THE MECHANISMS OF CHRONIC ALLOGRAFT INJURY

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CHRONIC ALLOGRAFT NEPHROPATHY INJURY

Terminology:
- Chronic allograft dysfunction (CAD)
- Chronic allograft nephropathy (CAN)
- Chronic allograft injury (CAI)
- Interstitial fibrosis and tubular atrophy (IF/TA), no evidence of any specific etiology

- Leading cause of allograft failure after patient death
- 40-50% CAI findings at 1 year protocol biopsies

Bohmig et al. Transplant Int 2005; 18: 131
Banff ‘05 Meeting Report: Differential Diagnosis of Chronic Allograft Injury and Elimination of Chronic Allograft Nephropathy (‘CAN’)


mediated rejection. Participation of B cells in allograft rejection and genomic markers of rejection were also major subjects addressed by the conference.

Key words: Banff classification, central slide review, scoring

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Table 1: Morphology of specific chronic diseases

<table>
<thead>
<tr>
<th>Etiology</th>
<th>Causes of IF/TA (non-rejection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic hypertension</td>
<td>Arterial/fibrointimal thickening with reduplication of elastica, usually with small artery and arteriolar hyaline changes.</td>
</tr>
<tr>
<td>CNI toxicity</td>
<td>Arteriolar hyalnosis with peripheral hyaline nodules and/or progressive increase in the absence of hypertension or diabetes. Tubular cell injury with isometric vacuolization.</td>
</tr>
<tr>
<td>Chronic obstruction</td>
<td>Marked tubular dilation. Large Tamm–Horsfall protein casts with extravasation into interstitium, and/or lymphatics.</td>
</tr>
<tr>
<td>Bacterial pyelonephritis</td>
<td>Intratubular and peritubular neutrophils, lymphoid follicle formation.</td>
</tr>
<tr>
<td>Viral infection</td>
<td>Viral inclusions on histology and immunohistology and/or electron microscopy.</td>
</tr>
</tbody>
</table>

1CNI, calcineurin inhibitor toxicity.
CHRONIC ALLOGRAFT INJURY

**Immunologic Factors**
- Acute or subclinical rejection
- HLA mismatch
- Donor specific antibodies

**Non-immunologic factors**
- Ischemia/reperfusion injury
- Delayed graft function
- Donor factors: Age
- Calcineurin inhibitor toxicity
- Viral nephritis (Polyoma, CMV)
- Post-transplant HTN
- Hyperlipidemia

**Inflammation and injury**
- Adhesion molecules, cytokines and growth factors

**Accelerated Senescence**
- Interstitial fibrosis, tubular atrophy, transplant glomerulopathy, and fibrous intimal thickening of arteries
Tissue Regeneration vs. Fibrosis: The Process of Wound Healing

Initiation Phase

- Ag dependent
- Ag independent

Fibrogenesis Phase

Matrix Phase

Polyoma Virus Nephropathy

- First reported in 1995 and associated with polyomavirus type BK. JC virus (PMLE) and SV 40 in same family
- 90% seroprevalence rate worldwide
- Mainly the disease of kidney tx patients. Association with anti-rejection treatment and the degree of immunosuppression
Polyoma Virus Nephropathy Cytopathic Changes and anti-SV40 staining
Cyclosporine-induced Nephrotoxicity

Mechanisms of Chronic Toxicity
- TGF-β induction
- Role of Ang II induction
- Oxidative stress
- Renal vasoconstriction
- EMT
Chronic Allograft Injury
Nodular Arteriolar Hyalinization
(Calcineurin Inhibitor Toxicity)
The natural history of chronic allograft nephropathy (Follow-up 119 kidney/pancreas transplant recipients by protocol biopsies up to 10 years)

*NEJM 2003;349:2326*

### Table 2. Cumulative Kaplan–Meier Estimates of the Prevalence of Histologic Diagnoses, According to the Time after Transplantation.

<table>
<thead>
<tr>
<th>Histologic Diagnosis</th>
<th>1 Yr</th>
<th>5 Yr</th>
<th>10 Yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic allograft nephropathy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Banff grade I</td>
<td>94.2</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Banff grade II or III</td>
<td>24.7</td>
<td>65.9</td>
<td>89.8</td>
</tr>
<tr>
<td>Calcineurin-inhibitor nephrotoxicity</td>
<td>76.4</td>
<td>93.5</td>
<td>96.8</td>
</tr>
<tr>
<td>Arteriolar hyalinosis</td>
<td>62.0</td>
<td>90.3</td>
<td>100.0</td>
</tr>
<tr>
<td>Striped fibrosis</td>
<td>33.2</td>
<td>68.3</td>
<td>87.3</td>
</tr>
<tr>
<td>Tubular microcalcification</td>
<td>42.7</td>
<td>67.2</td>
<td>78.5</td>
</tr>
</tbody>
</table>
Chronic renal failure after transplantation of a non-renal organ (69,321 persons who received transplantation in USA between 1990-2000)

Transplant Glomerulopathy
Peritubular Capillary Basement Membrane Multilayering
Chronic Allograft Arteriopathy
Antibody-Mediated Rejection

