The role of clinical and subclinical inflammation in kidney transplant biopsies

Christina Dörje

Faculty of Medicine

UNIVERSITY OF OSLO

Department of Nephrology

Department of Transplant Medicine

OSLO UNIVERSITY HOSPITAL RIKSHOSPITALET

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Abbreviations

ABOi	ABO incompatible
ABOc	ABO compatible
ABMR	Antibody-mediated rejection
aABMR	acute antibody-mediated rejection
aTCMR	acute T-cell mediated rejection
APC`s	antigen presenting cells
ATG	Thymyoglobuline
ATN	acute tubular injury
cABMR	chronic antibody-mediated rejection
CADI	Chronic Allograft Damage Index
CDC	cytotoxic crossmatch
CNI	calcineurin inhibitor
C4d	complement factor 4d
cTCMR	chronic T-cell mediated rejection
DD	deceased donor
DGF	delayed graft function
DSA	donor-specific antibody
HLA	human leucocyte antigen
IvIg	Immunoglobulin
LD	living donor
MFI	mean fluorescence intensity
MHC	major histo compability complex
MMF	mycophenolate mofetile
MPGN	membranoproliferative glomerulonephritis
MVI	microvascular inflammation
PE	plasma exchange
PRA	panel reactive HLA antibody
SCR	subclinical rejection
STAMP	Scandinacian Acceptable Mismatch Program
TCMR	T-cell mediated rejection
TG	Transplant glomerulopathy
TMA	thrombotic microangiopathy

List of Papers

I. Christina Dörje, Karsten Midtvedt, Hallvard Holdaas, Christian Naper, Erik H. Strøm, Ole Øyen, Torbjørn Leivestad, Tommy Aronsen, Trond Jenssen, Linda Flaa-Johnsen, Jørn Petter Lindahl, Anders Hartmann, Anna Varberg Reisæter. **Early and late acute antibody-mediated rejection in renal transplant recipients.**
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INTRODUCTION

1. BACKGROUND

Kidney transplantation is the best treatment option with respect to quality of life and survival for patients with end stage kidney failure. The superiority of transplantation above dialysis was convincingly shown in the landmark study of Wolfe et al. (*1*) and was later on reassured by a systematic literature review including almost 2 million patients (*2*). Transplantation offers a survival benefit compared to hemodialysis also in the older population age > 70 years, provided the recipients pass transplantation without early rejections (*3, 4*).

This survival benefit of transplantation versus dialysis was also corroborated in a multicenter retrospective study of 1025 immunological high risk patients sensitized to Human leukocyte antigen (HLA) matched to control persons, all receiving a living donor transplant (*5*). Kidney transplantation is cost effective compared to dialysis, especially after the first year post transplantation (*6*).

1.1 Kidney transplantation in Norway in historical perspective

The first successful kidney transplantation was performed in 1954 by the surgeon Joseph Murray at the Peter Bent Brigham hospital in Boston in a 23 year old patient who had an identical twin brother as donor. Joseph Murray was awarded the Nobel Prize in Medicine in 1990 (*7*).

Encouraged by this, the Norwegian surgeon Leif Efskind transplanted the first human kidney in Scandinavia only 2 years later in 1956 at Rikshospitalet in Oslo. After a pioneer period of kidney transplantation, the Norwegian national kidney

transplantation program started in 1969 at Rikshospitalet and Ullevål Hospital. From 1983 Rikshospitalet has been the only center for solid organ transplantation in Norway. The Scandinavian organization for organ exchange *Scandiatransplant* was established in 1969.

Tissue typing was at its early beginning in the1970`s and was a prerequisite for successful organ transplantation. The tissue typing laboratory was established at Rikshospitalet by Erik Thorsby in 1970, while the Norwegian Renal Registry was established by the enthusiast Torbjørn Leivestad, nephrologists and immunologist at the same hospital. The official founding took place at the annual meeting of the Norwegian Society of Nephrology in 1994. All patients in Norway starting renal replacement therapy, dialysis or transplantation, are included and followed in the registry with yearly data until death or migration. The high quality and completeness of data is facilitated by the network of Norwegian nephrology departments reporting to the registry (Annual Report 2014 at http://www.nephro.no/nnr/AARSM2014.pdf).

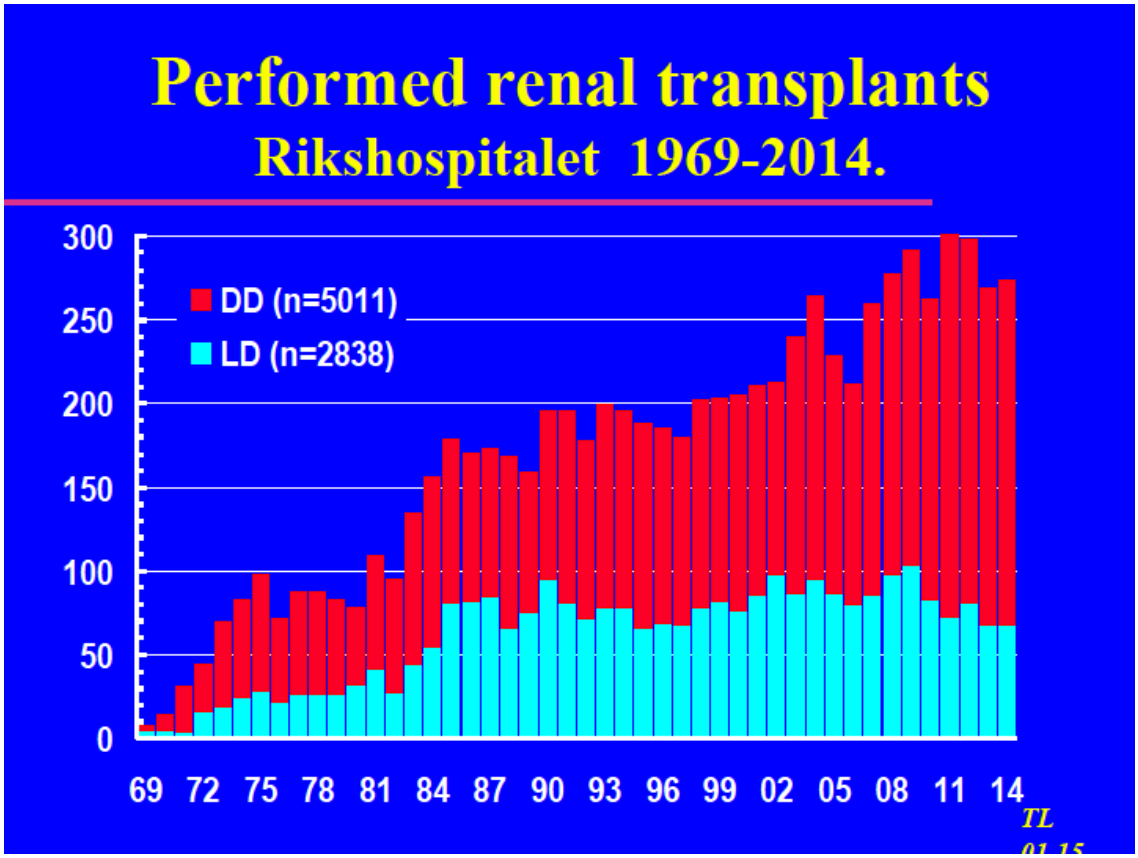
1.2 Kidney transplantation in Norway today

Rikshospitalet Oslo University Hospital is the national center for solid organ transplantation in Norway. It covers a well working network of 26 donor hospitals and 25 nephrological centers throughout Norway. Since the establishment of the renal transplant program the aim has been to offer transplantation to all patients with end stage renal disease who can benefit from the treatment not restricted by recipient's age or comorbidity. The Norwegian renal transplantation program also has the ambition to offer transplantation to those with less access to donor organs because of immunization.

A high rate of renal transplantation can be realized as both living donor (LD) and deceased donor (DD) programs have been actively pursued. Rikshospitalet is one of the largest transplantation centers worldwide with 250-300 kidney transplants/year (Figure 1), corresponding to 53.7 kidney transplantations /million inhabitants in 2014 (NEWSLETTER TRANSPLANT, International figures on donation and transplantation 2014, EDQ, Volume 20, 2015).

Approximately 10-15% of all kidney transplantations/ year at Rikshospitalet are immunological high risk transplantations with preformed HLA donor-specific antibodies (DSA) and/or panel reactive HLA antibodies (PRA) ≥20% or blood group antibodies (own data).

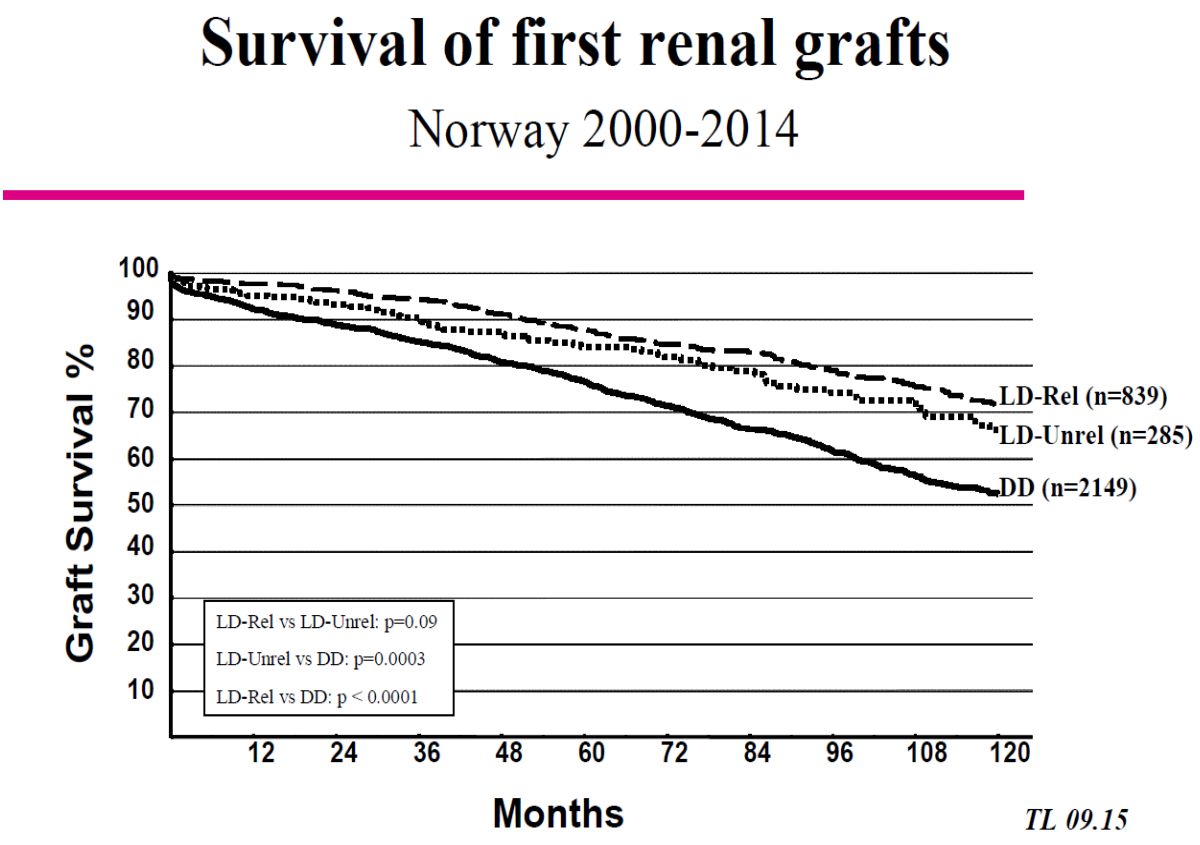
Figure 1. Renal transplants performed in Norway since 1969-2014



With courtesy Torbjørn Leivestad, data retrieved from the Norwegian Renal Registry

Norwegian nephrologists have since the beginning of the renal transplantation program in Norway always focused on a very active living donor program. LD transplantation provides a better outcome for recipients compared to DD transplantation, (Figure 2). It offers advantages such as the possibility of a planned preemptive transplantation, shorter ischemia time and good organ quality with little chronic changes in the transplant.

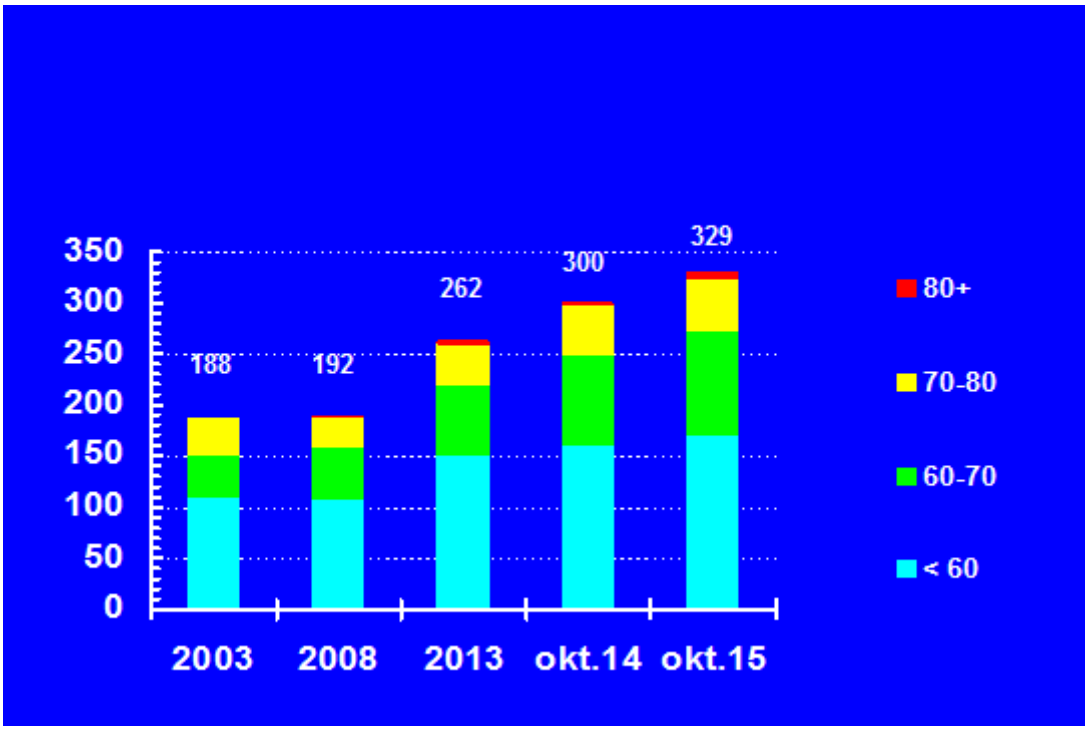
Figure 2. First graft survival - Living donor (LD) and deceased donor (DD)



With courtesy Torbjørn Leivestad, data retrieved from the Norwegian Renal Registry

Approximately 30-40% of the annual kidney transplants are living donations, which until recently has contributed to keep the Norwegian waiting list short compared to Europe and the USA. However during the last few years the Norwegian waiting list has also increased due to decrease in donation and expansion of the potential recipient pool by accepting patients with higher age, more comorbidities and higher GFR, (Figure 3).

