patients taken pre and post transplantation were analysed with the help of MSD Electrochemiluminescence (ECL). Therefore, 15ng of CD7 antigen was coated onto specific MSD ELISA plates and their Anti-CD7 antibody level was measured with the help of the MSD MESO QuickPlex SQ 120 Plate Reader.

All samples were run in duplicates and the mean of the two results was taken. For a better comparison between data with different means, we used the CV. For CD7, the overall CV came to 11% and Stdev was 1079.389.

As for CTLA4, we asked whether antibody levels pre and post transplant correlate with each other. All samples were plotted against each other and can be observed in the correlation plot in Figure 9.

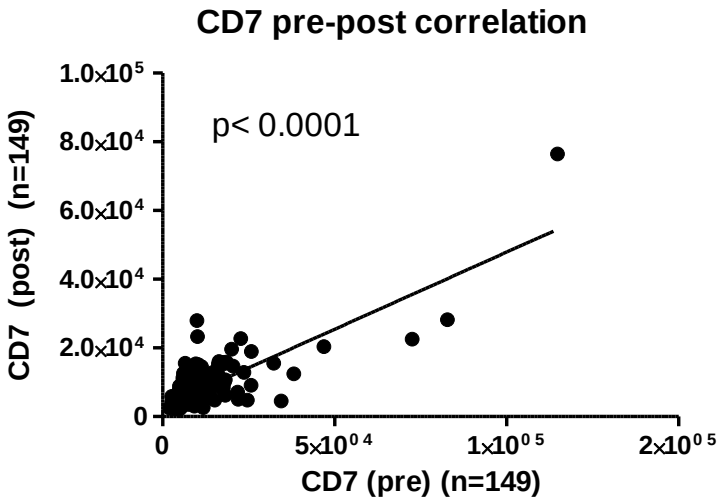

Figure 9: ***Correlation of Signals for CD7 from Samples taken Pre- and Post transplantation..*** *All 149 pre-samples were plotted against their corresponding post-transplant sample and expressed in this correlation graph.*

Table 4 Statistics to Correlation of Pre- and Post- samples for CD7

Number of XY Pairs	149
Pearson r	0.7963
95% confidence interval	0.7289 to 0.8485
P value (two-tailed)	< 0.0001
P value summary	***
Is the correlation significant? (alpha=0.05)	Yes
R square	0.6342

Also for CD7 we wanted to know whether there was a change in antibody concentration between blood taken prior to transplantation or after. A slight decrease in

signal was expected in post-transplant sera. Due to the sample date being after the transplantation and therefore after patients were treated with various immuno-suppressants, the antibody levels in the blood fell significantly.

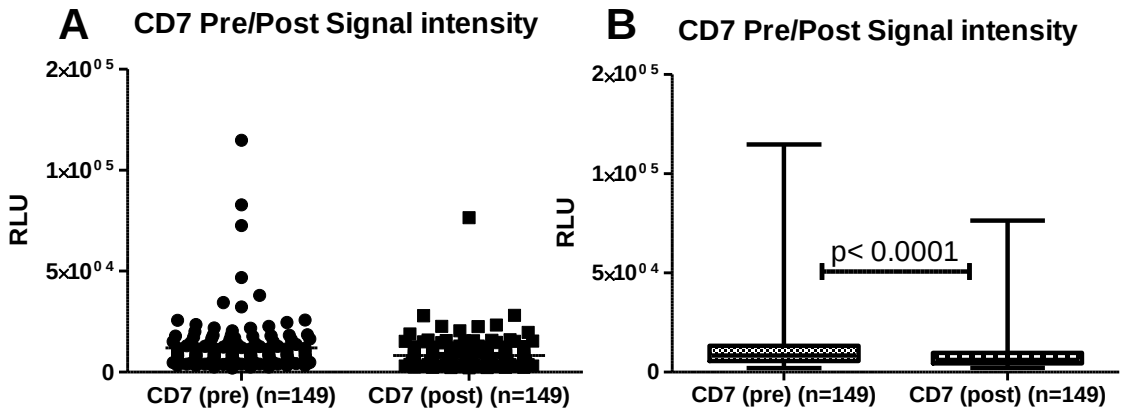

Figure 10 ***A)*** *Scatter Plot showing different Signal intensities Pre and Post Transplantation. Signal decreased significantly after transplantation.* ***B)*** *Block diagram with whiskers showing Min to Max values and 25% of Data in the Box*

Table 5: Statistical Data to compared pre- and post- transplant CD7 antibody levels

Mean ± SEM of column A	12016 ± 1105 N=149
Mean ± SEM of column B	8365 ± 623 N=149
Stdev Pre Transplant	13398.24
Stdev Post Transplant	7526.79
Difference between means	0.1695 ± 0.04111
95% confidence interval	0.08896 to 0.2501
R square	0.05331
Median Pre Transplant	8483.75
Median Post Transplant	6154.29

To answer whether there was a trend in high antibody levels pre transplant and the time of rejection post transplant. Again two approaches were made where first 48 samples were separated according to their signal intensity. Patients with higher CD7 antibody levels showed to develop rejection earlier than patients with a lower concentration. This can be observed in Figure 11.

