4-1-2016

Advances in allograft monitoring after intestinal transplantation.

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As submitted to:

Current Opinion in Organ Transplantation

ADVANCES IN ALLOGRAFT MONITORING AFTER INTESTINAL TRANSPLANTATION

And later published as:

Volume 21, Issue 2, pages: 165-170

April 2016

DOI: 10.1097/MOT.0000000000000279.

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WORD COUNT: 2500 words -without ABSTRACT and KEY POINTS

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KEYWORDS:

intestinal transplantation, non-invasive graft monitoring, zoom video-endoscopy, DSA

ABSTRACT:

Purpose of Review: The intestinal allograft, with an enormous lymphoid load, is a highly immunogenic organ which elicits a strong alloimmune response. In the early post transplant period, a robust graft biopsy protocol via a temporary ileostomy is utilized for surveillance to detect rejection. In the later post-transplant period, after enteral continuity is re-established, graft biopsies via a colonoscopy become more cumbersome. Alternative methods for intestinal allograft monitoring other than graft biopsy are of particular interest.

Recent Findings: Biomarkers and diagnostic tools such as Granzyme B, Perforin, fecal Calprotectin, Citrulline, donor specific antibody (DSA), and zoom video-endoscopy have all been studied for application as reliable methods of performing intestinal allograft surveillance. Each modality has the capability of monitoring a separate and unique process in the host-allograft immune response.

Summary: The goal to find a reliable , reproducible and non-invasive method for intestinal graft monitoring remains an elusive one. Many of the current modalities available only serve to act as complementary tests in conjunction with astute clinical observations. Graft biopsy remains the gold standard for monitoring the intestinal allograft.

KEY POINTS:

- Long-term outcome after intestinal transplantation is still negatively affected by rejection
- Better graft monitoring , other than biopsy, could reduce late allograft loss
- Video-endoscopy is a reliable diagnostic tool but in expert hands
- Molecules such as Granzyme B and Perforin, Citrulline or Calprotectin so far have shown questionable results in allograft surveillance

- DSAs are promising biomarkers but their management in clinical practice has still to be clarified

INTRODUCTION:

Intestinal transplantation (ITx) has shown steady improvements in graft and patient survival over the past 20 years, particularly with short-term results. Lasting improvements in long-term outcome, however, still remain a challenge. Late allograft loss due to acute rejection (AR) still remains a vexing problem, in particular due to the challenge of diagnosing AR in a stable recipient long removed from an aggressive, biopsy-driven, early post-transplant monitoring protocol. Biopsy is the gold standard for the diagnosis of ITx allograft pathology. Currently we lack a reliable biomarker able to forewarn of rejection episodes: we herein review recently published data of non-invasive allograft monitoring after ITx.

MONITORING OF THE IMMUNE SYSTEM:

In kidney or liver transplant, blood tests such as creatinine level or liver function tests are suitable to determine if there is graft dysfunction: such a simple and reproducible biomarker has yet to be discovered in ITx. Currently, ITx monitoring is based on protocol biopsies. However, this is an invasive procedure which requires a skilled operator as well as an experienced histopathologist. An ideal biomarker should be non-invasive, reproducible, and with a rapid result allowing for the initiation of prompt treatment.

- **Granzyme B and Perforin :**

Similarly to kidney transplantation, the Bologna group **(1-4)** analyzed the blood expression of Granzyme B (GB) and Perforin (PF) after ITx by real time polymerase chain reaction (PCR) on 32 recipients (494 blood samples) during episodes of AR, Epstein-Barr virus (EBV) or cytomegalovirus (CMV) infection, and post-transplant lymphoproliferative disease (PTLD). Both GB and PF are expressed in natural killer cells and cytotoxic T lymphocytes, acting as effector molecules for the induction of apoptosis in target cells. The inability to use the test in

the first 28 days because of variable expression was a limiting factor. Mean levels of GB and PF in the AR (GB=279.7; PF=256.7), PTLD (GB=199; PF=185.9), EBV (GB=133.2; PF=143.7), and CMV (GB=151.3; PF=144) groups were significantly higher than in the normal group (GB=100.1; PF=101.1) (all $P<0.05$, except for PF in CMV infection). Although the best accuracy was obtained for the diagnosis of AR, half of the samples were eliminated due to confounding conditions, limiting the clinical applicability. Sensitivity and specificity were 80% and 79% for GB and 70% and 79% for PF, respectively, and the area under the receiver-operator characteristics (ROC) curve was 0.87 for GB and 0.82 for PF. GB and PF levels rose in only 10 of 30 episodes of rejection from eight patients at a mean of 14 days (range 3–38) prior to AR. Their conclusion was that GB and PF are diagnostic markers of AR even though the levels also increase in case of viral infections or PTLD. GB and PF might act as predictive molecules but should be interpreted in a clinical, histological and virological context. Therefore, their utility is questionable.