Figure 3. Waiting list kidney transplantation in Norway from 2003-2015 with age distribution



With courtesy Torbjørn Leivestad, data retrieved from the Norwegian Renal Registry

A way to expand the LD pool and shorten the waiting list emerged with ABO incompatible (ABOi) transplantation. The improved outcome of ABOi transplantation reported by the Japanese in the early 2000`s encouraged a broader application around the world (*8, 9*).

We adopted the “Stockholm protocol”, a protocol including CD 20 monoclonal antibody (Rituximab) treatment to avoid splenectomy (*10-12*). A Swedish company developed antigen specific immunoadsorption columns; Glycosorb-ABO system (Glycorex Transplantation AB, Lund, Sweden) depleting blood group antibodies, thereby diminishing the risk of infectious and bleeding complications (*13, 14*). The first ABOi kidney transplantation in Norway was performed 1.11.2006. Until January 2016, 55 adults and 5 children have been transplanted across the ABO barrier in Norway.

The transplantation procedure and follow-up of recipients the first 8-10 weeks postoperatively is centralized at Rikshospitalet. Patients are referred from the local nephrologists to Rikshospitalet for indication kidney graft biopsies. Since 2009 protocol graft biopsies are performed at 6-8 weeks posttransplant and recipients are invited to a follow-up visit with a protocol biopsy one year after transplantation. The approximately annual number of kidney transplant biopsies taken at Rikshospitalet is 700-1000. All kidney transplant biopsies are analysed by the same 4 renal pathologists, who provide unique experience and expertise.

1.3 Biopsies of the kidney transplant

1.3.1 International kidney transplant biopsy classification system- short history of the Banff classification

The Banff classification was developed as a standardization of renal allograft biopsy interpretation to guide therapy and to establish an objective end point for clinical trials. The first Banff conference was held in 1991 and the results were published in 1993 (*15*). The Banff working report from 1997 was for a long period the key reference paper for kidney transplant biopsy evaluation, and the classification today is still based on this framework (*16*).

The Banff working groups consist of multidisciplinary specialists, which include the key opinion leaders in the field. The report is based on consensus discussions at the meeting, publication of and experience from international conferences and trials.

Table 1 shows an example of the key classification table in the report from 2009, which is still valid for the classification of Borderline -, T-cell mediated rejection (TCMR) and chronic changes like interstitial fibrosis and tubular atrophy (IFTA) (*17*). The classification has since the beginning been under continuous revision. The last Banff meeting reports from 2009 until 2013 have in particular revised the criteria for antibody-mediated rejection (ABMR) (*17-19*). The following detailed description of the criteria of TCMR and ABMR [see 1.4.1 and 1.4.2], refers to the Banff meeting report from 2013 (*19*). The last Banff meeting in 2015 did not make any major revisions to the Banff 2013 report (oral preliminary communication from the Banff conference in Vancouver 2015) (*20*).

As all histopathology-based classification systems also the Banff classifications has some limitations, which include potential for sampling errors, suboptimal

reproducibility, lack of application of morphometry with quantification of lesions and until recently the lack of integration of molecular and genomic data (*21*). Only a few studies have tried to evaluate the diagnostic impact on clinical outcome of the current Banff 2013 criteria for ABMR. De Serres et al. studied the revised Banff 2013 criteria compared to the Banff 2007 criteria for chronic ABMR and clinical outcome and found that only the change in C4d threshold but not the definition for microvascular inflammation (MVI) was associated with graft survival and kidney function (*22*).
All Banff scores for the different tissue components are reported in a categorical scoring system from 0 to 3. The scores are 0 (absent or negligible), 1 (mild), 2 (moderate) and 3 (severe).

Table 1. The Banff report 1997 with Banff '09 update

Table 1: Banff 97 diagnostic categories for renal allograft biopsies—Banff '09 update

1. Normal
2. Antibody-mediated changes (may coincide with categories 3, 4 and 5 and 6)
   Due to documentation of circulating antidonor antibody, C4d,[1] and allograft pathology

C4d deposition without morphologic evidence of active rejection

   C4d+, presence of circulating antidonor antibodies, no signs of acute or chronic TCMR or ABMR (i.e. g0, cg0, ptc0, no ptc lamination (<5 layers by electron microscopy), no ATN-like minimal inflammation). Cases with simultaneous borderline changes are considered as indeterminate

Acute antibody-mediated rejection[2]

   C4d+, presence of circulating antidonor antibodies, morphologic evidence of acute tissue injury, such as (Type/Grade)
   I. ATN-like minimal inflammation
   II. Capillary and or glomerular inflammation (ptc/g >0) and/or thromboses
   III. Arterial – v3

Chronic active antibody-mediated rejection[2]

   C4d+, presence of circulating antidonor antibodies, morphologic evidence of chronic tissue injury, such as glomerular double contours and/or peritubular capillary basement membrane multilayering and/or interstitial fibrosis/tubular atrophy and/or fibrous intimal thickening in arteries

3. Borderline changes: 'Suspicious' for acute T-cell mediated rejection (may coincide with categories 2 and 5, and 6)
   This category is used when no intimal arteritis is present, but there are foci of tubulitis (t1, t2 or t3) with minor interstitial infiltration (i0 or i1) or interstitial infiltration (i2, i3) with mild (t1) tubulitis
4. T-cell mediated rejection (TCMR, may coincide with categories 2 and 5 and 6)

Acute T-cell mediated rejection (Type/Grade:)

   IA. Cases with significant interstitial infiltration (>25% of parenchyma affected, i2 or i3) and foci of moderate tubulitis (t2)
   IB. Cases with significant interstitial infiltration (>25% of parenchyma affected, i2 or i3) and foci of severe tubulitis (t3)
   IIA. Cases with mild to moderate intimal arteritis (v1)
   IIB. Cases with severe intimal arteritis comprising >25% of the luminal area (v2)
   III. Cases with 'transmural' arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic inflammation (v3)

Chronic active T-cell mediated rejection

   'chronic allograft arteriopathy' (arterial intimal fibrosis with mononuclear cell infiltration in fibrosis, formation of neo-intima)

5. Interstitial fibrosis and tubular atrophy, no evidence of any specific etiology
   (may include nonspecific vascular and glomerular sclerosis, but severity graded by tubulointerstitial features)
   Grade
   I. Mild interstitial fibrosis and tubular atrophy (<25% of cortical area)
   II. Moderate interstitial fibrosis and tubular atrophy (26–50% of cortical area)
   III. Severe interstitial fibrosis and tubular atrophy/ loss (>50% of cortical area)
6. Other: Changes not considered to be due to rejection- acute and/or chronic (For diagnoses see table 14 in (49); may include isolated g, cg, or cv lesions and coincide with categories 2, 3, 4, and 5)

ATN, acute tubular necrosis.
The 2009 updates are underlined. All existing scoring categories (g, t, v, i, ptc, cg, ct, ci, cv, ah, mm) remain unchanged (45, 49).
[1]Please refer to Banff 2007 classification paper (45).
[2]Suspicious for antibody-mediated rejection if C4d (in the presence of antibody) or alloantibody (C4d+) not demonstrated in the presence of morphologic evidence of tissue injury.

Am J Transplant; 2010, *10:464* (*17*)

1.4 Rejection

A kidney graft biopsy remains the gold standard for the diagnosis of rejection (*23*). Rejection in solid organ transplantation can be divided into three main groups, dependent on the *time* of rejection after transplantation (see below). Acute and chronic rejections are further categorized according to what kind of *immunological mechanisms* dominate the rejection episode or in which *clinical setting* the rejection occurs [see 1.4.1-1.4.2].

1. Hyperacute rejection develops from minutes up to 24 hours after transplantation. It is mainly caused by preformed HLA antibodies or blood group antibodies, which by activation of the classical complement cascade, followed by endothelial necrosis, platelet deposition and local coagulation lead to immediate graft loss. An early case report in 1966 by Kissmeyer-Nielsen recognized hyperacute rejection with pre-existing antibodies against donor cells (*24*). The association between preformed HLA antibodies causing a positive cross-match and hyperacute rejection was further corroborated by Patel and Terasaki in their landmark study published in 1969 (*25*). In times of modern immunosuppression and advanced immunological work-up before transplantation this kind of rejection is rarely observed.

2. Acute rejection occurs usually from days to months after transplantations, especially during the first three months. In immunological high risk patients with preformed HLA DSA or blood group antibodies mainly acute antibody-mediated rejection (aABMR) dominates [see 1.4.2.1], while in the immunological low risk population acute T-cell mediated rejection (aTCMR) is most frequently diagnosed [see 1.4.1.1] (*26-28*).

3. Chronic rejection is a smoldering phenomenon observed from months up to years posttransplantation and often represents a mixture of T-cell and antibody-mediated injury patterns; whereas the antibody-mediated injuries often dominate the histological picture (*27*). The recent years have provided growing evidence for the risk of chronic rejection in the presence of HLA DSA both preformed and *de novo* (*26, 29*). The development of *de novo* DSA has been linked to unintentionally reduced immunosuppression caused by nonadherence (*29, 30*).

Frequently both T-cell and humoral mechanisms are involved in the process of allograft rejection. In the Banff classification the two immunological dominating mechanisms are described separately as **T-cell mediated (TCMR)** and **antibody-mediated rejection (ABMR)**.

Protocol biopsies are per definition planned biopsies at certain time points, with stable graft function, in our case 6-8 weeks and 1 year after transplantation. All transplantation centers with protocol biopsy programs have the opportunity to detect not only **clinical** but also **subclinical rejections (SCR)**.

1.4.1 T-cell mediated rejection (TCMR)

1.4.1.1 Acute T-cell mediated rejection (aTCMR)

Acute mild and moderate TCMR early after transplantation (<3 months posttransplant) was common in the earlier days of renal transplantation, but the numbers are constantly declining; with modern immunosuppression approximately around 15-20% (*31*).

Data from a large international multicenter register study indicate that a successfully treated acute rejection during the first 3 months after transplantation (not differentiated by TCMR or ABMR) had no influence on long-term graft survival, provided the recipient returned to a relatively good kidney function at 1 year posttransplant (creatinine ≤ 130 µmol/l). The long- term graft survival of treated acute rejections was mostly dependent on the time point after transplantation the rejection occurred. According to this registry data late rejections had the poorest prognosis (*32*). Another retrospective clinical study supported these findings for TCMR; late and vascular rejection affected graft survival unfavorable (*33*). Nevertheless the bad outcome of late rejections may be caused by unrecognized antibody- mediated injury or mixed rejections (*27, 34, 35*).

The lesions used by the Banff classification to score mild/ moderate TCMR are interstitial inflammation (Banff i-score) and tubulitis (Banff t-score). Severity of rejection is defined by the intensity of interstitial inflammation and tubulitis (Table 1, point 4).

Mild TCMR	**Banff IA**	i2 **t2** v0
		i3 **t2** v0
Moderate TCMR	**Banff IB**	i2 **t3** v0
		i3 **t3** v0

A more severe form of TCMR is the vascular rejection defined by vascular lesions/ intima arteritis (Banff v-score). Until Banff 2013 intima arteritis was only classified as part of the TCMR definition, but growing evidence, especially form a large French cohort study, has shown a connection to early ABMR in patients with preformed HLA DSA. Patients with vasculitis and ABMR had a 2.5 times higher risk of graft loss than patients with ABMR without vasculitis (*36*). Consequently vascular rejection was included in the histological injuries qualifying for ABMR definition (*19*).

Severe TCMR	**Banff II A**	i0-3 t 0-3 **v1**
	Banff II B	i0-3 t 0-3 **v2**
	Banff III	i0-3 t 0-3 **v3**

1.4.1.2. Chronic T-cell mediated rejection (cTCMR)

The chronic allograft arteriopathy was introduced in the Banff 2005 report as diagnostic criterion for chronic TCMR (*37*). New onset arterial intima fibrosis not present in the organ at the time of transplantation is today part of the general definition of chronic rejection both T-cell mediated and antibody-mediated (*19*), but a “pure” chronic TCMR are not seen in clinical practice (*35*).

1.4.2 Antibody-mediated rejection (ABMR)

1.4.2.1 Acute ABMR (aABMR)

Acute ABMR is a severe form of rejection with a poor prognosis without treatment (*38*). The reported aABMR incidence varies from 30-40% in HLA- sensitized patients and being as high as 32% early after transplantation in ABOi transplantation (*28, 39, 40*) compared to less than 1- 5% in an immunological low risk population. Acute ABMR is associated with pre-mature graft loss (*39, 40*).

Already in the 1960`s preexisting HLA antibodies were identified as cause for hyperacute rejection (*24, 25*). In the early 1990`s Halloran et al recognized the endothelial injury/ inflammation in the microvascular compartment peritubular capillaries (peritubular capillaritis; Banff ptc- score) and capillaries in the glomeruli (glomerulitis; Banff g-score) in the kidney graft as key pathological features for early aABMR in patients with preformed HLA class I antibodies (*41, 42*) .

Peritubular complement activation visualized by Complement factor 4d (C4d) staining in the biopsy is an indirect sign for antibody interaction with the endothelium. Feucht et al. demonstrated that C4d had a negative impact on graft survival and was associated with antibody presence (*43*). In 2005 the staining for C4d of the kidney transplant biopsy was established at Rikshospitalet as a routine.