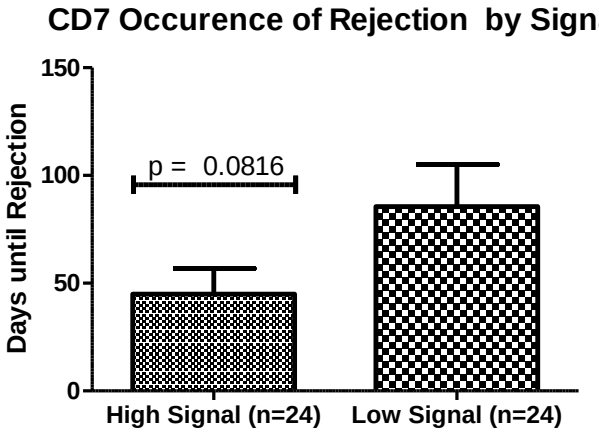

Figure 11 Signal strength split into high signal and low signal groups, plotted against days until rejectiond. High CD7 antibody levels apparently lead to a faster rejection of the graft.

To evaluate this finding further, 48 samples were split into rejection post transplant after 30 days and before 30 days. Figure 12 demonstrates the result of this analysis.

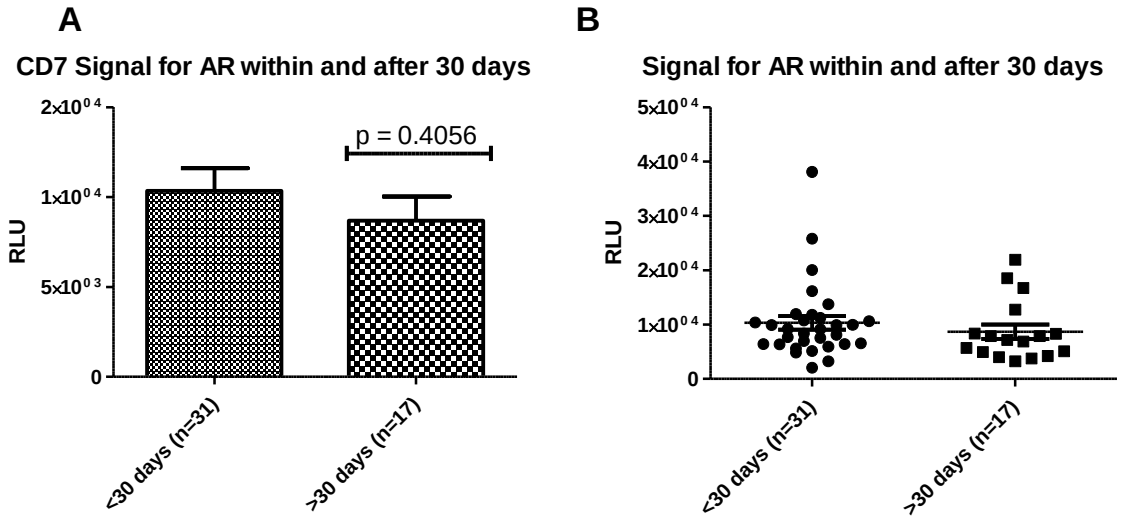

Figure 12 CD7 Signal of patients suffering graft rejection within 30 days post transplant and after 30 days. ***A)*** *Showing a box diagram with whiskers and* ***B)*** *showing the same data in a scatter diagram in order to visualize specific data points.*

As with CTLA4, we asked again what the signal difference between samples taken pre- and post transplant could say about rejection. Three approaches were made to find whether a decrease or increase in signal might lead to more or earlier rejection.

First the question was whether patients with a higher difference were more or less likely to reject their organ. Ultimately these findings had a p-value of 0.3403 and were therefore considered as non-significant.