- **Fecal Calprotectin:**

Fecal Calprotectin have been widely correlated with inflammatory bowel disease activity, proposing it as potential marker of ITx rejection. In an early Omaha study, fecal Calprotectin levels were measured from ileostomy of 68 recipients with AR (n =12), viral enteritis (n= 5), and nonspecific inflammation (n =16), and compared with 35 normal controls (**5**). During AR episodes, fecal Calprotectin was significantly higher than in viral enteritis or normal controls (198 mg/kg compared with 7 and 19 mg/kg, respectively (P = 0.0002)), and the cut-off level was 92 mg/kg with specificity of 77% and sensitivity of 83%, as suggested by receiver operator characteristics (ROC). In a follow-up study by the same group, published in 2011 **(6-7)**, 732 stool samples were collected from 72 recipients: due to significant inter-patient variability, defining an effective general "cutoff" level was difficult and not clinically applicable. A Miami study **(8)** measured fecal Calprotectin on 29 recipients and a cut-off level of 100 ng/mg was considered positive. Retrospective evaluation of 122 samples revealed non-specific results. The assay was sensitive to the identification of intestinal illnesses in general, but not useful in specifically distinguishing AR from these ongoing organic intestinal problems. Finally a report from Argentina **(9)** showed a good sensitivity but low specificity for the diagnosis of intestinal AR. High Calprotectin levels were also observed in other clinical conditions. In summary, a clear role for Calprotectin as a marker of rejection has yet to be realized.

- **Citrulline:**

Serum Citrulline is a non-protein amino acid deriving from glutamine conversion within enterocytes, studied as a marker of functional enterocyte mass in short bowel syndrome. The Miami group **(10-14)** analyzed 2135 dried blood spots from 57 intestinal transplant recipients at or beyond 3 months post-transplant. Lower Citrulline levels were found in presence of mild, moderate, or severe AR, bacteremia or respiratory infection, in the pediatric age group, and were dependent upon the time from transplant. Using a <13 vs. =/>13 μmoles/L as a cutoff point, levels =/>13 μmoles/L were associated with moderate or severe rejection. They confirmed their data in 2012 **(15)**, concluding that Citrulline in ITx may serve as a non invasive biomarker with excellent negative predictive values in the long term follow-up of pediatric recipients. On the contrary, a New York study **(16-18)** reported that Citrulline reflects the extent of mucosal injury regardless of the etiology, but does not seem to be a predictive marker for rejection or viral enteritis, as its values may decline only when diffuse mucosal damage has occurred. These last results were confirmed by a Madrid group **(19)**: they reported Citrulline as a sensitive and specific biomarker of the residual functional enterocyte load, related to enteric feeding tolerance, but its prognostic value as a rejection marker was questionable. Recently, a Bologna study (**20**) investigated Citrulline according to time from ITx, episodes of AR and creatinine clearance. Twenty-four adult recipients were prospectively studied. The results were compared with those of 19 healthy controls and of 29 patients with chronic renal failure. Citrulline sensitivity and specificity in detecting AR after the 45th post-operative day were 38% and 83% using Citrulline threshold observed in healthy controls, and 69% and 77% using Citrulline threshold adjusted for chronic renal failure degree. Their conclusion was that adjusting Citrulline threshold for chronic renal failure degree almost doubled the sensitivity of Citrulline as a non-invasive marker of AR in ITx. Summarizing the reported studies, Citrulline probably expresses an exclusionary role because its higher levels are unlikely correlated with severe AR, and its major limitation is a lack of forewarning of AR episodes.