In a French study population with preformed DSA C4d had however a low sensitivity for the diagnosis of ABMR (0.69) with a higher specificity (0.83) (*44*). The Banff initiative for quality assurance found a poor interinstitutional reproducibility for C4d scoring with kappa=0.17. This was caused by interobserver technical variability (*45*). Nevertheless, C4d has become one of the cornerstones of ABMR diagnosis. The latest Banff reports from 2011 and 2013 additionally defined a C4d negative ABMR type (*18, 19*).

During the last two decades the diagnostic criteria for ABMR have been under a continuous evolution. Our diagnostic tools improved by revised international pathological consensus guidelines by the Banff working group and by introducing the very sensitive and specific solid phase assays for HLA antibody detection. At Rikshospitalet an ELISA technique for HLA antibody detection was first introduced in 2000 (*46*), but was replaced by a flow cytometry based technique, the LUMINEX platform in 2007 (*47-50*). The Flow-cytometric cross match technique is however not used at our immunological laboratory.

1.4.2.2 Chronic ABMR (cABMR)

Chronic ABMR appears most often ≥ 1 year posttransplant, and reveals itself clinically with slowly decreasing kidney function and/ or proteinuria. The histological picture can be a mixture of T-cell and antibody-mediated injury, frequently associated with nonadherence and appearance of *de novo* DSA (*51*). It is postulated as a continuum developing from a subclinical to a clinical evident condition (*38*). The key/ hallmark lesion, transplant glomerulopathy (TG) (Banff score- cg), resembles features of membranoproliferative glomerulonephritis (MPGN) with duplication of the glomerular basement membranes ("tram-tracking") and mesangial proliferation. Accordingly transplant glomerulopathy is not a specific lesion, it can be observed in MPGN, chronic thrombotic microangiopathy (TMA), radiation injury and toxemia of pregnancy (*52*).

Patients with HLA DSA present at transplantation, especially patients in need for desensitization before transplantation, are also at risk for cABMR. In a desensitized group of patients transplanted at the Mayo Clinic, 21% and 55% of recipients developed TG at 1 year and 5 years respectively (*26*).

In this study there was a significant higher risk for TG in the group who had class II HLA DSA compared to class I HLA DSA (38% vs. 8%), which was associated with a higher risk for graft loss 7%/ year vs. 1.6%/ year respectively.

The Johns Hopkins group documented 25% TG at 1 year in their immunological high risk, desensitized patients (*53*). In another protocol biopsy study by the Mayo clinic 3% of the conventional patients developed TG at 1 year and 8% at 5 years (*28*).

Chronic ABMR has been recognized as the leading cause of late graft loss (*30, 34*). One cross sectional study of indication biopsies sampled up to 32 years posttransplant showed that 50% of late graft loss could be ascribed to cABMR (*29*).

Development of *de novo* DSA is associated with cABMR (*54, 55*). Impaired immunosuppression by nonadherence of the patient or physician-initiated immunosuppression minimization for medical indications, e.g. side effects like diarrhea, leukopenia, anemia and CMV infection has been associated with development of *de novo* DSA (*56, 57*).

1.4.2.3 BANFF CRITERIA for acute and chronic antibody-mediated (ABMR) rejection

The ***Banff classification for acute and chronic antibody-mediated rejection includes 3 criteria.*** All of these 3 criteria have to be present to make a definitive diagnosis of ABMR (*19*).

Acute ABMR (aABMR) criteria

1. <u>Histological tissue injury</u>

- ***Microvasculær inflammation (MVI) and injury***;
  typically glomerulitis (g) and/or peritubular capillaritis (ptc)
- ***Intimal or transmural arteritis*** v>0
- ***thrombotic microangiopathy*** (TMA)
- ***acute tubular injury*** (ATN) ≥2

2. <u>Evidence of antibody interaction with vascular endothelium</u>

- ***C4d peritubular positivity***
- at least moderate ***MVI (g + ptc ≥ 2)*** *

3. <u>Circulating donor-specific antibodies (DSA)</u> (HLA or other antigens)

***** Another evidence for interaction between DSA and the endothelium has emerged with using *endothelium activation and injury transcripts (ENDATs*) in centers, where this technology is available (*58*).

Chronic ABMR (cABMR) criteria

1. **Histologic tissue injury**
   - ***transplant glomerulopathy***, cg>0
   - ***peritubular multilayering of the peritubular capillaries on electron microscopy*** (EM)
   - ***arterial intima fibrosis*** of “new onset, cv>0”**

2. Same definition as with acute ABMR

3. Same definition as with acute ABMR

** acknowledged as lesion which could be seen in both chronic TCMR and chronic ABMR

1.5 Immunological barriers to transplantation the HLA and ABO system

1.5.1 The HLA system

The two antigen systems HLA and ABO are polymorphic and differ between individuals. They are the main immunological transplantation barriers detected by the recipient`s immune system as foreign antigens.

In the 1950`s and 1960` s little was known about the human leukocyte antigen (HLA) tissue type, blood group antigens and the causes of rejection. There were several milestone discoveries leading towards transplantation medicine as we know it today. The French surgeon, Alexis Carrel, introduced the idea that the cause of rejection

could be immunological in his publication of renal transplantation already in 1908 (*59*). In 1944 the English biologist Peter Medawar was the first one to define that rejection is an immunological reaction against foreign cells and tissue. He described the differences between first and second transplantation rejection and was awarded the Nobel Prize in Medicine in 1960 (*60*).

In 1956 the French immunologist Jean Dausset identified the first HLA, later called HLA A2 (*61*). He was awarded the Nobel Prize in Medicine in 1980.

The HLA system is part of the major histocompability complex (MHC) and plays a crucial role in regulating the immune response. The classical HLA genes are the most polymorphic in the human genome, with a large number of allelic variants at each locus (*62, 63*). The two HLA classes are expressed in different immunological cells; HLA class I (HLA-A, -B and –C) is expressed on all nucleated human cells and HLA class II (HLA-DR, -DQ, and –DP) is expressed additionally to HLA class I antigens on antigen-presenting cells (APCs) including B-cells, macrophages and dendritic cells. The HLA consists of amino acid strings of several polymorphic sites, which could act as targets, or epitopes, for antibody binding (*64*).

It was soon realized that matching for HLA antigens was important to avoid dysfunction and destruction of the graft (*65, 66*). Especially the matching for HLA-DR had a beneficial effect on graft survival (*67, 68*).

The role of HLA matching for graft survival and graft allocation policies are still a matter of debate. The improved immunosuppression strategies reduced the differences in graft survival between poorly and well HLA matched grafts, but the best results are still achieved by fully HLA matched grafts (*69, 70*). The revival of the HLA matching discussion is fueled by the emerging data on formation of *de novo* DSA, mainly antibodies towards HLA class II. Studies have shown reduced graft survival in

patients with *de novo* DSA (*57, 71, 72*). HLA mismatching is correlated to the risk of development of HLA antibodies posttransplant (*73*).

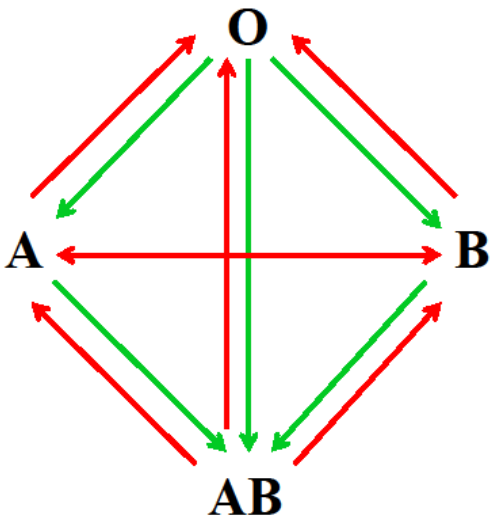
The HLA mismatching and nonadherence acted synergistic on the risk for graft loss (*74*). A recent study by Sapir-Pichhadze showed that HLA class II EPLET mismatch was an independent predictor for transplantat glomerulopathy (*75*).

1.5.2 The ABO system

The ABO blood group system was first detected by Landsteiner in 1900, who was awarded the Nobel Prize in Medicine in 1930 (*76*). It was recognized that cells and tissue only could be transferred across the blood groups as shown in Figure 4.

Alexandre et al. published 23 ABO incompatible kidney transplantations in 22 patients in Brussels in 1987. The recipients were preconditioned with DSPT (donor specific platelet transfusion), ALG (antilymphocyte polyclonal globulin), plasmapheresis and splenectomy (*77*). This paved the breakthrough for a broader use of ABOi transplantation worldwide.

Figure 4. Compatible and incompatible transplantation scenarios based on Karl Landsteiner`s blood group system



Green arrows illustrating compatible ABO transplantation scenarios, red arrows illustrating the ABO incompatible scenarios

Figure modified after Zschiedrich et al., (78)

1.6 Immunization towards HLA and ABO antigens

1.6.1 HLA antibodies

Immunization and antibody production towards HLA antigens can be caused by exposure to foreign HLA during pregnancy, with blood transfusions and former transplantation. For patients who are sensitized towards HLA before transplantation, it can be difficult to find suitable organs and the waiting time for transplantation can be long. If the HLA antibodies are directed towards the organ donor, donor-specific

antibodies (DSA), it will be an immunological high risk transplantation with increased risk of ABMR and inferior graft survival (*79-82*).

HLA DSA that develop after transplantation, *de novo* HLA DSA, are associated with impaired immunosuppression/ nonadherence or other preceding immunological events like subclinical or clinical rejection (*29, 57*). In non- selected immunological low risk populations the risk of developing *de novo* DSA during the first year posttransplant is estimated to be between 11-25% (*57, 83, 84*).

1.6.2 Blood group antibodies

The blood type antigens are present on cellular surfaces on plant and animal cells. Immunization takes place in the intestines during early infancy (*85*). Antibodies towards blood type antigens appear and could interact with donor antigen in case of ABOi transplantation and cause acute or chronic ABMR (*86-88*).

1.7 Renal transplantation programs for HLA immunized and ABOi recipients

All preconditioning and maintenance immunosuppression in HLA DSA positive and ABOi transplantation have two major goals:

1. Reduction of antibodies pretransplant
2. Prevention of antibody reproduction posttransplant and thereby prevention of antibody-mediated tissue injury

Kidney transplant patients with preformed HLA antibodies strong enough to cause a positive cytotoxic crossmatch (CDC) are currently only accepted for transplantation after desensitization therapy, which result in a negative cytotoxic crossmatch. Patients with a negative CDC crossmatch, but preformed HLA DSA can be accepted

for transplantation, with preconditioning treatment.

Without preconditioning therapy ABOi transplantation has in general a poor prognosis and leads to a high risk of accelerated rejection (*85, 89*). Blood group A2 is a subtype of blood group A, which has a much lower expression of A-antigen on the cell surfaces and is less immunogenic (*90, 91*). Transplantation with blood group A2 kidney donors has been performed without preconditioning (*85, 92, 93*).

1.7.1 Renal transplantation programs for patients highly immunized against HLA

Studies to modulate the immune responsiveness of the recipient started in the 1980`s with donor specific blood transfusions but only with a limited success (*94, 95*) . At Rikshospitalet the first desensitization study was performed in the period 1984-1993 with pretransplant plasma exchanges (PE) or immunoadsorption (IA) combined with cyclophosphamide and prednisolone (*96*).

Since the mid 1990`s the first reports of intravenous immunoglobulin (IvIg) used for desensitization emerged (*97, 98*). Currently different combinations of plasma exchange, low and high dose IvIg and Rituximab (anti-CD20 monoclonal antibody/ anti-B cell) are most frequently employed to desensitize patients (*99-104*). In some desensitizing protocols other agents such as Bortezomib (proteozome inhibitor/ anti-plasma cell) (*105, 106*) and complement inhibiting by Eculizimab (antiC5) (*107, 108*) have been developed and are applied by a limited number of transplant centers (*109*).

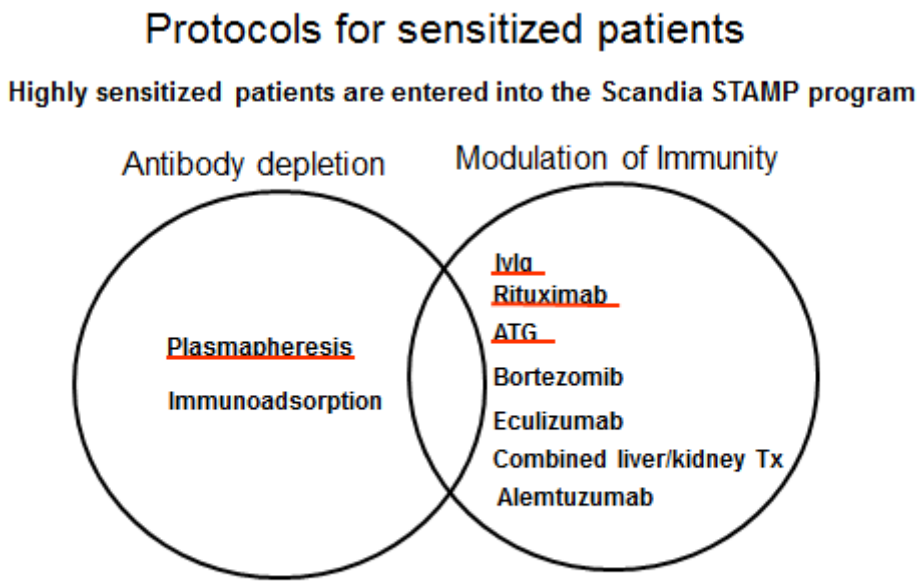
Other options for sensitized patients include paired kidney donation and the acceptable mismatch programs (*110, 111*). In 2009 Scandiatransplant introduced the

Scandinavian Acceptable Mismatch Program (STAMP) (*112*) inspired by the Eurotransplant Acceptable Mismatch Program (AMP) which started in 1989 (*113, 114*) .