3.3 IFI6

In the case of IFI6, 150 samples could be analysed. with the help of MSD Electrochemiluminescence. 15ng of IFI6 antigen were coated onto specific MSD ELISA plates and all samples were assessed to find Anti-IFI6 antibodies. All samples were run in duplicates and the mean of the two results was taken. The overall CV for this analysis was 4% and the Stdev was found to be 6364.162 RLU.

As for the antigens discussed previously, we asked whether antibody levels pre and post transplant correlate with each other. All samples were plotted against each in Figure 13.

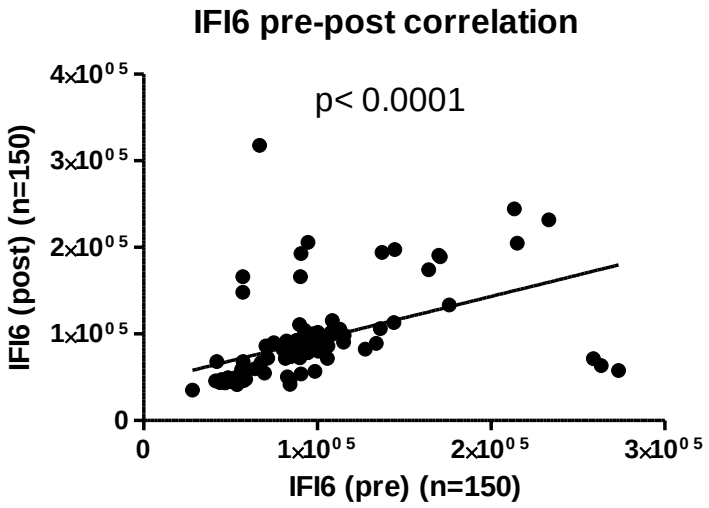

Figure 13 ***Correlation of Signals for IFI6 from Samples taken Pre- and Post transplantation.*** *All 150 pre-samples were plotted against their corresponding post-transplant sample and expressed in this correlation graph.*

Table 6 Statistics to Correlation of Pre- and Post- samples for IFI6

Number of XY Pairs	150
Pearson r	0.4702
95% confidence interval	0.3351 to 0.5863
P value (two-tailed)	< 0.0001
P value summary	***
Is the correlation significant? (alpha=0.05)	Yes
R square	0.2210

Change in antibody concentration between blood taken prior to transplantation and blood taken after transplantation was assessed again plotting results obtained from pre transplant sera against results obtained from post transplant sera.

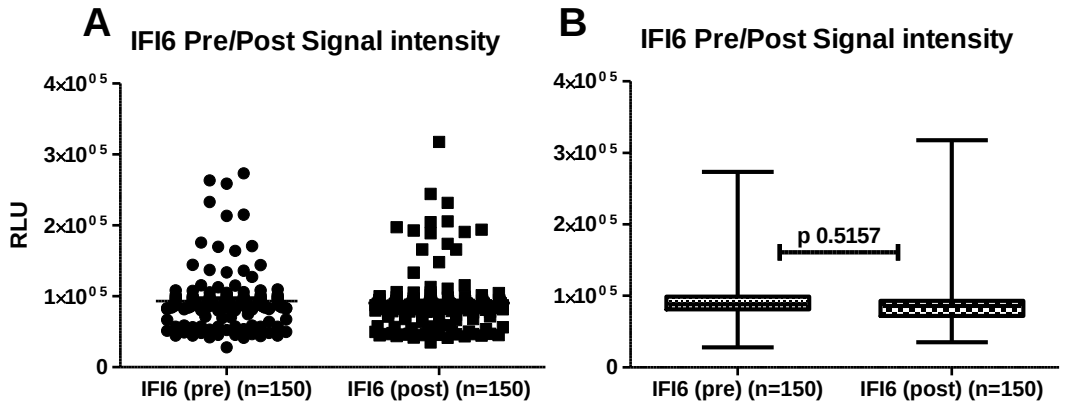

Figure 14 Scatter Plot showing different Signal intensities Pre and Post Transplantation. Signal was constant after transplantation but no significance could be found in this analysis. ***B)*** *Block diagram with whiskers showing Min to Max values and 25% of Data in the Box*

Table 7 Statistical Data to compared pre- and post- transplant IFI6 antibody levels

Mean ± SEM of column A	93437 ± 3252 N=150
Mean ± SEM of column B	90475 ± 3430 N=150
Stdev Pre Transplant	39693.45
Stdev Post Transplant	43493.71
Difference between means	2962 ± 4726
95% confidence interval	-6302 to 12226
R square	0.001316
Median Pre Transplant	88007.66
Median Post Transplant	85944.87

Also for IFI6 it was evaluated whether there was a trend in high antibody levels pre transplant and the time of rejection post transplant. Again two approaches were made where first 48 samples were separated according to their signal intensity. Patients with higher IFI6 antibody levels showed to develop rejection earlier than patients with a lower concentration.