Cellular Rejection

Anti-HLA-Ab → C1 → C4 → C4b + C4a → C3a + C3b → MAC → C4d

Donor-HLA → T-cell → PMN → Mø

C4d
# C4d+ PTC in Chronic Rejection

<table>
<thead>
<tr>
<th>Chronic Rejection (TGP/CAA)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mauiyyedi (Boston) 2001 CAA/TGP</td>
<td>38</td>
<td>61%</td>
</tr>
<tr>
<td>Regele (Vienna) 2002 TGP</td>
<td>58</td>
<td>67%</td>
</tr>
<tr>
<td>Mróz (Warsaw) 2003 CAA/TGP</td>
<td>6</td>
<td>83%</td>
</tr>
<tr>
<td>Vongwiwatana (Edmonton) 2004 TGP</td>
<td>24</td>
<td>25%</td>
</tr>
<tr>
<td>Sijpkens (Leiden) 2004 TGP (Glom C4d+)</td>
<td>10</td>
<td>40%</td>
</tr>
<tr>
<td>Herman (Leuven Bel) 2005 TGP</td>
<td>11</td>
<td>73%</td>
</tr>
<tr>
<td>Jeong (Seoul) 2005 CAA</td>
<td>24</td>
<td>21%</td>
</tr>
<tr>
<td>Aly (St Louis) 2005 TGP</td>
<td>20</td>
<td>0%</td>
</tr>
<tr>
<td><strong>Control Tx (No TGP/CAA)</strong></td>
<td><strong>281</strong></td>
<td><strong>7%</strong></td>
</tr>
<tr>
<td>Mauiyyedi (Boston) 2001</td>
<td>30</td>
<td>3%</td>
</tr>
<tr>
<td>Regele (Vienna) 2002</td>
<td>155</td>
<td>22%</td>
</tr>
<tr>
<td>Mróz (Warsaw) 2003</td>
<td>13</td>
<td>8%</td>
</tr>
<tr>
<td>Vongwiwatana (Edmonton) 2004 (IgAN)</td>
<td>19</td>
<td>0%</td>
</tr>
<tr>
<td>Sijpkens (Leiden) 2004</td>
<td>14</td>
<td>7%</td>
</tr>
<tr>
<td>Jeong (Seoul) CIT</td>
<td>16</td>
<td>6%</td>
</tr>
<tr>
<td>Aly (St Louis) 2005</td>
<td>34</td>
<td>6%</td>
</tr>
</tbody>
</table>
DIAGNOSTIC CRITERIA FOR CHRONIC REJECTION

AJT 2007; 7: 518

CHRONIC ACTIVE ANTIBODYMEDIATED REJECTION
- Allograft histopathology
  - Arterial intimal fibrosis
  - Duplication of glomerular basement membrane (transplant glomerulopathy)
  - Laminated PTC basement membrane
  - Interstitial fibrosis/tubular atrophy
- C4d in peritubular capillaries
- Serologic evidence of donor anti-HLA antibodies

CHRONIC ACTIVE T-CELL MEDIATED REJECTION
- Chronic allograft arteriopathy (arterial intimal fibrosis with mononuclear cell infiltration in fibrosis, formation of neo-intima
INCREASED TRANSPLANT GLOMERULOPATHY RATE IN DESENSITIZED PATIENTS
Gloor JM et al. AJT 2006; 6: 1842

- 1 year protocol kidney biopsies of 37 cross-match positive patients who underwent desensitization and 198 conventional allografts without DSA

- Acute antibody-mediated rejection
  - Desensitized patients 46%
  - Control group 2%

- Acute cellular rejection
  - Desensitized patients 16%
  - Control group 14%

- Transplant glomerulopathy
  - Desensitized patients 22%
  - Control group 8%
Differential Outcome in Three Types of AMR: The Mount Sinai Experience

Median SCr = 1.6 mg/dL (0.8-2.7 mg/dL)
TG – transplant glomerulopathy

Rafiq MA et al Clin Transpl 2009
Transplant Glomerulopathy: Subclinical Incidence and Association with Alloantibody


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Transplant glomerulopathy (TG) usually has been described as part of a constellation of late chronic histologic abnormalities associated with proteinuria and declining function. The current study used both protocol and clinically-indicated biopsies to investigate clinical and subclinical TG, their prognosis and possible association with alloantibody. We retrospectively studied 582 renal transplants with a negative pretransplant T-cell complement dependent cytotoxicity crossmatch. TG was diagnosed in 55 patients, 27 (49%) based on protocol biopsy in well-functioning grafts. The cumulative incidence of TG increased over time to 20% at 5 years. The prognosis of subclinical TG was equally as poor as TG diagnosed with graft dysfunction, with progressive worsening of histopathologic changes and function. Although TG was associated with both acute and chronic histologic abnormalities, 14.5% of TG biopsies showed no interstitial fibrosis or tubular atrophy, while 58% (7/12) of biopsies with severe TG showed only minimal abnormalities. TG was associated with acute rejection, pretransplant hepatitis C antibody positivity and anti-HLA antibodies (especially anti-Class II), with the risk increasing if the antibodies were donor specific. We suggest that subclinical TG is an under-recognized cause of antibody-mediated, chronic renal allograft injury which may be mechanistically distinct from other causes of nephropathy.

Introduction

Transplant glomerulopathy (TG) is a condition associated with poor outcome, characterized by duplication of glomerular basement membranes, mesangial matrix expansion, and mesangial cell interposition (1,2). Originally classified as a variant of chronic allograft nephropathy of unknown etiology, TG is now recognized with increased frequency in patients with a prior history of humoral rejection, and is also associated with deposition of the complement degradation product C4d, suggesting that TG may be one manifestation of antibody-mediated injury (3–5).