Highly sensitized patients in Norway who are otherwise eligible for renal transplantation will be considered for the STAMP program. We also run a local acceptable mismatch program (LAMP) with less rigid inclusion criteria.
Our current program for patients with preformed HLA DSA detected by the LUMINEX technique was introduced in 2007 and includes Rituximab, IvIg and high dose CNI. PRA positive patients with negative DSA receive induction with ATG since 2014.

Figure 5. Examples for different treatment options for sensitized patients

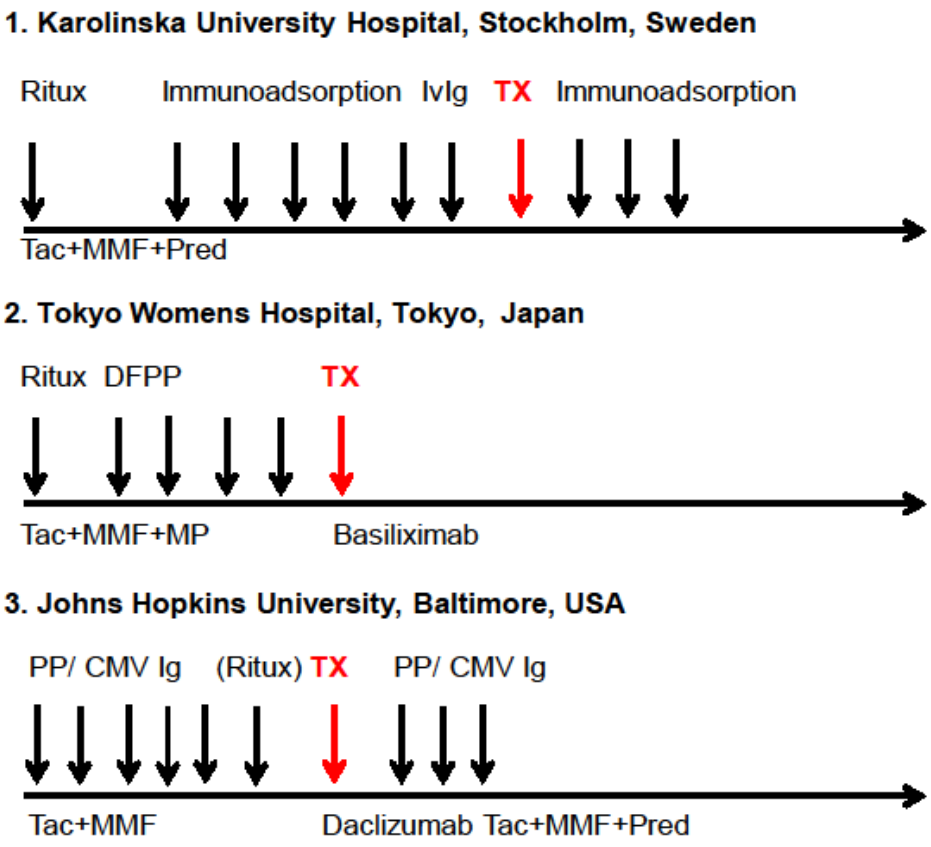
Treatments used at our center are underlined in red

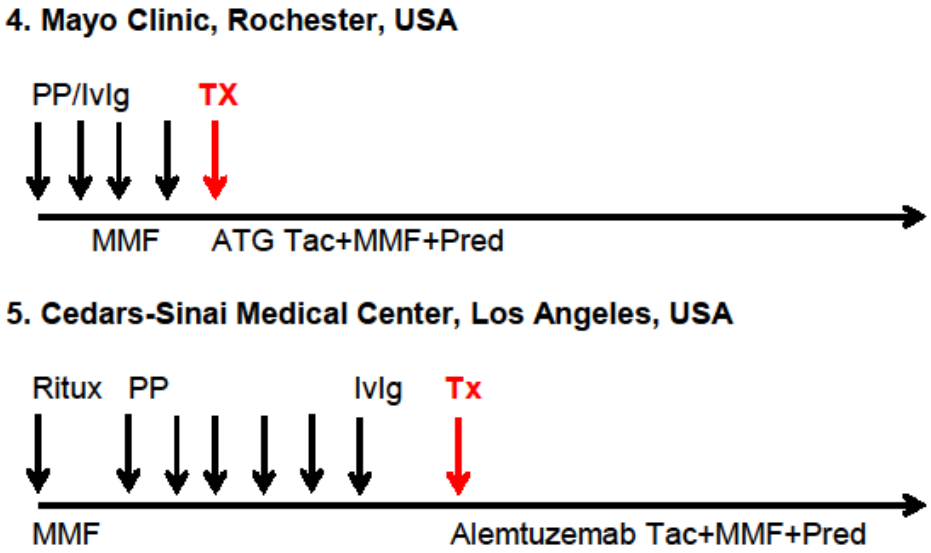


1.7.2 Programs for ABOi transplantation

Figure 6 gives an overview of the most frequently applied preconditioning programs in ABOi transplantation. At Rikshospitalet we introduced a protocol with antigen specific immunoadsorption with Glycosorb ABO columns, Rituximab and IvIg in 2006. This protocol was adapted from Karolinska University Hospital in Sweden (*11*).

Figure 6. Examples of different preconditioning treatments in ABOi transplantation





Legend Figure 6

Ritux, Rituximab; Tac, Tacrolimus ; MMF, Mycophenolate mofetil ; MP, Methylprednisolon; TX, transplantation; Pred, Prednisolon ; DFFP, Double filtration plasmapheresis ; PP, plasmapheresis; CMV Ig, Cytomegalovirus immunoglobulin; IvIg, immunoglobulin; ATG, Thymoglobulin (*11, 86, 115-117*)

Figure modified after Wongsaroj et al.(109)

1.8 Protocol biopsies

Protocol biopsies, also called standard of care surveillance biopsies, are typically performed from 1 month to 8 weeks and also at 1 year posttransplant. These biopsies have been useful in revealing subclinical rejection. Not only are subclinical TCMR and subclinical ABMR detected (*118*), but also subclinical Polyoma virus associated nephropathy (PVAN) and recurrent and de novo glomerulonephritis may be diagnosed (*119, 120*). Another important finding in protocol biopsies is development of accelerated interstitial fibrosis with severe arteriolar hyalinosis. This

finding can represent Calcineurin inhibitor (CNI) toxicity, although recent data outlined the lesion as non-specific (*121*). Donor age has also been associated independently with presence of medial arteriolar hyalinosis and vascular intima thickening in adult kidneys transplanted into pediatric recipients. Naesens et al. also demonstrated the independent role of higher donor age in the development of medial arteriolar hyalinosis two years posttransplantation (*122*).

The beneficial effects outweigh the possible complications of protocol biopsies as they provide the opportunity of individualized immunosuppressive therapy (*120, 123*).

1.8.1 Protocol biopsy findings in ABOi transplants

Despite the improved pretransplant preconditioning regimes (Figure 6), the ABOi transplanted patients experience an increased risk for early ABMR (*86, 124*). A question is whether this increased early injury could lead to more chronic changes and subclinical microvascular inflammation or tubulo-interstitial inflammation in the long run, e.g. one year after transplantation.

Positive C4d staining without inflammation and other signs of rejection in ABOi protocol biopsies has been interpreted as a sign of accommodation to the blood group antigens (*125*).

Table 2 shows the most important published data of one year biopsies in ABOi transplantation. The studies include small number of ABOi transplantations and the data sets are often incomplete. To study the effect of ABOi on subclinical and clinical antibody mediated tissue injury the recipients should be negative for HLA antibodies pretransplant. A weakness of several of the presented studies is that data of pretransplant HLA DSA is not presented or HLA DSA positive patients are included in the ABOi groups (*28, 87, 124*).

Table 2. Most important published 1 year protocol biopsy findings in ABOi recipients

Study	Study cohort	Biopsyfindings at 1 year		
	No. of patients enrolled (n) ***ABOi*** *ABOc*	***Transplant-glomerulopathy*** ***ABOi*** *ABOc*	***Banff chronicity scores*** ***ABOi*** *ABOc*	***C4d positivity*** ***ABOi*** *ABOc*
Bentall et al. (2014) *ABOi no preformed HLA DSA, HLA DSA data at 1 year NR*	**73** 652 **64** 597 alive at 1 year **37** 416 1 year protocol biopsy taken	**4/ 36** 13/ 416	**ci>0** **17/ 37** 225/ 416 **ct>0** **22/ 37** 289/ 416	**5/ 6** 1/ 19
Gloor et al. (2006) *HLA DSA data NR*	**24** 198	**3/ 24** 16/ 198	*Banff scores reported NS different*	**9/ 17** *ABOc NR*
Setoguchi et al. (2008) *different C4d definition negative < 25% focal 25-75% difffus/ bright >75% HLA DSA data NR*	**48** 133 **40** 95 biopsies at 6-12 months	**6/ 40** 7/ 95	**ct>0** **21/ 40** 68/ 95 all Banff sores reported only ct sig. higher in ABOc	**26/ 40** 4/ 95 diffuse **38/ 40** 10/ 95 diffus + focal
Montgomery et al. (2009) *no ABOc group*	11 XM+/ **60**	**9/ 36**	**36/ 60** presented with cg+ci+ct+cv SUM score	*NR*
Flint et al. (2011) *ABOi no HLA DSA*	**37** 52	**0/ 25** 1/ 46	**IFTA (ci+ct) >0** **7/ 25** 10/ 46	*NR*
Sanchez-Escuerdo et al. (2016)	**30** 146	**1/ 30** 1/ 146	**ci>0** **11/ 30** 55/ 146 **ct>0** **10/ 30** 58/ 146	**25/ 30** 3/ 146 **C4d>0**

ABO incompatible ABOi; *ABO compatible=ABOc; NR, not reported; XM+=Cross-match positive*; *NS= non-significant, ci = interstitial fibrosis; ct tubular atrophy*; *IFTA= interstitial fibrosis and tubular atrophy; MVI, micro vascular inflammation (28, 87, 116, 124, 126, 127)*

1.9 Inflammation in fibrotic areas of the kidney graft - "Another kind of Inflammation" apart from rejection

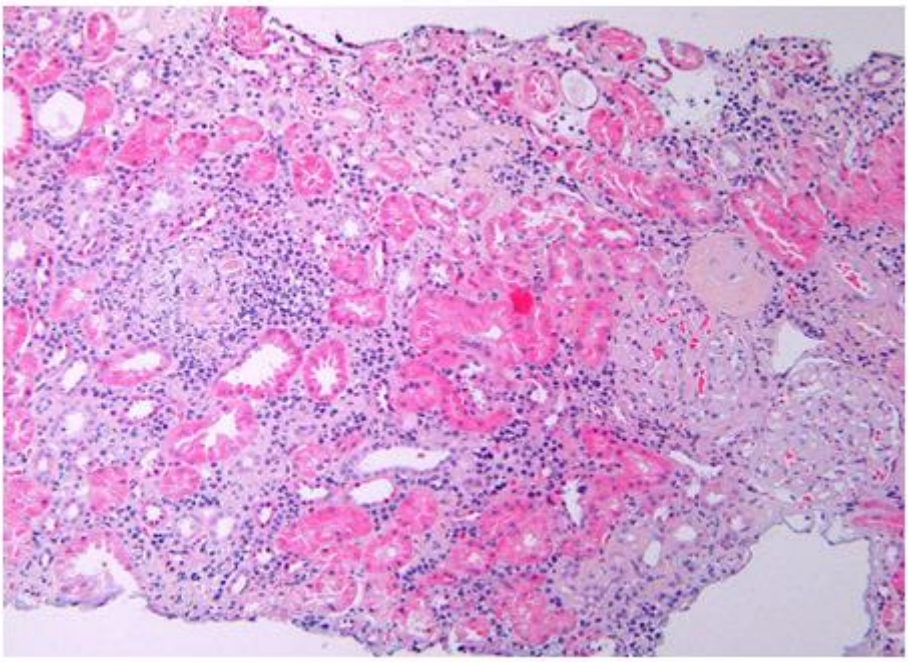
Interstitial inflammation (Banff i-score) was scored according to the Banff classification in the cortical kidney tissue not affected by fibrosis and tubular atrophy. Inflammation in fibrotic and scarred areas was considered non-specific. Some studies could however show an association between inflammation in the fibrotic and non-fibrotic areas of the kidney graft; called total inflammation (Banff ti-score) on one hand and graft outcome on the other (*128-130*). The Banff 2007 report proposed a Banff ti score as it seemed to be a better predictor for graft survival (*128, 131*). Nevertheless the Banff ti-score has not been broadly applied by pathologists.

One-year protocol biopsy studies have shown an inferior graft survival in kidney allografts with coexistence of interstitial inflammation and fibrosis (*120, 132-134*). Another study on early protocol biopsies (1-6 months postttransplant) by Moreso et al. showed that the coexistence of chronic changes and subclinical rejection (SCR) was an independent predictor of graft survival (*135*).

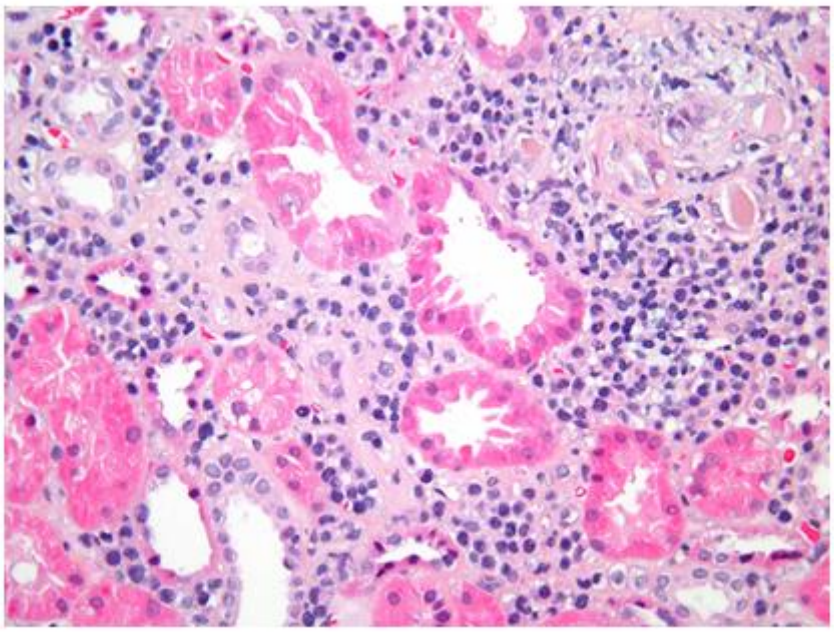
SCR was left untreated in this study and chronic changes at that time were defined as chronic allograft nephropathy (CAN) a definition abolished by Banff in 2005 (*37, 135*).