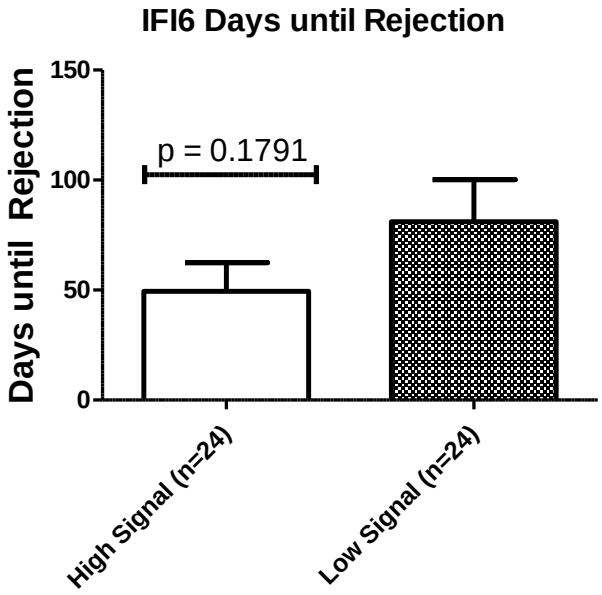

Figure 15 Signal strength split into high signal and low signal groups, plotted against days until rejection occured. High IFI6 antibody levels appear to lead to a faster rejection of the graft

To evaluate this finding further, 48 samples were into rejection post transplant after 30 days and before 30 days. Figure 13 demonstrates the result of this analysis.

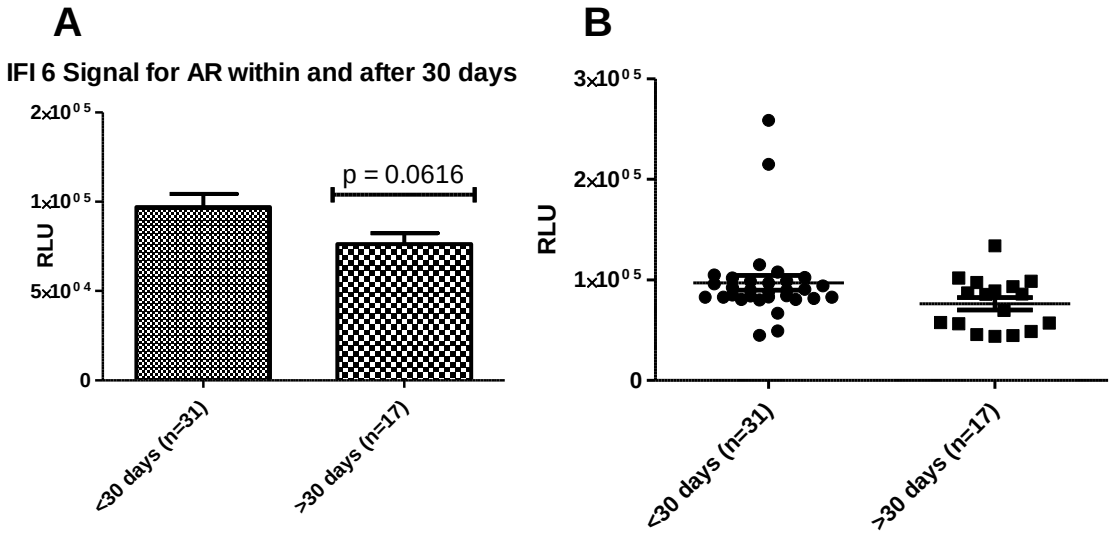

*Figure 16 IFI6 Signal of patients suffering graft rejection within 30 days post transplant and after 30 days. **A)** Showing a box diagram with whiskers and **B)** showing the same data in a scatter diagram in order to visualize specific data points.*

To further analyse this Antibody, the same three approaches conducted for the first two antibodies were repeated. The signal difference between samples taken pre- and post transplant was analysed. Looking at samples with a higher difference and seeing if they were more likely to reject their organ ultimately showed to have a p-value of 0.7012 and the idea was therefore rejected.