Previous reports of TG have commonly described TG as a late manifestation of allograft injury, occurring years after transplantation, typically associated with decreased function and proteinuria, as well as severe interstitial fibrosis and tubular atrophy (IF/TA) (2,6). These reports were based on biopsies performed to investigate renal allograft dysfunction. Recently we have identified the characteristic lesion of TG early following transplantation on protocol biopsies performed in patients with well-functioning allografts, often with minimal or no other histologic abnormalities (7). Despite the fact that this early presentation may be associated with relatively mild degrees of allograft dysfunction at diagnosis, these individuals have poor outcome (8). The purpose of this investigation is: (1) to determine the incidence of TG in a large cohort of kidney transplant recipients studied with surveillance biopsies to compare clinical and subclinical TG, (2) to evaluate allograft histology and function at the time of diagnosis of TG, (3) to assess the progression of histologic and functional abnormalities and (4) to investigate factors associated with the development of TG, including its association with anti-HLA antibodies.

Methods
Transplant Glomerulopathy, Late Antibody-Mediated Rejection and the ABCD Tetrad in Kidney Allograft Biopsies for Cause

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Introduction

Late kidney allograft loss remains common (1), with a rising recognition that alloantibody plays a role in this problem (2). One important phenotype of late kidney deterioration is the ABCD tetrad of transplant glomerulopathy (TGN), focal arteriolar nephrosclerosis (FAN), chronic tubulointerstitial fibrosis, and calcineurin inhibitor (CNi) nephrotoxicity (3). Multiple non-antibody mechanisms have been proposed to explain TGN (4); however, the role of alloantibody in late kidney loss is not well understood. A recent study found a strong association between antibody-positive (Ab+) and antibody-negative (Ab−) biopsies among 473 renal allograft biopsies (5).

Table 6: The association of TG (D) with anti-HLA antibody (A), PTCBMML (C) and C4d (C) in 45 cases in which both C4d staining and anti-HLA testing were available

<table>
<thead>
<tr>
<th>TG phenotype</th>
<th>n (%)</th>
<th>Post-transplant time (median, mos)</th>
<th>Recipient age at biopsy (years)</th>
<th>Donor age at donation (years)</th>
<th>Cg</th>
<th>mm</th>
<th>Cv</th>
</tr>
</thead>
<tbody>
<tr>
<td>'ABCD'</td>
<td>16 (36)</td>
<td>66 ± 73</td>
<td>47 ± 13</td>
<td>37 ± 13</td>
<td>1.8 ± 0.7</td>
<td>1.4 ± 0.7</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td>'ABD'</td>
<td>13 (29)</td>
<td>69 ± 65</td>
<td>49 ± 12</td>
<td>48 ± 13</td>
<td>0 ± 0</td>
<td>0.5 ± 0.7</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td>'ACD'</td>
<td>1 (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'AD'</td>
<td>2 (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'BCD'</td>
<td>1 (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'CD'</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'BD'</td>
<td>10 (22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'D'</td>
<td>2 (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7: Comparison of clinicopathologic features in TG biopsies vs. non-TG biopsies

<table>
<thead>
<tr>
<th></th>
<th>TG</th>
<th>non-TG</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>53</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Post-transplant time (median, mos)</td>
<td>66 ± 73</td>
<td>69 ± 65</td>
<td>NS</td>
</tr>
<tr>
<td>Recipient age at biopsy (years)</td>
<td>47 ± 13</td>
<td>49 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td>Donor age at donation (years)</td>
<td>37 ± 13</td>
<td>48 ± 13</td>
<td>0.001</td>
</tr>
<tr>
<td>Cg</td>
<td>1.8 ± 0.7</td>
<td>0 ± 0</td>
<td>0.000</td>
</tr>
<tr>
<td>mm</td>
<td>1.4 ± 0.7</td>
<td>0.5 ± 0.7</td>
<td>0.000</td>
</tr>
<tr>
<td>Cv</td>
<td>1.6 ± 0.8</td>
<td>1.5 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>PTCBMML</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of layers, median</td>
<td>5.0 ± 2.2</td>
<td>4.0 ± 2.4</td>
<td>NS</td>
</tr>
<tr>
<td>No PTCBMML, n (%)</td>
<td>5 (9%)</td>
<td>8 (25%)</td>
<td></td>
</tr>
<tr>
<td>2-4 Layers, n (%)</td>
<td>17 (32%)</td>
<td>9 (29%)</td>
<td></td>
</tr>
<tr>
<td>≥5 Layers, n (%)</td>
<td>31 (59%)</td>
<td>14 (45%)</td>
<td></td>
</tr>
<tr>
<td>C4d in peritubular capillaries</td>
<td>18 (36%)</td>
<td>1 (3%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Anti-HLA panel-reactive Ab\textsuperscript{1}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive, n</td>
<td>33</td>
<td>13</td>
<td>0.01</td>
</tr>
<tr>
<td>Negative, n</td>
<td>14</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Donor-specific anti-HLA Ab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive, n</td>
<td>28</td>
<td>7</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Anti-HLA antibody analysis was available in 47 of 53 TG biopsies.
Table 2. Histopathologic features of patients with CAN and TGP, anti-HLA antibodies, and graft outcome