Inflammation in early protocol biopsies may represent a tissue injury response to ischemic injury during the operation procedure, alternatively an alloimmune response or a "silent" interstitial cell infiltration or tubulitis, which may not proceed to a "full blown" TCMR (*136*). Inflammation is a two-sided sword, beneficial for repairing an injury, but detrimental when it is uncontrolled and leads to progressive fibrosis (*137*).

Picture 1+2 Example for a kidney transplant biopsy with extensive inflammation in interstitial fibrotic and tubular atrophic areas



Picture 2- higher magnification



With courtesy Professor Finn Reinholt

1.10 Interstitial fibrosis and chronic changes in the kidney graft

Fibrosis is a histological hallmark for chronic progressive kidney disease. Fibrosis in the transplanted organ is a result of dysregulation between wound healing and repair processes. Fibrosis development is a complex multistage inflammatory process which consists of injury to the tissue, recruitment of inflammatory cells, release of fibrogenic cytokines and activation of collagen producing cells (*137*). Fibrosis and tubular atrophy is an unspecific lesion. The potential injuries who could cause fibrosis in a kidney transplant are mainly immunological (rejection or recurrence of glomerulonephritis), but fibrosis can also be caused by ischemia-reperfusion at transplantation, hemodynamic changes (hypertension), toxicity (CNI toxicity and other drugs), infection (PVAN or bacterial pyelonephritis) or be metabolic (diabetic nephropathy) (*137*). Development of fibrosis in a kidney graft has to a large extent been contributed to CNI toxicity (*138-140*). Alloimmune triggered injury has received more attention in the later years.

Studies of kidney graft biopsies have indicated that early tubulo-interstitial inflammation is linked to IFTA progression and graft failure (*141-143*). There is still uncertainty whether the cause of IFTA leading to graft loss could be identified in most of the cases. Naesens et al. recently presented their data from a large indication biopsy cohort, including 1365 biopsies, where 31 % of biopsies were classified as IFTA of non-specific disease. Early global histological damage negatively affected long term graft survival (*144*). On the other hand El Zoghby et al. studied 1317 indication biopsies in an immunological low risk population, followed for 50.3 ±32.6 months. In patients with graft loss censored for death 47/153 (31%) were caused by IFTA. In a majority (81%), a specific cause for IFTA was identified. One hundred and

seventy-seven grafts were lost either due to death with functioning graft (n=138) which is the leading cause for graft loss or 39 due to primary non-functioning grafts (*145*).

Attention has been paid to the classification of rejection, but less effort has been spent on the evaluation of fibrosis. A review by Farris et al. shed some light on the different visual pathological tools of fibrosis assessment, and concluded that the conventional “eyeballing” by an experienced pathologist on samples stained with trichrome is satisfactory in estimating the amount of fibrotic tissue in the biopsy compared to other methods like computerized analysis and morphometry (*146*).

The Banff categorical classification from 0-3 is a very coarse classification. The Banff classification for chronic lesion evaluation includes:

interstitial fibrosis (ci)/ (IF)

tubular atrophy (ct)/ (TA)

glomerular sclerosis (gs)

transplant glomerulopathy (cg)

intima fibrosis (cv)

arteriolo hyalinosis (ah)

mesangial matrix (mm)

Constructing sum scores of the individual elements of the Banff classification can be useful. There are different histological scoring systems.

In paper #2 a sum score was applied for hyalinosis and scarring (ci+ ct+ cv+ ah)

which was proposed by Sis et al. representing the cumulative injury over time (*147*). Table 3 gives an overview of the definitions of the Banff chronic lesion scores, Chronic Allograft Damage Score (CADI) (*148, 149*) and the modified CADI score used at Rikshospitalet.

Table 3

Classification of the different thresholds for chronic damage scores in the Banff classification, Chronic Allograft Damage Index (CADI) and the modified Rikshospitalet (RH)/ CADI score

	Element	Banff 2013	CADI[a]	RH/CADI
•	**1. Fibrosis, ci**	ci: 0–5, 6–25, 26–50, >50% of cortex	**1.** 0, 1–25, 26–50, >50%	**ci**
•	**2. Tubular atrophy, ct**	ct: 0, 1–25, 26–50, >50%	**2.** 0, 1–15, 16–30, >30%	**ct**
•	**3. Interstitial inflammation, i**	i: <10, 10–25, 26–50, >50% (excluding areas with fibrosis)	**3.** 0, 1-25, 26–50, >50% (no exclusions mentioned)	**i**
•	**4. Arterial intimal thickening, cv**	cv 0, 1–25, 26–50, >50%	**4.** cv, at least one artery	cv
•	**Atherosclerosis**	in most involved artery		
•	**5. Glomerular sclerosis, gs**	Percent sclerotic %, no score	**5.** Periodic-acid Schiff and trichrome 0,1-15,16–50,>50% of affected glomeruli	0, 1-15, 16-50,>50%
•	**6. Mesangial matrix, mm**	0, 1–25, 26–50, >50% of	**6.** mm	mm
•	**7. Hyalinosis, ah**	0, mild to moderate more than one arteriole, moderate to severe, severe	not scored	**ah**
•	**Transplant gomerulopathy**	cg:<10, 10–25, 26–50, >50% GBM duplication in most severly affected glomerulus	Used in as a substitute for glomerulosclerosis in one CADI study	cg/ no score

CADI original includes element 1-6; total score 18,
Modified Rikshospitalet (RH) CADI; RH used Banff compared to CADI original for these elements
RH CADI includes element 1-7; total score 21

Modified after Colvin « CADI, Canti, Calvi" I sing of CADI, but am cautious, Transplantation», March 27, 2007, 83:6, 677-678
a From Yilmaz et al. Supplemental Material, available online at www.transplantjournal.com.

Reference (*148-150*)

2. AIMS OF THE STUDIES and RESEARCH QUESTIONS

2.1 Paper I

Since the Banff conference 2011 in Paris suggested two distinct phenotypes of ABMR; the *early ABMR* mainly linked to the immunological high risk population with preformed HLA DSA and the *late ABMR* linked to development of *de novo* HLA DSA and nonadherence, we wanted to test the hypothesis in our patient cohort (*18*).

2.2 Paper II

Higher risk of early aABMR in ABOi transplantation could lead to more chronic changes and eventually inflammatory injury one year after transplantation.
The aim of paper #2 was to study one year protocol biopsies for inflammation and chronic changes from living donor ABOi patients and living ABOc patients, both without HLA DSA.

2.3 Paper III

Previous reports are conflicting whether early inflammation in protocol biopsies predicts fibrosis in the long run, they disregard inflammation in fibrotic areas**.** The aim of paper #3 was to assess the association between subclinical inflammation, both in intact areas and fibrotic areas of the kidney cortex in early 6 week protocol biopsies with fibrosis progression during the first year after transplantation.

3. PATIENTS and METHODS

3.1 Paper I - ***Early*** **versus** ***late acute antibody-mediated rejection***

3.2 Paper II + III **One-year protocol biopsies from** ***ABO-incompatible*** **compared to a matched cohort** ***ABO-compatible*** **renal allografts**

Total inflammation in early protocol biopsies and fibrosis one year posttransplant

Data collection

Clinical data were obtained by chart review in all 3 papers. The follow-up data were retrieved from the Norwegian Renal Registry. Follow-up time in paper #1 was defined as time from diagnosis of acute ABMR to death or end of study. Paper #2 and #3 collected data from the first year after transplantation.

3.1 Paper I- *Early* versus *late acute antibody-mediated rejection*

3.1.1 Study Population

All renal transplant recipients with a biopsy verified acute ABMR (aABMR) between January 1st 2005 and December 31st 2010 were included in this study and were followed until 31st December 2011. January 1st 2005 was set as start of inclusion because C4d staining was available from that time point as standard procedure.

Sixty-seven renal transplants in 65 recipients transplanted from May 2000 until May 2010 were included. Two groups were defined; one group with *early aABMR* detected before three months and one group with *late aABMR* detected more than three months post-transplant. We excluded ABO incompatible kidney transplants, children under the age of 15 and combined transplantations, except simultaneous kidney and pancreas transplantation.

From January the 1st 2005 to December 31st 2010 a total of 1534 renal transplantations were performed at Rikshospitalet. During this period we identified 376 rejection episodes that were not diagnosed as antibody-mediated. Of these, 276 episodes occurred within 3 months and 100 episodes beyond 3 months post transplantation. Graft survival of recipients in these two subgroups was used for comparison of outcome for *early* and *late aABMR* episodes.

3.1.2 Nonadherence definition

Nonadherence was self-reported by patients and/or based on measurements of inappropriate low drug trough levels or drug metabolites (Tacrolimus <3 µg/l until 2008 afterwards <1 µg/l, CyA<25 µg/l, Everolimus <2 µg/l, Sirolimus <2 µg/l and MMF <0.2 mg/l). Some recipients experienced insufficient dosages of immunosuppression due to non-adequate adjusting of medication. This was documented by collecting hospital records, in combination with measurement of low trough drug levels as defined above.

3.1.3 Acute ABMR diagnosis and definition

A raise in serum creatinine level of 20% (excluding other causes) or delayed graft function (DGF) (defined as need of dialysis within the first week after transplantation) warranted a graft biopsy. Biopsies were graded according to Banff 97 classification with updates until 2011(*16-18, 37, 131, 151*). All patients experienced graft dysfunction. The diagnosis of suspicious aABMR was made if at least 2 out of the 3 following criteria were present and definitive for ABMR if all criteria were fulfilled: 1) C4d positivity 2) histopathological evidence of acute antibody-mediated injury or 3) circulating DSA.

3.1.4 Histocompability testing

Pretransplantation donor and recipient HLA typing (A, B and DR) was performed. HLA-C, -DQ and DP typing was not regularly performed. DQ typing was introduced regularly from 2009. Cytotoxic T- and B-cell crossmatches were negative at transplantation in all recipients. All donor-recipient pairs were blood group compatible. All recipient sera were tested for the presence of complement activating HLA antibodies with a panel of B-cells (CDC-PRA) pretransplantation. Additionally, a generic ELISA test was used for screening for IgG antibodies reactive with HLA molecules. Gradually during 2007 testing on the Luminex platform LX200, replaced the ELISA technique. Identification of HLA class I and class II IgG antibody specificities was done using single antigen coated flow beads (SAFB). We used 1000 mean fluorescence intensity (MFI) as a cutoff value.

3.1.5 Immunosuppression and treatment of TCMR and ABMR

Standard immunosuppression consisted of calcineurine inhibitor (CNI) cyclosporine or tacrolimus, mycofenolate mofetile (MMF) and steroids. From Jan 1st 2007 induction with basiliximab, 20 mg iv day 0 and 4, was added to the protocol and the CNI target concentration lowered. CNI, mostly tacrolimus was started day 0.

Before 2007 HLA sensitized patients with LD received cyclophosphamide 1 mg/kg bodyweight and 15 mg prednisolone for 2 weeks before transplantation. From 2007 all HLA sensitized patients, PRA > 20 % and/or DSA, received 1 dosage of Rituximab 375 mg/m^2 and IvIg 400mg/kg bodyweight for 5 consecutive days following engraftment. Two recipients were CDC crossmatch positive before desensitization and received plasmapheresis and IvIg before transplantation.

Acute rejections were treated with intravenous methylprednisolone, followed by an oral tapering of Prednisolon. In case of steroid resistant rejection rATG and/or OKT3 up to 2009 (Muromonab CD3, Jansen-Cilag, Switzerland) was administered (*152, 153*). Acute ABMR was additionally treated with plasmapheresis and/or IvIg. Routinely 5 plasmapheresis sessions treating one plasmavolume were performed with replacement preferentially 4% albumine in Ringer. Plasmapheresis was contraindicated in presence of bleeding complications. IvIg was then given as 400 mg/ kg bodyweight, 3–5 dosages. In therapy resistant cases rituximab was considered.

3.2 Paper II +III

Paper #2 **One-year protocol biopsies from *ABO-incompatible* compared to a matched cohort *ABO-compatible* renal allografts**

Paper #3 **Total inflammation in early protocol biopsies and fibrosis one year posttransplant**

3.2.1 Study Population

The population undergoing renal transplantation from January 2009 to December 2012 forming the basis for paper #2 and #3. In the period 1156 renal transplantations were performed at Rikshospitalet, 341 were LD transplantations.

In **paper #2** eighty adult recipients of LD kidney transplants without presence of preformed HLA DSA or panel reactive antibodies (PRA) >20% at transplantation and with a valid 1 year protocol biopsy were included (ABOi study group (n=20)/ABOc controls (n=60), (Figure 7).

ABOc recipients were first matched for the period of transplantation, 2009-2010 or 2011-2012 and for donor age ± 5 years and then randomly selected 3:1 from LD transplantation with a 1 year protocol biopsy.