3.4 TRIM21

The last antigen studied was TRIM21. Optimization experiments showed that the best results could be achieved by coating 10ng of this protein. 150 samples could be analysed. with the help of MSD Electrochemiluminescence. All samples were run in duplicates and the mean of the two results was taken. The overall CV for this analysis was 4% and the mean stdev was found to be 4074.106RLU

Again we wanted to see whether antibody levels pre and post transplant correlate with each other. All samples were plotted against each in Figure 17.

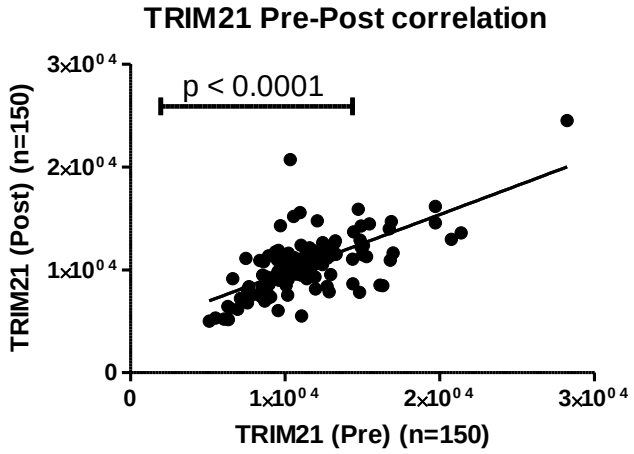

Figure 17 ***Correlation of Signals from Samples taken Pre- and Post transplantation.*** *All 150 pre-samples were plotted against their corresponding post-transplant sample and expressed in this correlation graph.*

Table 8: Statistics to Correlation of Pre- and Post- samples

Number of XY Pairs	153
Pearson r	0.6253
95% confidence interval	0.5180 to 0.7132
P value (two-tailed)	< 0.0001
P value summary	***
Is the correlation significant? (alpha=0.05)	Yes
R square	0.3910

Again a slight decrease in signal was expected in post-transplant sera compared to pre transplant sera. If this was true is shown in the following Figure 18.

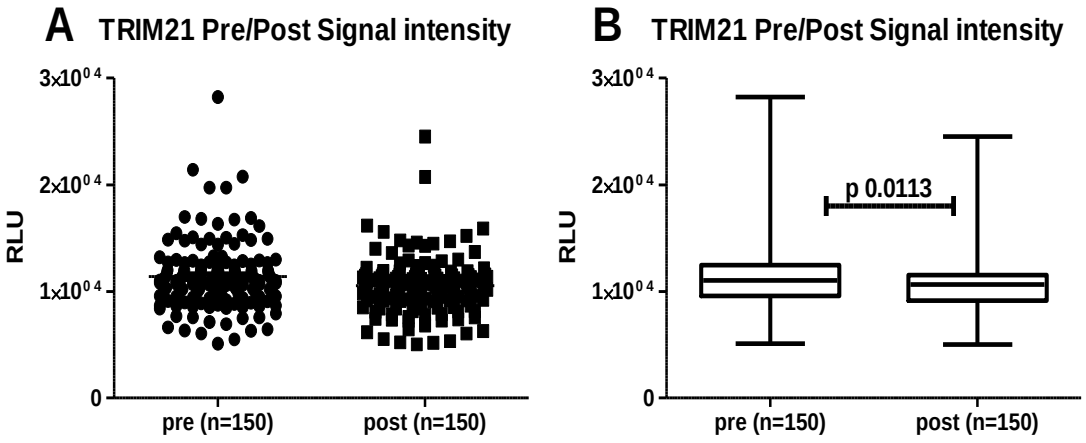

Figure 18 ***A)*** *Scatter Plot showing different Signal intensities Pre and Post Transplantation. Signal decreased significantly.* ***B)*** *Block diagram with whiskers showing Min to Max values and 25% of Data in the Box*

Table 9: Statistical Data to compared pre- and post- transplant antibody levels

Mean ± SEM of column A	11360 ± 255.2 N=150
Mean ± SEM of column B	10511 ± 214.1 N=150
Difference between means	848.7 ± 333.1
95% confidence interval	195.8 to 1502
R square	0.02132
Median Pre Transplant	11021.15
Median Post Transplant	10635.75
Stdev Pre Transplant	3115.35
Stdev Post Transplant	2617.43

Whether there was a trend to be seen in high antibody levels pre transplant and the time of rejection post transplant was the next question. To evaluate this, two approaches were made. First 48 samples were split into two groups according to their signal intensity. Patients with higher TRIM21 antibody levels in their sera showed to develop rejection earlier than patients with a lower concentration. This can be observed in the following Figure 26.