<table>
<thead>
<tr>
<th>Feature</th>
<th>CAN (51 Patients)</th>
<th>TGP (36 Patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAN grade per Banff 97 (n [%])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>23 (45)</td>
<td>9 (25)</td>
</tr>
<tr>
<td>II</td>
<td>21 (41)</td>
<td>21 (58)</td>
</tr>
<tr>
<td>III</td>
<td>7 (14)</td>
<td>6 (17)</td>
</tr>
<tr>
<td>TGP grade (n [%])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>6 (17)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>9 (25)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>13 (36)</td>
<td></td>
</tr>
<tr>
<td>electron microscopy alone</td>
<td>8 (22)</td>
<td></td>
</tr>
<tr>
<td>C4d positivity (diffuse; %)</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>C4d positivity (focal; %)</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Nodular arteriolar hyalinosis (%)</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>DSA (%)a</td>
<td>33</td>
<td>36</td>
</tr>
<tr>
<td>anti-HLA class I</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>anti-HLA class II</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>both classes I and II</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Non-DSA (%)a</td>
<td>23</td>
<td>29</td>
</tr>
<tr>
<td>No anti-HLA antibodies (%)</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Graft loss (%)</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td>Graft loss time after biopsy (yr; mean ± SD)</td>
<td>0.7 ± 0.7</td>
<td>1.1 ± 1.1</td>
</tr>
</tbody>
</table>
Intragraft detection of anti HLA Abs
Martin L et al, Transplantation 2003; 76: 395-400

Eluates from 20 kidney allografts removed for CAN
IgG anti Class I and/or anti Class II (Flow PRA beads)

Serum
1 yr post Tpl Transplantectomy 4 wks after TE
42.1% 31.6% 73.6%

DSA found in both, eluates and serum of 11/15 patients
Glomerulopathy

IgG  CD3  ICOS

CXCR3  Mig  IP-10
Predominant Th1 and Cytotoxic Phenotype in Biopsies from Renal Transplant Recipients with Transplant Glomerulopathy

S. Horns\textsuperscript{a,†}, H. Mansour\textsuperscript{a,†}, D. Desvaux\textsuperscript{a}, C. Diet\textsuperscript{a}, M. Hazan\textsuperscript{c}, M. Buchler\textsuperscript{b}, Y. Lebranchu\textsuperscript{b}, D. Buob\textsuperscript{d}, C. Badoual\textsuperscript{f}, M. Matignon\textsuperscript{g}, V. Audard\textsuperscript{b}, P. Lang\textsuperscript{a} and P. Grimbert\textsuperscript{a,*}

Key words: Chronic transplant glomerulopathy, immune function, renal transplant, renal transplant pathology

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Figure 3: Expression levels of nine genes (encoding Foxp3, GB, IFN\textgamma{}, T-bet, GATA3, IL-4, IL23, CD20 and ROR\gamma{}) in biopsy samples from patients with TGP with (C4d+) or without (C4d−) C4d staining in peritubular capillaries.
11 of 16 patients with TGP and 3 of 16 patients with CAN but without TGP had circulating antibodies reactive with GBM isolates.

7 of those 11 patients anti-GBM activity was identified as towards “agrin”
TRANSPLANT GLOMERULOPATHY

- Probably multifactorial
  - C4d+/DSA+= chronic active antibody-mediated rejection
  - C4d-/DSA- with significant tubulointerstitial and/or glomerular infiltrates= chronic active T cell-mediated rejection
  - C4d-/DSA- without any infiltrates= endothelial damage due to CNI or autoantibodies
CLINICAL SIGNIFICANCE OF INFILTRATES IN FIBROtic AREA

COSIO FG et al. AJT 2005; 5:2464
**DIAGNOSTIC CRITERIA FOR CHRONIC REJECTION**

**CHRONIC ACTIVE ANTIBODYMEDIATED REJECTION**

- Allograft histopathology
  - Arterial intimal fibrosis
  - Transplant glomerulopathy
  - Laminated PTC basement membrane
  - Interstitial fibrosis/tubular atrophy
- C4d in peritubular capillaries
- Serologic evidence of donor anti-HLA antibodies

**CHRONIC ACTIVE T-CELL MEDIATED REJECTION**

- Chronic allograft arteriopathy (arterial intimal fibrosis with mononuclear cell infiltration in fibrosis, formation of neo-intima)
- C4d-/DSA- transplant glomerulopathy with significant tubulointerstitial and/or glomerular infiltrates???
IS KIDNEY TRANSPLANT BIOPSY THE GOLD STANDARD FOR ALLOGRAFT INJURY AND THE FUNCTION?

- Allograft injury is patchy and heterogenous (sampling error)
- Subjectivity in scoring, inter- and intra-observer variability (45-70% and 80-85%, respectively)
- Diagnostic uncertainty between borderline and grade I acute rejection, or grade I to II acute rejection
- Significance of infiltrates at fibrotic areas?
- Mechanisms of IF/TA (immunologic vs non-immunologic)
- Isolated v lesions (one lymphocyte underneath the arterial endothelium)
- Clinical significance of C4d+ lesions without tissue injury, or focal C4d+ or glomerular C4d+
- Molecular heterogeneity of the lesions (molecular subtypes of rejection)
GENECHIP TECHNOLOGY

- Has the power to measure the expression of thousands of human genes simultaneously
- Microarrays, have been successfully used to analyze the gene expression patterns of different types of cancers and inflammatory diseases for diagnosis, and to predict prognosis and response to therapy
- Hypothesis-generating, rather than hypothesis-driven
- To elucidate biomarkers or footprints, a set of genes, that could be used to identify subgroups, to predict the clinical outcome or response to treatment
Cells

Poly (A)^+ RNA

cDNA

Add polyA Controls

Wash & Stain (16 hours)

Scan (75 minutes)

Add Oligo B2 & Staggered Spike Controls

Add Oligo B2 & Staggered Spike Controls

Biotin - labeled cRNA transcript

Fragment heat, Mg^{2+}

IVT

Biotin-UTP

Biotin-CTP

Hybridize (8 minutes)
RNA fragments with fluorescent tags from sample to be tested

RNA fragment hybridizes with DNA on GeneChip
Data Analysis of Microarrays

- Data normalization (to compare expression levels)
- Data filtering (to eliminate genes expressed below a certain threshold)
- Statistical Analysis of Microarrays
- Pattern identification
  - Unsupervised (no prior knowledge of the data is used in the analysis)
    - Clustering algorithms (Hierarchical Clustering, $k$-means Clustering, Self-Organizing Maps)
    - Singular Value Decomposition
  - Supervised
- Gene family and pathway analysis (Gene Ontology)
- Literature mining
7 patients (4 males and 3 females, ages 30-54) with the diagnosis of AR IIa or IIb and 3 patients (all male, ages 34-51) with normal histopathology were included in this study.