Figure 7. Study design paper #2, retrospective matched cohort

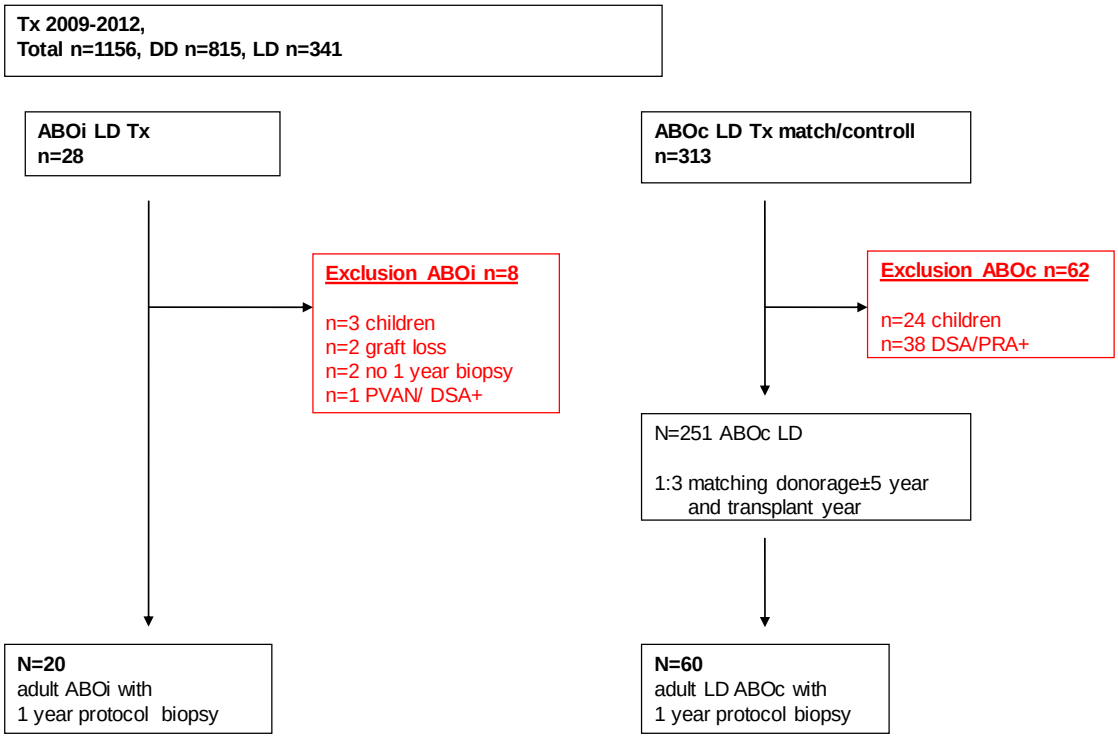


Figure 7 legend

LD living donor, DD deceased donor, Tx transplantation, ABOi ABO incompatible, ABOc ABO compatible, PVAN Polyomavirus associated nephropathy

In **paper #3** a subgroup transplanted in 2010 was chosen. In 2010 254 adult renal transplantations were performed. One hundred and fifty-six single adult ABO compatible renal transplant recipients with an adequate 6 week and 1 year kidney transplant biopsy (312 kidney transplant biopsies) were included in this study.

3.2.2 Protocol Biopsy

Protocol kidney transplant biopsies were performed at 6 weeks ± 2 weeks and 1 year ± 2 months posttransplantation, during a period of stable graft function without acute graft failure or recent immunological event. Two cores were obtained with ultrasound guidance using an 18 gauge spring-loaded biopsy gun: one core was fixed in 4% buffered formalin, embedded into paraffin wax, sectioned, and stained (hematoxylin/eosin/saffron, periodic acid-Schiff and trichrome) for conventional histology; the other core was frozen without prior fixation, sectioned and subjected to indirect immunofluorescence staining for complement. When the endothelium of the peritubular capillaries in >50% of the cortical area showed linear staining (Banff grade 3) the C4d staining was recorded as positive.

In paper #2 only 3 ABOc 1 year protocol biopsies were scored for C4d in paraffin embedded material, all negative (grade zero). In paper #2 and 3 an adequate biopsy was defined as a specimen with a minimum of seven glomeruli plus at least one artery. Patients with protocol-biopsies not fulfilling these criteria were excluded from the studies. All biopsies were graded according to the revised Banff 2007 classification and updates (*17-19, 131*).

Paper #2

Based on the Banff scoring system three sums of scores were constructed, representing tubulointerstitial inflammation (tubulitis(t)+interstitial inflammation(i)=0, vs.>0), microvascular inflammation (peritubular capillaritis(ptc)+glomerulitis(g)=0, vs.>0, scarring/ hyalinosis (interstitial fibrosis(ci)+tubular atrophy(ct)+intima fibrosis(cv)+arteriolar hyalinosis(ah)≤1, vs.>1 (*147*).

Paper #3

All kidney transplant biopsies were coded and reassessed by 2 experienced renal pathologists (Finn Reinholt and Helge Scott) sitting together at a 2-headed microscope. The sections were examined at medium power magnification (20 x objective) and each parameter was evaluated in each field of vision by semi-quantitative eyeballing. Subsequently, the mean score for all fields of vision in the core was calculated and recorded as the overall score for the parameter in question. In addition to the conventional Banff score 0-3, we examined whether a more detailed visual fibrosis and inflammation score with categorization in 10% intervals could improve resolution in grading the lesions.

The Chronic Allograft Damage Index (CADI) (score 0-21) was calculated as a summarized score by combining the Banff scores for interstitial fibrosis (ci), tubular atrophy (ct), interstitial inflammation (i), arterial intimal thickening (cv), mesangial matrix (mm), arteriolar hyalinosis (ah) and glomerular sclerosis (gs) categorized as (score 0, 0%; score 1, 1-15%; score 2, 16-50% and score 3, > 50%). Additional inflammation inside and outside fibrotic areas and fibrosis were scored in a 10-grade semi-quantitative eyeballing system 0% to 100%.

Thus, we recorded *interstitial fibrosis/ chronic changes in 4 ways:*

1. according to the *Banff system (Banff ci-score)*
2. in a *10-grade interval fibrosis score*
3. as a *calculated Banff IFTA score* and
4. as a *modified CADI score* 1 year posttransplant.

These serve as possible surrogate end points for long term graft survival (*150, 154*). The delta fibrosis from six weeks to 1 year posttransplant was the difference between

score at 6 weeks to 1 year. Delta 10% fibrosis >0 or delta Banff ci fibrosis>0 represent progression of fibrosis during this time period.

Inflammation was scored both in non-fibrotic and fibrotic areas. Banff interstitial inflammation (Banff i-score) was scored in non-fibrotic cortical areas. Total inflammation was scored in intact /non-fibrotic cortex as well as in cortex with fibrosis/scarring (*128*). Both the current Banff score 0-3 and the more detailed inflammation score in 10% intervals were employed.

The total inflammation of the entire cortex in % was calculated from the scoring in 10% steps by following equation: Total inflammation of the entire cortex (%) = (fibrotic area of the entire cortex % x inflammation in fibrosis %)/100 + (non-fibrotic area of the entire cortex % x inflammation outside fibrosis %)/100.

3.2.3 Definition and treatment of subclinical and clinical TCMR and ABMR in ABOi and ABOc patients

Subclinical Borderline rejections, defined by a Banff score of at least i0t1 did not receive any additional treatment. The subclinical and clinical TCMR, defined as Banff IA (at least i2t2) received methylprednisolone i.v. The details of our protocol for treatment of clinical TCMR and ABMR have been described for paper #1.

Clinical and subclinical ABMR/mixed rejection diagnosis in ABOi and ABOc were classified according to the Banff 2013 report (*19*). The treatment of subclinical ABMR was individualized.

3.2.4 Histocompability Testing

Histocompability testing was done as referred to in paper #1. HLA-C and DP typing was performed from 2009 if the recipient had anti HLA-C or HLA-DP antibodies detected by LUMINEX.

3.2.5 Titration of ABO blood group antibodies and ABOi immunoadsorption protocol

Anti-A or anti-B titers of the recipient were determined using gel hemagglutination titration technique, both for immunoglobulin (Ig) M and IgG. Titrations were performed using microtubes of saline (NaCl) gel card and Liss/Coombs gel card, respectively. Recipients` anti-A/anti-B were tested against donor erythrocytes by direct agglutination in NaCl for titration of IgM and the indirect agglutination using indirect antiglobulin test for IgG (*155*) .

ABO antigen-specific immunoadsorption (GlycosorbABO®) was performed preoperatively until ABO IgG/IgM titers were <1:8, routinely with 4 treatment sessions, or as needed (*14*). Immunoadsorption posttransplant was performed on demand if a titer rise ≥ 1:32 appeared <3 weeks posttransplantation or as rejection therapy.

3.2.6 Immunosuppression

Standard immunosuppression in all patients consisted of induction with basiliximab, CNI, MMF and prednisolone as described in paper #1 from 2007 for standard immunological risk/ ABOc patients and immunological high risk patients. For ABOi

and HLA incompatible patients the target concentrations for CNI were higher.
In ABOi and HLA incompatible LD transplantation one i.v. dosage of anti-CD20 monoclonal antibody Rituximab 375 mg/m^2 was administered 4 weeks prior to transplantation. In ABOi transplantation oral immunosuppression with MMF 1g x 2 and prednisolone 30 mg was started on day -14. On day -1 IvIg 500 mg/kg bodyweight was administered. From May 2011 until June 2012 rituximab and IvIg were not used for induction of ABOi patients, 5/20 ABOi were transplanted by this protocol. In addition 3 ABOi patients received only IvIg induction. Due to 2 early severe acute ABMR, not included in the study population, we returned to our previously used protocol.

3.3 Statistical Analysis

In paper #1 and #3 data are reported as mean (SD), median (total range) or frequencies (%). Groups (early vs. late acute ABMR in paper #1) were compared using independent sample *t*-test or Mann-Whitney *U* test for continuous variables, as appropriate. A t-test can be used to determine if the mean values from two sets of continuous data are significantly different from each other, meaning that the probability that the observed difference between the two data sets is due to chance and not to the fact that they are inherently different is less than five per cent. To apply the t-test both data sets must be normally distributed. A normal (or Gaussian) distribution is classically expressed by a bell shaped curve that is bilateral symmetrical. If a variable is normally distributed the mean and median values will be approximately equal. The Mann-Whitney *U* test is a non-parametric test based on

ranks and can to be used for comparison of two independent data sets, if the continuous variables are not normally distributed, or if the group is small (between 20 and 30 cases). The test assumptions are that the groups are independent and that the data are randomly sampled (*156*).

The Chi-square test was used to assess if the frequency of a categorical variable is significantly different between two independent groups. If the expected number in any category is less than five the Fisher`s exact test was applied for categorical variables both in paper #1 and #3.

In paper #1 a survival analysis was performed by the Kaplan-Meier method using the log-rank test for significance. A *P* value less than 0.05 was considered significant. Survival analyses were applied to examine the time between entry into the study (aABMR) and the occurrence of an event (death or graft loss). The time to event is seldom normally distributed and therefore a non-parametric statistic test is used to estimate the survival function. Patients who do not experience the event are called “censored”. Patient survival time was calculated from the time of rejection to death, graft survival time from acute rejection to death or graft loss (defined as need for dialysis and retransplantation). For both endpoints survival time was censored at study end.

The Kaplan-Meier method is used for the estimation of survival probability between two independent groups.

In paper #2 protocol biopsy findings at 1 year and categorical data of the two groups were compared using conditional logistic regression. The conditional logistic regression is a variant of logistic regression. It is most commonly used in the analysis of matched case-control studies. This statistical test describes the relationship

between one independent (explanatory) variables and a categorical dependent (outcome) variable. Continuous data were analyzed with repeated measures ANOVA and if not normally distributed with Friedman's test.

In paper #3 protocol biopsy findings at 6 weeks and 1 year posttransplant were compared with the non-parametric paired Wilcoxon signed rank test. The Wilcoxon signed rank test is the alternative for non-normally distributed variables as opposed to the parametric paired t-test which demands independent sampling, normal distribution, and similar variance in each group.

The Wilcoxon signed rank test is a statistical hypothesis test used when comparing repeated measurements on a single sample, in our case biopsy parameters at 6 weeks and 1 year. It is a paired difference test, which assesses whether the mean ranks differ.

The assumptions are that the data are paired and derives from the same population. The data have to be measured at least on an ordinal scale and cannot be nominal and each pair is to be chosen independently and randomly.

Total inflammation scored by inflammation in % of the entire cortex and Banff total inflammation (ti score) at 6 weeks were analyzed with linear univariate regression towards Δ10 % fibrosis score at one year. Clinical risk factors were analyzed with linear univariate regression towards Δ 10% fibrosis score and Δ ci Banff fibrosis score at one year. This statistical test describes the relationship between one independent (explanatory) variables and a continuous dependent (outcome) variable. As our sample sizes were rather small there is a possible risk for a type II error with false negative results.

Statistical analyses were performed using SPSS ® (SPSS 20.0, Chicago, IL). The Kaplan–Meier survival analyses were implemented using STATA/SE ® 12.0 (StataCorp, College Station, TX).

3.4 Ethical considerations in general

The studies were approved by the institutional review board and the South-Eastern Regional Committee for Medical and Health Research Ethics in Norway and was performed in accordance with the Declaration of Helsinki and is consistent with the Principles of the Declaration of Istanbul on Organ Trafficking and Transplant Tourism. All patients gave their written informed consent to participate in the study.

4. RESULTS

4.1 Paper I - *Early* versus *late acute antibody-mediated rejection*

4.2 Paper II - One-year protocol biopsies from *ABO-incompatible* compared to a matched cohort *ABO-compatible* renal allografts

4.3 Paper III - Total inflammation in early protocol biopsies and fibrosis one year posttransplant

4.1 Paper I - *Early* versus *late acute antibody-mediated rejection*

We identified 67 cases of acute antibody-mediated rejection (aABMR), 40 *early aABMR* (<3 months after transplantation) and *27 late aABMR* (>3 months). Graft survival was also compared to recipients with *acute early non-ABMR* (n=276) or *acute late non-ABMR* (n=100).

Recipients with *late aABMR* had significantly reduced graft survival compared with recipients who experienced *early aABMR* (*P*<0.001, log rank test; 40% versus 75% graft survival at 4 years; HR 3.72; 95% CI 1.65-8.42).

The *late aABMR* graft survival was also inferior to *late non-ABMR*, (*P*=0.008).

The patient characteristics were different in the *early* vs. the *late aABMR* group. In the *early aABMR* group more recipients were presensitized to HLA (22/40 (55%) vs. 4/27 (15%), *P*=0.001) and the majority were women (25/40 (63%) vs. 9/27 (34%), *P*= 0.03).