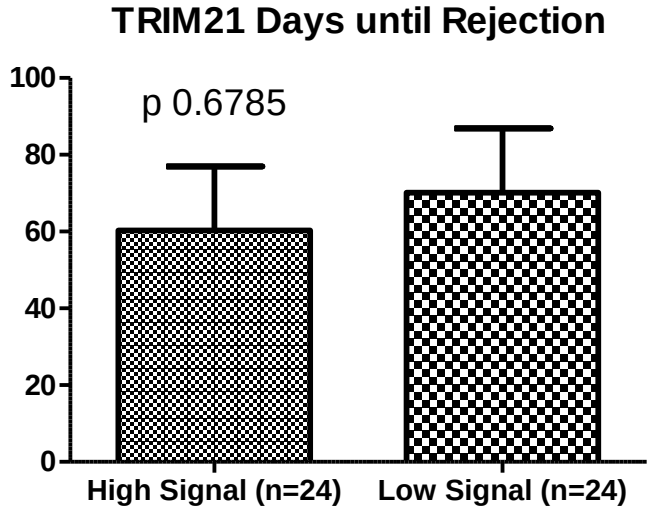

Figure 19: TRIM21 Signal intensities pre transplant correlated to days until rejection.

As a second approach the same 48 samples were divided according to their time of rejection post transplant. Acute rejection (AR) during the first 30 days after surgery, also showed to have a higher level of Anti-TRIM21 antibody pre- transplant.

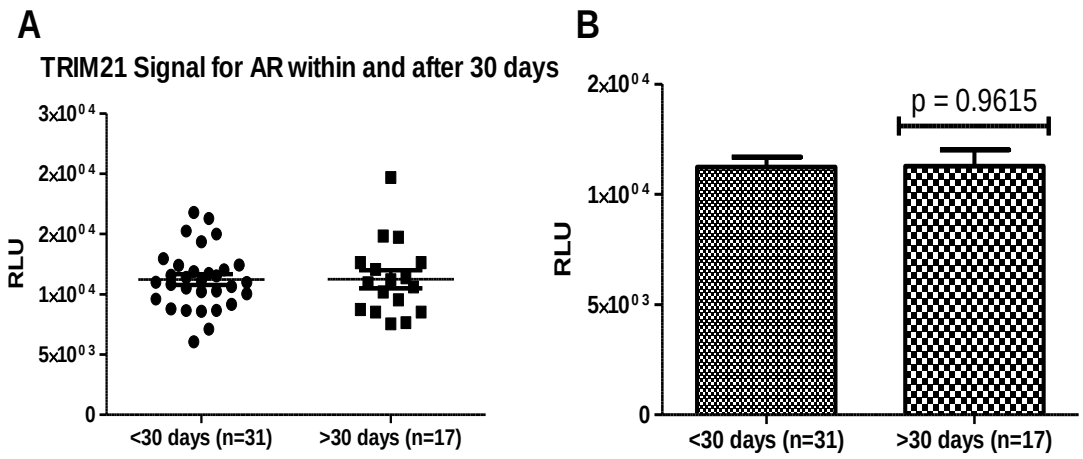

Figure 20 TRIM21 Signal of patients suffering graft rejection within 30 days post transplant and after 30 days. ***A)*** *Showing a scatter diagram visualizing individual samples and* ***B)*** *showing a box diagram.*

Once more the signal difference between samples taken pre- and post transplant were analyzed in terms of what they could say about rejection. Three approaches were made to find whether a decrease or increase in signal might lead to more or earlier rejection. First the question was whether patients with a higher difference were more or less likely to reject their organ. Ultimately these findings had a p-value of 0.3403 and were therefore considered as non-significant.

4 Discussion

The clinical importance of HLA-specific antibodies to graft rejection is well established. However, several studies have proven non-HLA-specific antibodies to negatively influence graft survival. In the worst cases, the graft can be lost hyperacutely or acute. Often, non-HLA-specific antibodies react with donor endothelial cells. Over the last 25 years transplant clinicians have tried to find a way to test for the presence of AECA before transplantation.

AECAs may be found in as many as 25% of kidney transplant recipients with negative conventional T- and B-lymphocyte crossmatch and are strongly correlated with graft dysfunction or rejection. Methods currently used to detect AECAs are laborious, time-consuming, and impractical for use as clinical routine diagnostics [51]

In this study an approach was made to detect AECAs and to see what outcome high antibody levels pre transplant have.