All of them were on triple immunosuppressive medications with CsA, MMF and prednisone.

CsA levels were in target range.

Biopsies were done within 7-47 days after transplantation.

Affymetrix GeneChip® Hu6800 system (mRNA for 6,800 full length human genes) were used.
59 allograft kidney biopsies from 50 pediatric patients and 8 donor biopsies were analyzed by cDNA arrays.

While the gene expression patterns suggested at least 3 possible distinct subtypes of acute rejection, 19 CAN biopsy samples were clustered together without demonstrating any subset.
AIMS OF THE STUDY

- To characterize gene expression patterns in transplant kidneys with CAN by comparing them to the expression patterns of transplant kidneys with normal histopathology.
- To correlate gene expression profiles in CAN with clinical features including positive donor specific antibodies, C4d staining, transplant glomerulopathy, and nodular arteriolar hyalinization.
**PATIENT CHARACTERISTICS**

<table>
<thead>
<tr>
<th>Sex/Race</th>
<th>Age</th>
<th>Tx Type</th>
<th>Years</th>
<th>S Cr</th>
<th>UA</th>
<th>1997 Banff</th>
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<tbody>
<tr>
<td>D1</td>
<td>F/H</td>
<td>33</td>
<td>LR</td>
<td>11</td>
<td>3.1</td>
<td>1.3 g</td>
</tr>
<tr>
<td>D2</td>
<td>M/H</td>
<td>42</td>
<td>LUR</td>
<td>5</td>
<td>2.5</td>
<td>3+</td>
</tr>
<tr>
<td>D3</td>
<td>M/W</td>
<td>50</td>
<td>CAD</td>
<td>4</td>
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<td>negative</td>
</tr>
<tr>
<td>D4</td>
<td>M/H</td>
<td>47</td>
<td>LUR</td>
<td>5</td>
<td>2.9</td>
<td>1+</td>
</tr>
<tr>
<td>D5</td>
<td>M/W</td>
<td>31</td>
<td>LR</td>
<td>16</td>
<td>3.6</td>
<td>1.5 g</td>
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<tr>
<td>D6</td>
<td>M/W</td>
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<td>2.1</td>
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<tr>
<td>D7</td>
<td>M/W</td>
<td>64</td>
<td>CAD</td>
<td>2</td>
<td>2.3</td>
<td>3+</td>
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<tr>
<td>D8</td>
<td>M/B</td>
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<td>1.8</td>
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<tr>
<td>D9</td>
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<tr>
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<td>53</td>
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<tr>
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<tr>
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<td>3+</td>
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<td>34</td>
<td>CAD</td>
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<tr>
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<tr>
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<td>3.5</td>
<td>2+</td>
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<tr>
<td>C1</td>
<td>F/B</td>
<td>28</td>
<td>LR</td>
<td>3 mos</td>
<td>1.3</td>
<td>30</td>
</tr>
<tr>
<td>C2</td>
<td>M/H</td>
<td>31</td>
<td>LR</td>
<td>13 mos</td>
<td>1.3</td>
<td>30</td>
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<td>30</td>
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<tr>
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<td>58</td>
<td>LR</td>
<td>7 wks</td>
<td>1.7</td>
<td>30</td>
</tr>
<tr>
<td>C5</td>
<td>F/B</td>
<td>36</td>
<td>LR</td>
<td>13 mos</td>
<td>1.1</td>
<td>negative</td>
</tr>
<tr>
<td>C6</td>
<td>M/H</td>
<td>53</td>
<td>CAD</td>
<td>3 weeks</td>
<td>1.1</td>
<td>negative</td>
</tr>
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## RESULTS

<table>
<thead>
<tr>
<th>Donor</th>
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<th>C4d</th>
<th>Nodular Arteriolar Hyalinization</th>
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<tbody>
<tr>
<td>D1</td>
<td>Neg</td>
<td>Neg</td>
<td>Yes</td>
</tr>
<tr>
<td>D2</td>
<td>Neg</td>
<td>Neg</td>
<td>Yes</td>
</tr>
<tr>
<td>D3</td>
<td>Neg</td>
<td>Neg</td>
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</tr>
<tr>
<td>D4</td>
<td>Neg</td>
<td>Neg</td>
<td>Yes</td>
</tr>
<tr>
<td>D5</td>
<td>DR8 Pos</td>
<td>Pos</td>
<td>Yes</td>
</tr>
<tr>
<td>D6</td>
<td>A2 Neg</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>D9</td>
<td>Neg</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>D13</td>
<td>Neg</td>
<td>Neg</td>
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</tr>
<tr>
<td>D14</td>
<td>Neg</td>
<td>Neg</td>
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</tr>
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<td>D15</td>
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<td>No</td>
</tr>
<tr>
<td>D16</td>
<td>ND Neg</td>
<td>Neg</td>
<td>Yes</td>
</tr>
</tbody>
</table>
RESULTS

455 probe sets representing 324 genes were differentially expressed in CAN biopsies compared to controls. While 212 genes were upregulated, 112 genes were downregulated. Biological functions of differentially expressed genes:

- Cellular metabolism: 171 (53%)
- Cellular communication: 105 (32%)
- Cell growth/maintenance: 103 (32%)
- Signal transduction: 79 (24%)
- Cell adhesion: 39 (9%)
- Immune response: 31 (9%)
- Apoptosis: 12 (4%)
- Humoral defense mechanism: 5 (2%)
- Complement: 5 (2%)
RESULTS

Selected upregulated genes related to immunology:

- Transforming growth factor-β induced factor and thrombospondin-1, which played role in TGF-β signaling pathway, and platelet derived growth factor-C
- The chemokine CXCL-6, and the adhesion molecules vascular cell adhesion molecule, cell adhesion molecule with homology to L1CAM, activated leukocyte cell adhesion molecule and selectin P
- The complement-related upregulated genes were complement component 4A and 4B, I factor, B factor (properdin), and clusterin (complement lysis inhibitor)
- The other upregulated immune response related genes were; MHC class II DR β4, and DPα1, interferon-α inducible protein, interferon induced protein 44, IL-17B receptor, natural killer cell transcript 4, CD24, and CD59
RESULTS

Other selected genes:

- Genes related to fibrosis and extracellular matrix deposition, such as; versican (chondroitin sulfate proteoglycan 2), integrin-beta 3 and 6, matrix metalloproteinase 7, 9 and 10, laminin, fibronectin, tenascin, glypican 3 and 4, collagen type IV alpha 2, disintegrin, and cadherin 2 and 6

- claudin 3, annexin A1, A3 and A4, connexin, which are related to tight junction between cells
RESULTS

Selected downregulated genes:

- Significant number of downregulated genes were podocyte related, such as; podocin, nephrin, Wilms tumor 1, podocalyxin, and synaptopodin, or renal-specific genes; renin, calbindin, adrenomedullin, stanniocalcin, and kininogen
- Vascular endothelial growth factor, epidermal growth factor, and fibroblast growth factors 1 and 9, insulin-like growth factor binding protein 3 and 5
CONCLUSIONS

A number of genes are differentially expressed in biopsies with CAN compared to normal transplant kidney biopsies by microarrays.

- Some of the upregulated genes are related to fibrosis (MMP-7, 9, and 10, laminin, fibronectin, and tenascin) or factors/cytokines, which induce fibrosis (TGF-β, thrombospondin-1, and PDGF).
- VEGF, EGF, FGF-1 and 9 were downregulated in CAN.
- Hierarchical cluster analyses did not show distinct subsets of CAN.
**PATHOGENESIS-BASED TRANSCRIPT SETS**

Halloran group, Un of Alberta, Edmonton, CANADA

- Affymetrix U1333Plus 2.0 microarrays
- Pathogenesis-based transcripts were defined by experimental mouse kidney transplants and in cell cultures
  - Cytotoxic-T cell-associated transcripts (CAT) (n=203)
  - Interferon-gamma and rejection-induced transcripts (GRIT) (n=68)
  - B cell associated transcripts (BAT) (n=146)
  - Immunoglobulin transcripts (IGT) (n=136)
  - Kidney transcripts (n=64)
Diagnosing Rejection in Renal Transplants: A Comparison of Molecular- and Histopathology-Based Approaches

J. Reeve a,b, G. Einecke b,c, M. Mengel a,b, B. Sis a,b, N. Kayser b, B. Kaplan d and P. F. H

Introduction

The transcriptome has considerable potential for improving biopsy diagnoses. However, to realize this potential the relationship between the molecular phenotype of disease and histopathology must be established. We assessed 186 consecutive clinically indicated kidney transplant biopsies using microarrays, and built a classifier to distinguish rejection from nonrejection using predictive analysis of microarrays (PAM). Most genes selected by PAM were interferon-γ-inducible or cytotoxic T-cell associated, for example, CXCL9, CXCL11, GBP1 and INDO. We then compared the PAM diagnoses to those from histopathology, which are based on the Banff diagnostic criteria. Disagreement occurred in approximately 20% of diagnoses, principally because of idiosyncratic limitations in the histopathology scoring system. The problematic diagnosis of ‘borderline rejection’ was resolved by PAM into two distinct classes, rejection and nonrejection. The diagnostic discrepancies between Banff and PAM in these cases were largely due to the Banff system’s requirement for a tubulitis threshold in defining rejection. By examining the discrepancies between gene expression and histopathology, we provide external validation of the main features of the histopathology diagnostic criteria (the Banff consensus system), recommend improvements and outline a pathway for introducing molecular measurements.
Expression of B Cell and Immunoglobulin Transcripts Is a Feature of Inflammation in Late Allografts

G. Einecke, J. Reeve, M. Mengel, B. Sis, S. Bunnag, T. F. Mueller and P. F. Halloran*

Division of Nephrology and Transplantation Immunology, Department of Medicine, University of Alberta, Edmonton, Canada
*Corresponding author: P. F. Halloran, phil.halloran@ualberta.ca

lin genes and B-cell markers was associated with steroid resistance and graft loss (1). Similarly, inferior outcomes were reported for TCMR cases with plasma cell-rich infiltrates (4,5). In protocol biopsies, B-cell-rich infiltrates are associated with interstitial fibrosis and tubular atrophy and are a negative prognostic indicator (6). However, the association of lymphoid clusters or CD20+ B-cell infiltrates with inferior graft survival in biopsies for cause was not confirmed by all studies (7–11). The ambiguous results