- **Donor Specific Antibodies:**

Donor specific antibodies (DSAs) represent the humoral alloimmunity against donor antigens: it has been widely established in other solid organ transplants that DSAs contribute to rejection and late allograft loss. After ITx, pre-transplant (pre-formed) DSAs are present in

nearly one-third of patients and post-transplant (de novo) DSAs develop in up to 40% of recipients. Recent literature shows that pre-formed and/or de novo DSAs are correlated with ITx rejection and allograft loss. A Pittsburgh report in 2000 **(21)** addressed the impact of DSAs, reporting that a positive T-cell lymphocytotoxic cross-match increases the frequency and severity of rejection after ITx, particularly with isolated bowel graft. In 2010, the Los Angeles group **(22)** showed that DSAs were associated with higher allograft loss and mortality while a Miami study **(23)** correlated DSAs with more frequent and severe episodes of rejection (P=0.041). In the Miami report DSAs were pre-formed (n=5, anti-human leukocyte antigen class II=3, anti-class I and II=2), de novo (n=4, 15.25±4.72 days after transplantation, anti-class II=1, and anti-class I and II=3) and never (n=6). Among 63 biopsies, 30 (47.6%) had significant correlations with positive DSA (kappa=0.30, P<0.001) and manifested severe rejection grade (P=0.009). In 2012, a Pittsburgh study **(24)** analyzed 194 primary adult recipients: complement-dependent lymphocytotoxic cross-match (CDC-XM) was positive in 55 (28%). HLA-DSA was detectable before transplant in 49 (31%) recipients with 19 continuing to have circulating antibodies. Another 19 (18%) developed de novo DSAs. Ninety percent of patients with pre-formed DSAs harbored HLA Class-I, whereas 74% of recipients with de novo antibodies had Class-II. Most antibody-mediated rejection (AMR) cases occurred in the first 3 months after transplant and patients with persistent DSAs exhibited the highest risk of AMR. Pre-formed DSAs significantly ($p < 0.05$) increased the risk of AR. Persistent and de novo HLA-DSA significantly ($p < 0.001$) increased risk of chronic rejection and associated graft loss. Inclusion of the liver was a significant predictor of better outcome ($p = 0.004$, HR = 0.347) with significant clearance of pre-formed antibodies ($p = 0.04$, OR = 56) and lower induction of de novo DSAs ($p = 0.07$, OR = 24). The Berlin group **(25)** recently confirmed that the development of de novo DSAs following ITx is often associated with AR, observing that the number of mismatched antigens and epitopes correlates with the probability of developing de novo DSAs. Different results were shown in a 2013 study by the Indianapolis group **(26)**: of 131 intestinal/multivisceral transplants, 27 (21%) had a positive flow cytometry cross-match (FCXM). Positive cross-match was not associated with an increased incidence of AR and graft loss (30% and 37% vs. 29% and 47%; P=0.94 and 0.35, respectively). This effect was maintained in liver-excluding transplants. The authors attributed their positive outcome to the immunosuppressive regimen based on induction by thymoglobulin and rituximab, and maintenance with tacrolimus and prednisone plus monthly doses of anti-interleukin-2 receptor antibody (IL-2RA) for the recipients of liver-free allografts. However, cross-matching patients was based on the less sensitive FCXM assay compared to a Luminex-based assay, thus

may be underestimating the impact of DSAs **(23)**. The virtual cross-match (VXM), in which known recipient HLA antibodies are prospectively compared to donor HLA type, provides one mean to optimize organ allocation and minimize immunologic risk. In fact, the VXM was recently shown **(27)** by the Washington DC group to allow for successful isolated ITx with acceptable short-term outcomes in sensitized patients. A number of papers have been presented at the recent Intestinal Small Bowel Transplant Symposium (ISBTS) held in June 2015 in Argentina. A Los Angeles report **(28)** confirmed that the presence of pre-formed or de novo DSAs leads to inferior survival outcomes following ITx. A study from the Washington DC group **(29)** showed that the development of de novo DSAs occurs at a rapid and high incidence after ITx. Their monitoring included weekly DSA analysis for 2 months, then monthly testing for the remaining year. If de novo DSAs appeared, then weekly monitoring was reinstituted. De novo DSAs developed in 32% recipients at a median time of 22.5 days after transplant. Liver inclusion was not associated with any difference in the development of de novo DSAs (p =0. 21) as confirmed by another paper from the Palo Alto group **(30)**. The Indiana team also performed DSA monitoring and noted that the development of de novo DSA had a trend towards the development of chronic rejection (14% vs 5%, p = 0.21) and graft loss due to AR (18% vs 7%, p= 0.14) **(31).** Boluda et al. from Madrid **(32)** showed that the presence of DSAs is linked to re-transplantation, being unusual in candidates for first transplant. In summary, there is still some debate going on regarding the impact of DSAs and its use as an ITx graft monitoring tool, but a large amount of evidence shows that the presence of de novo or pre-formed DSAs is involved negatively in long-term graft survival. New therapeutic strategies have been developed in recent years **(33-36)** to treat DSAs, but their description is beyond the scope of the present review.