4.1 CTLA4

The first findings that CTLA4 antibody levels decrease in samples taken post transplantation were expected and could be explained easily due to the fact that all patients were receiving some form of immunosuppression. Therefore, most showed to have a lower concentration after treatment.

In the case of CTLA4, a possible predictive characteristic has been found. Samples taken before transplantation were tested for their Anti-CTLA4 Antibody levels and then analysed regarding their time until rejection. The trend observed was that graft rejection occurred faster within patients presenting a high concentration of CTLA4 antibody prior to receiving their organ. However, due to the small amount of samples that could be administered in this interest, further analyses are necessary. Importantly more data points are needed to validate this finding. Additionally, the line drawn at 30 days to split the groups into early AMR and “late” AMR was chosen just for a broad analysis and without an actual threshold. The obtained p-

values of 0.0296 and 0.0229 indicate that these findings are significant and encourage this theory additionally.

Considering that different patients might generally have different amounts of antibody to begin with, we analyzed the change of antibody signal in pre- and post-transplant samples. Unfortunately it could not be proven that a decrease or increase in CTLA4 antibody would more likely lead to rejection. This can either be true or there were not enough samples to come to a clear result in this finding. It might also be that the current ELISA techniques used are not sensitive enough.

In the next analysis, since a decrease was generally expected, high and low decreases were considered. A high decrease in CTLA4 levels lead to later rejection than samples that had only a low decrease. This strengthens CTLA4s potential to predict earlier rejection further and might also indicate that some patients correspond better to the given treatment. Further investigation regarding treatment might be very interesting at this point but could unfortunately not be conducted in this study.

Furthermore we wanted to know whether there was a difference between samples that had an increase and samples that had a decrease. Unfortunately only little samples actually had an increase in antibodies post-transplant.. Although the p-value for this analysis is above 0.05 and was therefore seen as non-significant, a trend towards faster rejection in patients that had a CTLA4 antibody level increase could be observed. Eventually due to wrong or non-sufficient treatment ultimately more antibodies were produced and the graft was rejected faster. As mentioned before, further investigation regarding treatment would be interesting.

4.2 CD7

Not only for CTLA4 but also for CD7 Antibodies, a high correlation could be found between samples taken pre and post transplant. Because the samples were run on different ELISA plates on different days, this is a very important finding and

gives us the proof that the results obtained are consistent throughout days and assays.

Signal intensities widely decreased post-transplant. This again can be concluded by the immunosuppressive treatment patients received.

As in CTLA4, also for CD7 we compared high signal samples vs low signal samples pre transplant and a trend towards earlier rejection could be observed in any sample that showed to have higher CD7 levels before surgery. Also analyzing signal intensities of grafts rejected within 30 days and after 30 days showed the same tendency. However, the p-value does indicate no significance and unfortunately it was not possible for us to collect enough data to further evaluate this question.

4.3 IFI6

Antibody levels decrease again in samples taken post transplantation. As patients were treated with immunosuppressants this finding was expected and could be explained. Therefore, most showed to have a lower concentration after treatment. The results for IFI6, however show a more or less constant antibody level post transplant. This might tell us something about the immunosuppressants not being able to fully block IFI6 activities. Further analysis in this point including more samples but also analysis testing immunosuppressants against their activity towards IFI6 would be very interesting.

In the case of IFI6, just as for CTLA4 and CD7, a possible predictive characteristic has been found regarding time of rejection. Samples taken pre transplantation were tested for their Anti-IFI6 Antibody levels and then analysed regarding their time until rejection. The trend that could be observed was that graft rejection occurred faster within patients presenting a higher amount of IFI6 ab prior to receiving their organ. The p-value for this finding is too high to be considered significant. However, a trend can be clearly observed in both methods used to approach this question. Due to the small amount of samples that could be administered in this interest, further analyses are necessary. Of course the line drawn at 30 days to

split the groups into early AMR and “late” AMR was just taken for a quick analysis and without an actual threshold.

For every antibody tested different patients might generally have different amounts to begin with. The change of antibody signal in pre- and post- transplant samples was therefore tried to evaluate. Unfortunately it could not be proven that a decrease or increase in antibody would more likely lead to rejection. This either simply be true or there were not enough samples to come to a clear result.

4.4 TRIM21

Correlation plots for this antibody shows to have high significance. This could be observed for all antibodies tested and let us suggest that we can relie on these data. This kind of observation was important since no one has ever tested for these certain antibodies and it was unclear what could be expected.

As for all other, time of rejection was correlated to anti-TRIM21 antibody levels pre transplant. The results show a smaller trend than has already been observed for the three previously tested antibodies CTLA4, CD7 and IFI6.