The *late aABMR* group was characterized by younger recipient age (37.9±12.9 vs. 50.9±11.6 years; *P*<0.001), increased occurrence of *de novo* DSA (14/27 (52%) vs. 5/40 (13%), *P*=0.001) and nonadherence/suboptimal immunosuppression (15/27

(56%) vs. 0; *P*<0.001). In the *late acute ABMR* group15/27 (56%) patients had at referral low drug trough levels. Of these 15 patients, eight developed *de novo* DSA. Ten recipients self-reported nonadherence (age 26.6±9.5 years). In five recipients the immunosuppression was not adequately adjusted by the nephrologist. In the *late aABMR* group 9/27 (33%) patients were classified as young adults (15-30 years) at transplantation and none in the *early aABMR* group.

4.2 Paper II - One-year protocol biopsies from *ABO-incompatible* compared to a matched cohort *ABO-compatible* renal allografts

The aim of this study was to evaluate protocol biopsy findings at one year in *ABOi* compared to *ABOc* recipients. One year protocol biopsies were scored according to Banff criteria. Three sum scores for tubulointerstitial inflammation, microvascular inflammation and scarring/hyalinosis were applied. There were no differences in Banff scores or sum scores between *ABOi* and *ABOc* groups, except for C4d positivity, which was more frequent in the *ABOi* group. Both inflammatory and scarring mean scores were low.

Subclinical rejection at one year was 6/20 (30%) vs. 11/60 (18%) in *ABOi* vs. *ABOc* groups. Subclinical ABMR was diagnosed in 1/20 (5%) vs. 4/60 (7%) respectively. All recipients in both groups with subclinical ABMR developed *de novo* HLA DSA. None of the protocol biopsies at one year displayed transplant glomerulopathy.

During the first 6 months after transplantation biopsy proven acute clinical rejections were diagnosed in 4/20 (20%) of *ABOi* patients compared to 9/60 (15%) in *ABOc*

patients. In the *ABOi* group there were 2 acute ABMR, one Banff IA and one Banff borderline rejection. The 9 rejection episodes in the ABOc group were all TCMR.

4.3 Paper III - Total inflammation in early protocol biopsies and fibrosis one year posttransplant

The study included 156 patients transplanted in 2010 with a 6 week and 1 year protocol biopsy (312 biopsies).

The included patient group compared to the excluded patient group had significantly more living donors 54/156 (35%) vs. 20/98 (20%, *P*=0.02) and less DR mismatches 92/156 (59%) vs. 70/98 (71%), *P*=0.04.Table 4.

Fibrosis increased significantly from week 6 to 1 year both with the 10-grade scoring system from 0.69±1.07 to 1.45±1.86, (mean±SD), *P*<0.001 and by conventional Banff interstitial fibrosis (ci) scoring from 0.81±0.65 to 1.13±0.87, *P*<0.001.

Fibrosis progression from 6 weeks to 1 year posttransplant was detected in significantly more recipients by the use of the 10% interval fibrosis score (defined by Δ10% fibrosis>0) than by the conventional Banff interstitial fibrosis (ci) score (defined by Δci>0), 63/155 (40.4%) versus 55/156 (35.5%), *p*<0.001 respectively.

No significant positive association was found between inflammation at week 6 with progression of fibrosis at one year posttransplant, neither scored by Banff total inflammation score (ti) nor by inflammation in % of the entire cortex in the 6 weeks biopsies. Fibrosis progression was scored by change of the conventional Banff (ci) score or the 10 grade fibrosis score from 6 weeks to 1 year.

Fibrosis was overall low grade, but moderate /severe fibrosis defined as Banff ci≥2 was found in 19/156 (12.1%) biopsies at week 6 and in 45 (28.8%) biopsies at 1 year, *P*<0.001. Chronic allograft damage score (CADI score 0-21) was low and not significantly changed from week 6 (mean 3.4±2.1) to 1 year (mean 3.8±2.6).

Forty-eight patients experienced one or more biopsy proven subclinical or clinical rejection episodes, Table 4.

Table 4.

Demographics baseline and 1year included and excluded patients paper #3

	All patients n=254	**Included patients n=156**	**Excluded patients n=98**	***p***
Baseline data				
Recipient gender (male), n (%)	179 (70.5)	114 (73.1)	65 (66.3)	0.3
Donor gender (male), n (%)	132 (52.2)	79 (50.6)	53 (54.1)	0.6
Recipient age (yr), mean (±SD)	53.8 (±14.4)	54.1(±13.8)	52.7 (±15.4)	0.4
Donor age (yr), mean (±SD)	49.9 (± 14.9)	50.6 (±13.9)	48.7 (±16.4)	0.3
Living Donor Tx, n (%)	74 (29.1)	54 (34.6)	20 (20.4)	**0.02**
Preemptive Tx, n (%)	69 (27.2)	44 (28.2)	25 (25.5)	0.5
Cold Ischemia time, hrs, median (range)	10.4 (0.8-28,4)	10.0 (0.8-24.4)	10.5 (0.9-28.4)	0.4*
Delayed graft function, n (%)	23 (9.1)	14 (9.0)	9 (9.2)	0.95
HLA A+B mm ≥2, n (%)	188 (74)	114 (73.1)	74 (71.4)	0.7
HLA DR mismatch ≥1, n (%)	162 (63.8)	92 (59.0)	70 (71.4)	**0.04**
Graft number ≥ 2, n (%)	36 (14.2)	19 (12.2)	17 (17.3	0.3
CMV D+→R-, n (%)	44 (17.3)	25 (16)	19 (19.4)	0.6
Prefomed DSA at Tx, n (%)	21 (8.3)	16 (10.3)	5 (5.1)	0.2

	All patients n=254	**Included patients n=156**	**Excluded patients n=98**	***p***
DATA ≤ first year posttransplantation				
CMV PCR positive, n (%)	81 (31.8)	43 (27.6)	38 (38.7)	0.06
PVAN, n (%)	4 (1.6)	4(5.9)	0	0.3**
Recurrence/ de novo GN, n (%)	11 (4.3)	7 (4.5)	4 (4.1)	1.0**
De novo DSA, n (%) at 6 weeks at 1 year at 6 weeks+1 year	18 (7.1) 4 (1.6) 11 (4.3) 3 (1.2)	12 (7.7) 3 (1.9) 7 (4.5) 2 (1.3)	6 (6.1) 1 (1.1) 4 (4.5) 1 (1.1)	0.6 1.0** 1.0** 1.0**
Patients with a biopsy proven rejection (BPAR) *,** n (%) subclinical 6 weeks/ 1 year clinical	79 (30.9) 22 (8.7) 57 (22.4)	48 (30.8) 17 (10.9) 31(19.9)	31 (31.6) 5 (5.1) 26 (26.5)	0.8
Graft loss	2 (0.8)	0	2 (2)	0.2**
Death	8 (3.1)	0	8 (8.2)	**0.01****

Mean (±SD), medians (range) or proportions as appropriate. Test of group differences included/ excluded by t-test, Mann Whitney U test* or Chi-Square test, if expected count less than 5 Fisher`s exact test**; Tx, transplant; CMV, Cytomegalo virus; DSA, donor specific antibody; HLA, human leucocyte antigen; mm, mismatch; PVAN , Polyomavirus associated nephropathy; dn GN, de novo glomerulonephritis; BPAR*** both clinical and subclinical

5. DISCUSSION

5.1 GENERAL DISCUSSION

We have explored the role of clinical and subclinical inflammation in kidney graft biopsies, both in patients with standard immunological risk and patients crossing the immunological barriers.

Organ availability

The shortage of donor organs and increasing waiting lists worldwide created the need for expansion of the LD pool. ABOi and HLA incompatible transplantation will provide donors, both living and deceased to extend the number of recipients. This is a benefit for the transplant programs and for the individual patients. A significant proportion of recipients on the waiting list is broadly sensitized to HLA and will have to wait longer for an acceptable graft or may never be transplanted (*5, 157*). The recipients crossing the immunological barrier by ABOi and HLA incompatible transplantation face an increased risk of ABMR and premature graft loss (*28, 39, 40, 87, 124*). In case of ABOi transplantation the increased risk for ABMR caused by blood group antibodies only seem to affect the early period after transplantation (*158*). We have had a program for HLA sensitized patients since 1984 with the attitude that transplantation is preferable to dialysis even in patients with higher risks (*96*). This policy is supported by two publications (*5, 157*), where the most recent by Orandi et al. (*5*) demonstrates that patients transplanted across HLA immunization with a LD had superior survival compared to two matched control groups. The *wait-list or transplant control group* consists of wait-listed patients, some of who received a transplant from a deceased donor. The second control group, the *waiting list only control group* consists of wait-listed patients, who never underwent transplantation.

Acceptable mismatch programs and donor exchange programs are different strategies to provide grafts to highly sensitized patients (*110, 112, 113*). In Scandinavia STAMP and a paired exchange program, recently initiated in Sweden, increases the possibility to find a suitable graft for highly immunized recipients.

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Protocol biopsies

Despite the fact of dramatically decreasing early clinical rejection rates from 80% in the early 1960ies to currently around 15% and decreasing SCR rates from 30-46% to 5 -17% (*140, 159-161*), the long term graft survival rate beyond 5 years is still unchanged (*162, 163*). Studies have indicated that early protocol biopsies and treatment of SCR, can improve outcomes (*159, 161, 164*). A recent paper showed that early SCR was associated to a higher risk of chronic ABMR (*165*). However, the declining prevalence of early SCR in the standard risk patients reduces the benefit of protocol biopsies for this patient group (*160*).

In centers like ours, which transplant across the HLA and ABO barrier, subclinical antibody-mediated rejection can be diagnosed by protocol biopsies both at 6 weeks and one year posttransplant (*28, 53, 87, 118*). Enforced and adjusted immunosuppression may reduce development of chronic antibody-mediated damage of the graft. In a recent paper from the Mayo clinic the one year protocol biopsy results could be related to graft function and survival. Identification of risk factors for unfavorable histology at one year altered the treatment protocols (*120*).

There is a well-documented association between early tubulo-interstitial inflammation and progression of IFTA and development of graft failure (*141-143*). The properties and significance of early inflammation in protocol biopsies is not completely

understood. Early inflammation could represent an alloimmune response, a tissue injury response to the ischemic injury during transplantation or a silent interstitial and tubulus inflammation, which resolves without causing any damage (*136*).
The major complication rate after kidney graft biopsies is low, between 0.4-1% in the literature. Moreover there is a low risk for serious bleeding complications (*166-168*).

The beneficial effects appear to outweigh the possible complications of protocol biopsies as they provide the opportunity of individualized immunosuppression and thus improve graft and patient survival (*169*). The one year protocol biopsy seems to be the key for long term prognosis (*120, 133, 134*).

5.2 Paper I- *Early* versus *late acute antibody-mediated rejection*

Increased risk of aABMR in patients sensitized to HLA is well known (*28, 39, 40*), but immediate graft loss is usually avoided by contemporary treatment protocols. However, antibody-mediated graft injury is a major contributor to late graft loss (*29, 30, 34*). Fifty-five percent in the *early aABMR* group had preformed HLA DSA. According to protocol these patients receive enforced induction and maintenance immunosuppression. Forty-five percent of the patients with *early aABMR* did not have detectable HLA antibodies before transplantation, but 13% had developed *de novo* HLA DSA at diagnosis of *early aABMR*. In the *early aABMR* group 32% of the patients were immunological low risk at transplantation and *de novo* DSA was not detected. A possible cause for this could be that non-HLA DSA was present (*170, 171*).
Antibody-mediated tissue injury is described as a smoldering process that can be subclinical before clinical findings appear (*38, 118*). Acute and chronic changes of ABMR often coincide and may represent a continuum (*38, 172, 173*).

In the *late aABMR* we diagnosed transplant glomerulopathy (TG) in 10/27 (37%) biopsies. TG is known as a negative prognostic factor (*174*). The inferior graft survival in the *late aABMR* group may be explained by the chronic changes in the graft.

At the time of transplantation the majority of the patients in the *late aABMR* group was immunologically a low-risk group, but 14/27 (52%) had developed *de novo* HLA DSA by the time of clinical rejection. Patients were younger in the *late aABMR* group and nonadherence/ suboptimal immunosuppression was documented in 15/27 (56%) recipients. Nonadherence as a major problem in transplant recipients has recently come to attention (*175-179*). Adolescents in transition from pediatric care to adult care are at particular risk (*180-182*).

Limitation and strength

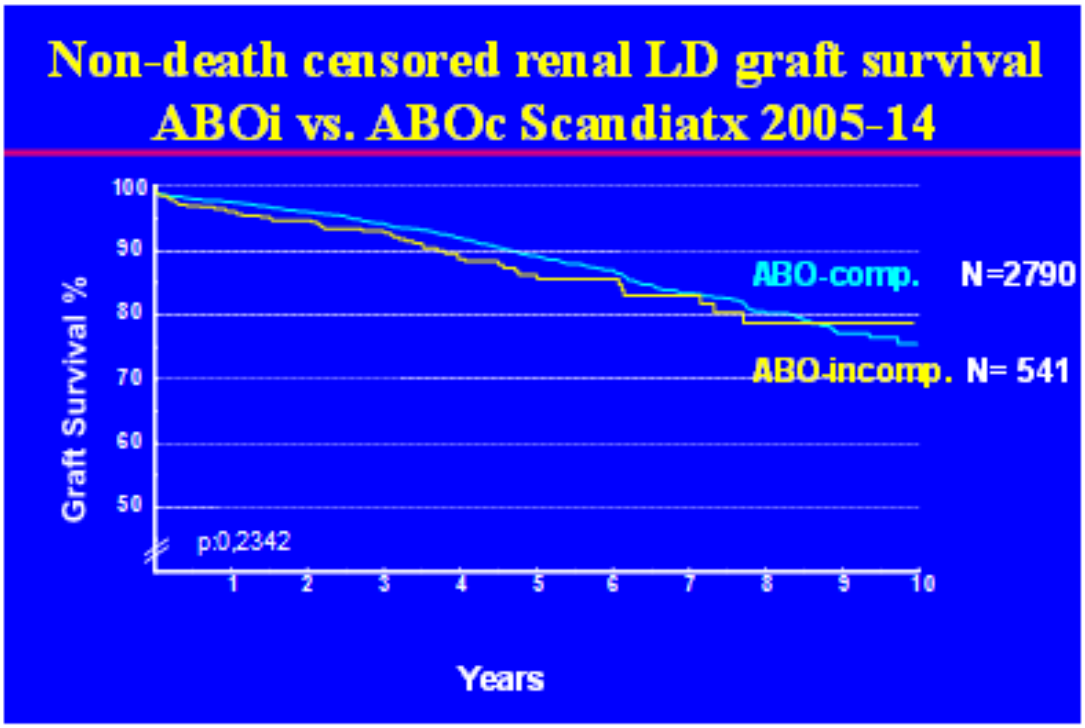
The retrospective design and rather small patient groups limit the interpretation of our findings. The strength of our study is the 100% completeness of follow-up data.