Figure 3: Relationship between BAT and IGT scores and the degree of interstitial inflammation and fibrosis. We analyzed the relationship between BAT and IGT transcript levels and histologic scores for interstitial inflammation and interstitial fibrosis. Because BAT and IGT scores are time dependent, the analysis is shown separately for early (<5 months) and late biopsies (≥5 months posttransplant). Interstitial inflammation (i-score) and fibrosis (ci-score) are scored as 0, 1, 2 or 3 according to severity of the lesion; BAT and IGT scores are illustrated for each of these categories. Symbols illustrate BAT or IGT scores in individual biopsies, lines represent the median BAT or IGT score in each category.
Gene expression profiles of 17 protocol kidney biopsies at 6 months after transplantation were studied by microarrays to predict the development of CAN at 12 months. Patients were on RAD B251 study (Certican versus Cellcept with Neoral and prednisone). 9 patients developed CAN at 12 months (All were on Certican). 10 genes might predict the development of chronic rejection (8 upregulated, 2 downregulated)

- Scherer et al. Transplantation 2003; 75: 1323

2 year protocol biopsies of 48 patients enrolled in a randomized study comparing CsA+MMF to Sirolimus+ MMF were studied by microarrays and showed that 379 differentially expressed genes and 97% of the genes were upregulated in the CsA-treated patient biopsies. Comparison of combined Banff 2 and 3 biopsies to Banff 0 biopsies in revealed significant upregulation of genes responsible for immune/inflammation and fibrous/tissue remodeling

- Flechner et al. AJT 2004; 4: 1776
MICROARRAY STUDIES IN CHRONIC ALLOGRAFT INJURY

Gene expression profiles of 11 biopsies with CAN were compared to 7 normal kidneys by microarrays. Genes related to fibrosis, extracellular matrix deposition, and immune responses were found upregulated in CAN.

- **Mas et al. Transplantation 2007; 83: 448**

- 15 uncomplicated living donor kidney transplant biopsies were taken at baseline and 1 year after tx. 8 developed subclinical fibrosis and microarray results showed increased expression of genes related to fibrosis, inflammation, and response to injury in biopsies with subclinical fibrosis.

- **Park et al. Transplantation 2007; 83: 1466**

- 59 protocol biopsies of 18 patients at baseline, and 1, 3, and 12 months after transplantation were studied by cDNA microarrays. Profibrotic genes were expressed before interstitial fibrosis were observed at sequential biopsy.

- **Vitalone et al. Transplantation 2010; 89: 537**
LIMITATIONS OF MICROARRAY STUDIES IN CAN

- Weak overlap exists between gene lists from different studies.
- GeneChip technology has been used in CAN patients in a limited number of studies involving limited number of patients (< 20).
- Difficulty in evaluating vast quantities of data to reach a meaningful conclusion in a limited number of heterogeneous group of patients and immunosuppressive medications for a very heterogeneous disease like CAN.
- Tissue sampling differences, as biopsy samples contain a mixture of different cell and tissue types, involving varying proportions of muscle, capsule, cortex and medulla of the kidney.
- Different microarrays, experimental settings, data analysis, thresholds for data filtering, and statistical analysis.
LIMITATIONS OF MICROARRAY STUDIES

- The data analysis of microarrays is still in its infancy and there is no universally accepted method.
- Too much data? An array of 10,000 elements, even at 95% confidence ($p<0.05$), 500 significant genes may be found purely by chance.
- Gene expression levels do not correlate with protein levels (post-translational modifications can not be measured by microarrays).
- Validation (RT-PCR, immunohistopathology).
A meta-analysis of kidney microarray datasets: investigation of cytokine gene detection and correlation with rt-PCR and detection thresholds

Walter D Park* and Mark D Stegall

Address: Department of Surgery, Mayo Clinic College of Medicine, Rochester, MN, USA
Email: Walter D Park* - park.walter@mayo.edu; Mark D Stegall - stegall.mark@mayo.edu
* Corresponding author

Published: 30 March 2007
Received: 13 November 2006
Accepted: 30 March 2007


Table 2: Cytokine genes examined by microarray and rt-PCR in Mayo Clinic kidney transplant specimens.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Probe Set ID</th>
<th>BLAST of Consensus Probe Sequence OK?</th>
<th>Microarray detection</th>
<th>rt-PCR detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>%P of Mayo samples (n = 30)</td>
<td>Min Detection p-value</td>
</tr>
<tr>
<td>1 TNF-α</td>
<td>207113_s_at</td>
<td>Yes</td>
<td>0.0%</td>
<td>0.0806</td>
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<tr>
<td>2 IFNγ</td>
<td>210354_at</td>
<td>Yes</td>
<td>0.0%</td>
<td>0.0586</td>
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<tr>
<td>3 IL-6</td>
<td>205207_at</td>
<td>Yes</td>
<td>70.0%</td>
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<tr>
<td>4 IL-10</td>
<td>207433_at</td>
<td>Yes</td>
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<td>0.1116</td>
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<tr>
<td>5 TGF-β II</td>
<td>205085_s_at</td>
<td>Yes</td>
<td>0.0%</td>
<td>0.1497</td>
</tr>
<tr>
<td></td>
<td>205084_at</td>
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<tr>
<td>6 FoxP3</td>
<td>224211_at</td>
<td>Yes</td>
<td>0.0%</td>
<td>0.2742</td>
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<tr>
<td></td>
<td>221333_at</td>
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<td></td>
<td>221334_s_at</td>
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<td>7 IFNγ-R1</td>
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<tr>
<td></td>
<td>242903_at</td>
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<td></td>
<td>211676_s_at</td>
<td>Yes</td>
<td>96.7%</td>
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</tbody>
</table>