VISUAL MONITORING OF THE GRAFT:

Visual graft surveillance is the easiest one over other forms of monitoring after solid organ transplantation, due to the graft ileostomy manufactured for biopsy. Ideally, endoscopy itself through the ileostomy (or, when the stoma has been taken down, a gastroscopy or colonoscopy) should be able to monitor the graft, avoiding the more invasive biopsy procedure with the possible hazards of mucosal hematomas, bleeding and perforations, especially in small and/or friable grafts. The Pittsburgh group examined **(37)** this issue, documenting that endoscopy alone would have missed 37% of cases of AR in 1273 endoscopies performed in 41 children. In their initial study they used fiber-optic endoscopy,

but 8 years later **(38)**, with the improved magnification and optical resolution, new video endoscopes have shown advantages of modern technology in detecting AR after ITx.

- **Zoom Videoendoscopy:**

In 2006, the Miami group **(38)** analyzed the video-endoscopic diagnosis of rejection, comparing it to histological findings. Adult patients showed a sensitivity of 45%, a specificity of 98%, a positive predictive value of 82%, and a negative predictive value of 88%. The most intriguing aspect of this study was that, on 59 distinct occasions in which the results were endoscopy-negative but biopsy-positive for AR, rejections were not treated on the basis of clinical evaluation and 58 (98%) resolved without further therapy. More recently a Bologna study **(39-40)** evaluated 52 protocol endoscopies over a period of 2 years on 17 adult recipients with more than 5 years follow-up. All the 52 endoscopic findings were comparable to biopsy-definitive results (specificity was 98%): only 1 case of mild enteritis and 1 case of EBV chronic infection at biopsy were not diagnosed by endoscopy, while no episodes of rejection were diagnosed either by biopsy or endoscopy because the study group was represented by stable recipients in long-term follow-up. Conversely, the Pittsburgh group in 2012 **(41)** reported different results, analyzing a number of adult endoscopies performed in 66 asymptomatic ‘‘surveillance’’ recipients and 71 symptomatic recipients. For surveillance patients, 125 ileoscopies were performed to collect 590 biopsies, and for the symptomatic group 229 ileoscopies and jejunoscopies were conducted to obtain 434 biopsies. The sensitivity and specificity of endoscopic visualization in detecting AR was 50% and 91.5% for the surveillance group and 43% and 67% for the symptomatic patients respectively. In surveillance, visual impression alone would have missed three cases of moderate and no cases of severe AR, while in the symptomatic group visual inspection alone would have missed 20 cases of moderate AR. Their conclusion was that in symptomatic patients, visual inspection detected all cases of severe rejection but would have missed patients with early readily treatable rejection, supporting the current clinical practice of ileoscopic biopsy for graft surveillance in asymptomatic patients.

CONCLUSION:

In conclusion, over the last 15 years there have been many attempts to find an alternative to biopsy for prevention and/or diagnosis of AR, in order to improve long-term graft survival after intestinal transplantation (**Table 1**). Serum or fecal biomarkers (Granzyme B and Perforin, Citrulline, Calprotectin) have been analyzed by many groups and, although promising to monitor the immune system, so far they have missed their role substituting the gold standard biopsy. Recently, DSAs have shown a link to graft survival, but their clinical significance in a day-by-day laboratory monitoring is still far from being fully understood. Zoom video-endoscopy has been identified as a useful tool to support biopsy findings, but its exclusionary role as non-invasive marker of the intestinal graft probably requires technology innovations still not available on the scientific market.

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ACKNOWLEDGEMENTS: We wish to thank **Ms. Claudia Cirillo** for her English language editing of our text.

FINANCIAL SUPPORT AND SPONSORSHIP : none

CONFLICTS OF INTEREST: none

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