5.3 Paper II- One-year protocol biopsies from *ABO-incompatible* compared to a matched cohort *ABO-compatible* renal allografts

There is evidence from Japanese and USA registry studies (*116, 158, 183*) and from the Scandinavian experience (Figure 8, unpublished data) that *ABOi* transplantation has long term prognosis comparable to *ABOc* renal transplantation. Protocol biopsy findings at one year are associated with long term outcomes (*120, 133, 134*). We

found a low incidence and no difference in inflammatory and chronic changes in one year protocol biopsies in *ABOi* and *ABOc* transplantation. These findings support similar prognosis in *ABOi* and *ABOc* transplantations from one year.

Figure 8. Non-death censored renal LD graft survival ABOi vs. ABOc, Scandiatransplantdata from 2005 -2014



With courtesy to the Nordic Kidney Group, Scandiatransplant , unpublished data

Several studies show a higher rate of acute antibody-mediated rejections and graft loss the first year in *ABOi* compared to *ABOc* transplantation (*158, 184*). Only grafts

reaching one year survival were included in the study. In the current study we detected 10% acute ABMR in the *ABOi* group and none in the *ABOc* group within the first year posttransplantation. Thus, in *ABOi* recipients there is an increased risk of acute antibody-mediated rejection and graft loss, despite a preconditioning regimen.

A recent study on surveillance biopsy data 1 year and 5 years after transplantation, presented TG in 3% and 8% respectively in *ABOc* recipients, 11% and 12% respectively in *ABOi* recipients and 29% and 58% respectively in *cross-match positive* recipients (*28*). In *ABOi* recipients the incidence of TG at one year varies from zero to 15% (*28, 87, 124, 126*). In the present study we found no TG, neither in the *ABOi* nor in the *ABOc* recipients. The study population was recruited between 2009 and 2012 and was thoroughly screened for HLA DSA before transplantation. The rate of early acute ABMR was low. This may explain the lack of TG in our study. There was no difference in the rate of microinflammation or subclinical ABMR in the *ABOi* and *ABOc* groups. Seven patients (1/20 *ABOi* and 6/60 *ABOc*) developed *de novo* HLA DSA within 1 year posttransplant. All patients with subclinical ABMR acquired *de novo* HLA DSA. Recent publications in unselected low risk populations demonstrate that 11-35% of patients develop *de novo* DSA during the first years posttransplant (*56, 57, 84, 185*) and that these patients experience a higher rate of acute rejections (*56, 185, 186*). Data on *de novo* HLA DSA in ABOi recipients are scarce. In the present study *de novo* HLA DSA, and not blood group antibodies, appears to be a risk factor for development of microvascular inflammation and subclinical ABMR at 1 year.

The preconditioning treatment for ABOi is more complicated, intensive and costly, compared to standard transplantation. When patients are evaluated for ABOi

transplantation it is important to consider the alternatives.

Limitation and strength

The design of our study with a matched control group from a recent time period is a strength. No patients with preformed HLA DSA were included in any of the groups. Most studies do not provide data on HLA antibody status pretransplant, and they may have mixed study groups, with both DSA and ABOi. Our data on biopsy findings and on *de novo* HLA DSA are close to complete. Data on protocol biopsies together with *de novo* DSA in ABOi patients are rarely published (*127, 187*). The small size of the ABOi group (n=20) is a limitation. There is also a shortcoming that the protocol biopsy program was started first in 2009, while the ABOi program was started in 2006.

5.4 Paper III- Total inflammation in early protocol biopsies and and fibrosis one year posttransplant

The main finding in paper # 3 was that inflammation score in protocol biopsies at week 6 was not a predictor of progression of fibrosis at one year posttransplant. This observation was not dependent on method used to assess inflammation and fibrosis; neither a more sensitive visual scoring in a 10 grade system nor the conventional Banff scoring was able to detect fibrosis progression related to inflammation at week six.

Studies which showed a correlation between early subclinical tubulo-interstitial inflammation and fibrosis progression did not asses inflammation in fibrotic areas (*135, 140, 141, 159*). Mengel et al. demonstrated that total inflammation including inflammation in fibrotic and tubulo- atrophic areas were a better predictor for T-cell

burden and graft survival compared to interstitial inflammation alone (*128*). These findings were corroborated by Mannon et al. who showed that inflammation in tubulo-atrophic areas and fibrosis correlated strongly with death censored graft survival (*129*). Both studies were based on indication biopsies obtained at median 19 months and mean 7.1 ±5.9 years posttransplant, respectively (*128, 129*). Not surprisingly these biopsies display a higher degree of inflammation and chronic changes and did not represent findings in early protocol biopsies. Park et al. applied the Banff total inflammation score in a 1 year protocol biopsy study in an immunological low-risk cohort. They showed increased inflammatory cells (T-cells and macrophages). Both inflammation in fibrotic and non-fibrotic areas correlated with a deleterious effect on kidney function and reduced graft survival at 5 years (*134*). Using the Banff total inflammation score compared to Banff traditional i- score, did not substantially alter the results in their study. Our results indicate that the Banff ti score in early protocol biopsies is not useful to predict progression of fibrosis one year posttransplant.
In accordance to other studies, we observed a relatively low grade fibrosis and tubular atrophy (IFTA) and an unchanged low CADI score at one year (*188*).

Fibrosis is a key histological correlate for chronic progressive kidney disease and fibrosis in 1 year biopsies is associated with graft failure (*133*).
In the last two decades the degree of fibrosis in early biopsies has declined partly due to improved immunosuppression with lower rates of acute rejection and partly due to immunosuppression protocols with lower CNI exposure (*139, 160, 167, 189-191*).
The rejection rate in our study was reduced compared to earlier eras, clinical 31

(19.9%) and subclinical 17 (10.9%) (*96, 192*). Since the introduction of Basiliximab in 2007, the CNI levels have been reduced.

The optimal assessment of fibrosis in kidney transplant biopsies is still a matter of debate. Visual assessment of trichrome-stained slides which is the standard of practice (*193*) has shown poor reproducibility in multicenter studies (*194, 195*).
A recent multicenter study which assessed trichrome, periodic acid-Schiff and computer-assisted quantification of collagen III immunohistochemistry for interstitial fibrosis (IF)/ tubular atrophy % (TA%) scoring and Banff total cortical inflammation score (ti), concluded that visual assessment of fibrosis varied among observers and had a weaker correlation with organ function compared to computer-assisted quantification (*196*) . The study concluded that collagen III immunohistochemistry could potentially accomplish standardized assessment in multicenter settings.
We chose visual assessment of trichrome slides for evaluation of fibrosis. In the hands of experienced pathologists this approach has turned out nearly as reproducible as morphometry (*146*).
Attention has been focused on methods for estimating/measuring fibrosis (*146, 197, 198*) and less on methods for estimating/measuring inflammation (*128, 129*) . In the current study we did not only include total inflammation scoring, but we did also pay attention to detailed estimation of inflammation by using a 10-grade scale.

Limitation and strength

There are some limitations to our study. It is a single center study which could be underpowered to show the impact of inflammation on fibrosis progression. Our methodology gives no further information on the immunological origin of the cell infiltrates. The strength of our study is the meticulous approach for a more precise measurement of fibrosis and inflammation in all compartments of a kidney transplant biopsy at the same time.

6.0 CONCLUSIONS

6.1 Paper I- *Early* versus *late acute antibody-mediated rejection*

Our study indicates that *late aABMR*, when compared to *early aABMR*, has inferior graft survival and is characterized by younger recipient age, more frequent nonadherence or suboptimal immunosuppression and development of *de novo* DSA. Nonadherence is a challenge in younger adults.

6.2 Paper II- One-year protocol biopsies from *ABO-incompatible* compared to a matched cohort *ABO-compatible* renal allografts

One year biopsies of *ABOi* and *ABOc* controls matched for donor age and transplantation year did not differ in inflammation, SCR and scarring. The chronic changes/scarring, although affecting the majority of patients, were low grade. Microvascular inflammation >0 was low grade and only found in 5% and 8% respectively, mainly in patients with *de novo* HLA DSA. No development of TG was found in any of the groups. If early graft losses caused by ABMR can be avoided, our findings suggest similar long term results for *ABOi* and *ABOc* recipient.
ABOi transplantation has good results, but there is still a need to reduce the early ABMR and early graft losses.

6.3 Paper III - Total inflammation in early protocol biopsies and fibrosis one year posttransplant

Inflammation grade measured in fibrotic/atrophic and non-fibrotic/atrophic areas in kidney graft protocol biopsies at 6 weeks post-transplant did not predict fibrosis progression at one year.

7. FUTURE PERSPECTIVES

7.1 Paper I- *Early* versus *late acute antibody-mediated rejection*

A phenotype with young age and *de n*ovo DSA has been defined for *late aABMR*. Results of this and other studies have drawn our attention to the problem of patient adherence to immunosuppressive medication. Tools to detect and instruments to reduce nonadherence are needed. Two clinical studies with focus on adherence have been initiated in our department. One study evaluates the Basel Assessment of Adherence to Immunosuppressive Medication Scale (BAASIS) , variance of tacrolimus concentration and assessment of treating physician to detect nonadherence (*199*). The other ongoing study evaluates the effect of an education program for patients. We now advise simple measures as alarm on mobile phone, more frequent visit at outpatient clinic, telephone contact from transplant nurse for recipients at risk, especially young adults and patients with low or high variation in CNI levels.

The study also increased our awareness for the problem of reduction of immunosuppression by nephrologists, usually initiated by CMV infection, polyoma virus infection or side effects. The clinical reason for reduction often has been transient, while the immunosuppression has not been restored to levels per protocol. The one year visit and protocol biopsy at the transplant center is useful to avoid under- and over-immunosuppression.

Our understanding of what properties define DSA with deleterious effect on the graft and what should be avoided is still incomplete. IgG subclass, complement binding

and MFI levels have been studied, but no firm conclusion has been reached (*200-202*). Most transplant centers will avoid transplantation with a positive CDC crossmatch and/or high antibody levels (MFI). Better definition of non- acceptable mismatches and antibodies is an urgent need for the future.

Acute ABMR could also be caused by non-HLA donor-specific antibodies. A test for endothel antibodies (XM One) and angiotensin receptor antibodies (ATR1R-ab) are available and used at some centers (*203*). Running extended testing on all patients is costly and requires extra resources. Introduction of these methods in routine work-up needs careful evaluation.

Desensitizing and induction protocols have improved the outcome of immunological high risk patients (*96, 99, 101, 157*), but there is still a risk of early severe aABMR and of chronic ABMR with reduced graft survival (*26*). The protocols in use have not been evaluated by randomized, controlled studies. Correspondingly, there are no approved treatment protocols for aABMR and cABMR, urging for studies and new treatment concepts. New drugs in the field are for example complement inhibition with C5 inhibitor (Eculizumab) or C1 esterase inhibitor (Berinert), antibody cleavage by IgG Endopeptidase, Bortezomib and IL-6 antibody (Tocilizumab) (*106, 108, 204-206*).

Therefore future research should focus on improved adherence in the care of transplanted patients, especially in young adults and on multicenter studies of treatment for acute and chronic ABMR.

7.2 Paper II- One-year protocol biopsies from *ABO-incompatible* compared to a matched cohort *ABO-compatible* renal allografts

Within Scandiatransplant most centers use modifications of the protocol for ABOi transplantation published by Tyden et al. (*11*). The graft survival for the Scandiatransplant ABOi cohort from 2005-2014 is shown in Figure 8 (unpublished).

Findings in paper #2 reassured us that renal transplantation across the ABO barrier do not lead to more microinflammatory injury causing chronic changes at one year posttransplantation, if the early acute ABMR is treated and best avoided.

Nevertheless mechanisms leading to early ABMR in ABOi renal transplants are not fully understood and need further research. There is an increased risk for early ABMR and graft loss (*87, 158*). In our experience careful monitoring after ABOi transplantation is necessary.

Furthermore the study has emphasized the importance for both patient-groups ABOc and ABOi recipients to develop strategies to avoid *de n*ovo HLA DSA formation.

Strategies to avoid *de novo* DSA development in the future should focus on HLA matching (*207, 208*) and improvement in patients adherence and doctors understanding of importance for maintenance immunosuppression.

HLA incompatibility is a more difficult immunological barrier to surpass than ABO incompatibility (*28*).

In the choice of living donor, ABOi should be preferred to HLA DSA.

The challenge is now to detect the cause of early severe ABMR in ABOi transplantation and to identify the circumstances, which lead to these detrimental events.

7.3 Paper III-Total inflammation in early protocol biopsies and fibrosis one year posttransplant

Fibrosis is a key histological correlate for chronic progressive kidney disease.

The negative findings in paper III should not discourage further research of inflammation in fibrosis. We need to try to define the nature of inflammatory cells in the graft and their biological effects more thoroughly. A pure macroscopic descriptive approach is not enough to fully understand the complex multifactorial mechanisms of fibrosis development (*137*).

The importance of early total inflammation is not well studied yet. In our study it is not a predictor of fibrosis at one year. Early inflammation, especially in coexistence with fibrosis is associated with appearance of *de novo* DSA (*209, 210*). These data support the treatment of early subclinical inflammation, but the degree of inflammation in need for treatment and the treatment modality should be the aim of future research.

One year is a rather short period to study fibrosis progression and it could add value to our study to repeat biopsies and clinical work-up at a later follow-up. We have investigated the prognostic value of the Omega-3 fatty acids for fibrosis progression in this cohort (manuscript in preparation).

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Appendix

Paper 1-3