a Contains probesets for the FoxP3 gene which overlap with the JM2 gene.
b Contains probesets for the IFNγ-Receptor I gene that specifically bind the DNA sequence of the gene.
<table>
<thead>
<tr>
<th>Time point</th>
<th>Transplant Or Biopsy (Day 0)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
<th>24</th>
<th>Unscheduled Visit</th>
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<td>02</td>
<td>03</td>
<td>04</td>
<td>05</td>
<td>06</td>
<td>07</td>
<td>08</td>
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<tr>
<td>Anti-HLA Antibody (Luminex)</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>Non-HLA Antibody</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>Isolation of peripheral blood cells</td>
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<td>X</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
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<td>x</td>
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<tr>
<td>Urine- mRNA Profiling</td>
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<td>X</td>
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<td>Biopsy- Histopathology and Genomics</td>
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<tr>
<td>eGFR (MDRD)</td>
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<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
R/O obstruction or arterial stenosis with kidney Doppler U/S
R/O prerenal factors (dehydration, hypotension, etc.)
R/O high CNI levels
Perform Tx kidney biopsy if 3 factors above are ruled out
Check BKV PCR and Luminex DSA
R/O recurrent or denovo glomerulonephritis
R/O viral nephritis (polyoma, CMV)
Approach to a Patient with Biopsy

Findings of Chronic Allograft Injury

- Polyoma nephropathy (2-5%)
- De novo GN/recurrence (2-5%)
- Chronic Pyelonephritis (1-3%)
- Acute rejection after first year (2-3%)

10-20%
Approach to a Patient with Biopsy Findings of Chronic Allograft Injury

CAAMR →
- TGP/C4d + /DSA + → 10-20%

CACMR →
- CAA (by Banff)
- TGP/ C4d - /DSA - → ?
- IF/TA with infiltrates → ? → 60-70%

CNI toxicity →
- Multinodular hyalinosis → ?
- Stripe fibrosis → ?

IF/TA etiol unclear
6 pediatric patients with CAMR received IVIG 1 g/kg for 4 consecutive weeks, followed by a single dose of Rituximab (375 mg/m2)

4/6 patients had improved or stabilized GFR at 1 year after treatment

Only 1/6 patient lost DSA, 3 persisted, and 2 increased reactivity

- Billing H et al. Transplantation 2008; 86: 1214

4 adult patients with CAMR received IV steroids 500-1,000 mg qd X 3-5 days, Rituximab 375 mg/m2 one dose on day 1, IVIG 400 mg/kg on day 2 and 5

Improved kidney function in all patients and DSA was reduced in 2 patients

One patient had acute rejection 12 months later

One patient had severe Rituximab associated lung toxicity

- Behr T et al. Transplantation 2009 87: 1837
CHRONIC ANTIBODY MEDIATED REJECTION

- Patients immunosuppressive treatment should be tacrolimus and MMF/EC-MPS.
- Tacrolimus dose will be adjusted to keep target levels between 5-7 ng/ml and the dose of MMF/EC-MPS will be kept at 1000/720 mg twice a day and prednisone 5-10 mg once a day.
- Patients with CrCL > 25-30 ml and biopsy results < grade III fibrosis are treated with Rituximab and IVIG.

Treatment protocol:

- Day 0: IVIG 1 gram/kg
- Day 15: Rituximab 375 mg/m²
- Day 30: IVIG 1 gram/kg

Repeat Luminex Single Antigen Beads starting 2 weeks after the second IVIG and once a month for 3 consecutive months along with serum creatinine levels and spot urine protein/creatinine ratio.
CHRONIC CELLULAR REJECTION

- Patients with IF/TA and significant infiltrates at tubulointerstitial or fibrotic area (>25%) and/or transplant glomerulopathy without C4d staining and no DSA, or chronic allograft arteriopathy
- Tacrolimus dose will be adjusted to keep target levels between 5-7 ng/ml
- The dose of MMF/EC-MPS will be kept at 1000/720 mg bid.
- Prednisone 5-10 mg qd. No pulse steroid treatment
Calcineurin Inhibitor Withdrawal Meta-analysis


Difference in Proportion with Acute Rejection

\[ \text{Pooled difference} = 0.11 \]
\[ (0.06-0.66), \ p<0.001 \]
\[ X^2 = 64.9, \ p<0.001 \]
RAPAMYCIN IN CHRONIC ALLOGRAFT NEPHROPATHY

- 59 patients with CAN switched to rapamycin from CNI
- 54% responded and had improved GFR
- Proteinuria over 800 mg/day is an independent variable for not responding to treatment
- Randomized, controlled, single center trial in 38 patients with CAN between 6 months to 8 years after transplantation demonstrated improved GFR in patients switched to rapamycin from CNIs
- No rejection occurred in both groups
- No difference in proteinuria in both groups
CNI TOXICITY:

- Patients with IF/TA and without any significant infiltrates at tubulointerstitial or fibrotic area (<25%) with or without histopathologic findings of arterial hyalinosis and strip fibrosis suggesting CNI toxicity are considered for this group.

- There should not be any findings of acute rejection or transplant glomerulopathy, or C4d positivity. Patients preferably should not have previous history of acute rejection, PRA should be < 10%, and no DSA by Luminex. 24 hour urine protein should be < 500 mg.

- First we minimize the CNI dose by keeping tacrolimus levels 3-5 and cyclosporine levels 70-100 along with full dose MMF/EC-MPS(1000/720).

- If there is no clinical improvement, tacrolimus can be switched to sirolimus treatment. Sirolimus target level is 10-15 ng/ml.