Acute rejection (AR) during the first 30 days after surgery, showed to have only a little difference in TRIM21 levels. These two findings only show a weak trend. The p-values for TRIM21 unfortunately resulted to be very high. Of course the observable trends are not enough to conclude anything. In the case of TRIM21 further analysis are very much wanted to either proof or disproof it’s predictive character.

Due to the fact that antibodies and especially IgG are very sticky proteins this study was difficult to conduct. Although already a very specific Electrochemiluminescence assay method was chosen, whereby washing steps are less important due to the fact that only antibodies in close proximity to the electrodes get excited and detectable, it might be that the assay is simply not applicable for TRIM21.

4.5 Overall Image

The big picture obtained from these four antibodies tested is that all showed to have a correlation with earlier rejection. However, high levels do not necessarily lead to rejection. The chances for samples that present elevated antibody concentrations were about 50/50 to reject within the first year. However, it is suggested that if a patient presents a certain degree of any or all of these antibodies, he/she is more likely to reject the graft earlier. Now maybe this finding could be put together with other analysis methods and other biomarkers for rejection that have already been found, to find a threshold of a specific amount of days until it can be said that rejection is less likely to occur. In a more practical sense that would mean if there is a patient that disposes of a high level of CTLA4 and CD7 antibodies (as an example) in his/her serum pre transplant and other tests have not shown any reasons to suggest early rejection so far, this patient could be considered as to be over the hump, corresponding well to his/her given treatment and less likely to reject the graft if he/she has not shown any signs of rejection until day XX.

When looking at antibody levels comparing AMR and TCMR a strong trend could be seen for antibodies CTLA4, CD7 and IFI6. Patients showing to possess a higher amount of these antibodies, tend to suffer AMR rather than TCMR. This of course was expected but also indicates that these certain antibodies are playing an important role in AMR. However it must be taken under account that for TCMR only very few samples were available (n=3). Unfortunately 68 of the 150 patients tested, also showed to have persistent DSAs in addition to non-HLA antibodies post-transplant. Therefore it cannot be concluded clearly that the tested antibodies were the only reason rejection occurred and that DSA might have contributed to rejection.

In the case of TRIM21 it is not clear whether it is a bad indicator or if, and this is presumed, the small amount of TCMR patients, were simply not enough to give reliable results. To confirm this, another trial with an equal amount of TCMR and AMR samples should be conducted. Also group of DSA negative patients should be included for further analysis.

HLA mismatches were detected in almost all the patients. However, patients presenting a higher amount of any of the four tested antibodies, also showed to have a higher HLA mismatch. This accounts especially for HLA class 1. In this study presence of non-HLA antibodies prior to transplant and their impact on rejection was administered. The existence of these antibodies prior to donor selection is therefore independent on the HLA mismatch. However, if patients that were tested positive to our non-HLA antibodies were transplanted with a higher mismatch, then we found that rejection occurs even faster among patients possessing HLA-mismatches and non-HLA antibodies together. This let's again suggest an important role for non-HLA antibodies in graft rejection.

When looking at the PRA that represents the percentage of the population to which the patient will react via pre-existing antibodies, no correlation to higher or lower levels of our four tested antibodies could be found. This finding lets us suggest that injury or inflammation that leads to non-HLA antibody formation may not be the same type as the one that leads to HLA antibody formation.

5 Conclusion

This study was a small follow-up study of a study prior published by Annette Jackson's group at John Hopkins hospital, trying to validate more AECAs and finding correlations to patient's clinical outcomes.

The study conducted prior to this, was published upon its completion after three years of hard work. In order to further validate our findings more samples will have to be included from larger clinical trials. Samples at the time of rejection would be helpful to correlate the antibody level with acute rejection episodes. With such an approach, samples could be assayed prior to rejection to test the hypothesis that these antibody levels are increased prior to rejection and might have predictive characteristics towards rejection. Unfortunately we did not have access to samples at the time of rejection.

However, we could validate the association of antibody level with early or late rejection possibility based on the level. Future approaches would include the finding of a threshold in antibody levels. What is considered elevated? The problem in testing for antibodies is that there are no standards, no "housekeeping" antibody that can be compared with as in genetics. Another approach would be including other antibodies found in Annette Jackson's study and correlating them to our findings. Maybe there are other antibodies with even better predictive properties. Of course including more samples with more data points as mentioned before is necessary. A multicenter approach to further evaluate the findings so far, is desirable.

For now only a small step has been taken but a possible future as biomarkers is seen possible.

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