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Advances in diagnostics for transplant rejection

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Abstract

Introduction—Identification of allograft injury, including acute clinical and subclinical injury, is vital in increasing the longevity of the transplanted organ. Acute rejection, which occurs as a result of a variety of immune and non-immune factors including the infiltration of immune cells and antibodies to the donor specific epitopes, poses a significant risk to the organ. Recent years have marked an increase in the discovery of new genomic, transcriptomic, and proteomic biomarkers in molecular diagnostics, which offer better potential for personalized management of the transplanted organ by providing earlier detection of rejection episodes.

Areas Covered—This review was compiled from key word searches of full-text publications relevant to the field as well as relevant articles from reference lists and review articles.

Expert Commentary—Many of the recent advancements in the molecular diagnostics of allograft injury show much promise, but before they can be fully realized further validation in larger sample sets must be conducted. Additionally, for better informed therapeutic decisions, more work must be completed to differentiate between different causes of injury. Moreover, the diagnostics field is looking at methodologies that allow for multiplexing, the ability to identify multiple targets simultaneously, in order to provide more robust biomarkers and better understanding.

Keywords

molecular diagnostics; acute rejection; graft monitoring; genomics; transcriptomics; proteomics; personalized transplant management; indicators of immune response; organ transplantation

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Declaration of Interest

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1.0 Introduction

Within transplant medicine, the risk of allograft rejection is one of the major hurdles that must be overcome.[1] In recent years, there has been an increase in the survival rates of solid organ transplant recipients due to advancements in organ procurement, surgical methods, and immunosuppressive therapy. Despite this progress, transplanted organs still experience injury caused by immune and non-immune related factors that include rejection due to infiltrating immune cells or antibodies against donor specific epitopes in the transplanted organ, viral infection, and drug toxicity due to the long term use of immunosuppressive drugs. Part, if not the majority of this injury is caused by immune and non-immune related factors and can be avoided by the timely detection of such events in their early stage. Current diagnostic approaches lack the necessary sensitivity and selectivity required to accurately identify these events early enough so that the damage to the transplanted organ or “graft” can be avoided. Microscopic evaluation of biopsies, which is used as the current gold standard method to date, leaves much room for improvement. Current efforts are focused on increasing specificity and sensitivity, and reducing the need for invasive procedures that carry an additional risk of infection and morbidity.[2,3] Therefore, there is a need for more sensitive, noninvasive diagnostics tools or assays that identify allograft health status post-transplant. Such diagnostics may facilitate personalized therapeutic strategies and can be used as adjunct tests with current methods to provide more robust analyses.[4]

Early identification of allograft injury not only allows for timely intervention but can also be used for patient-specific customization of immunosuppressive drugs to optimize graft outcome.[5] Significant innovation in our ability to assess global molecular profiling through different “omics” methods, such as genomics, transcriptomics, proteomics, and metabolomics, has offered new and exciting opportunities for the discovery and validation of potential surrogate biomarkers that could be used towards improved diagnostics. These diagnostics can target biomarkers of biological and more specifically, immunological activity, as upon organ transplantation, the immune response impacts both the humoral and cellular immune system[6]. This results in a cascade of events which manifest in various biomolecules (genes, RNAs, proteins, metabolites, circulating cell free DNA, etc.) that can be captured using state-of-the-art molecular profiling methods available to us today. These potential surrogate biomarkers are the result of undergoing accelerated immune activity that results in perturbation of biological processes resulting in altered levels of nucleotides (DNA, circulating cell free DNA and RNA), gene products (proteins) and metabolites. Understanding the correlation between their presence or lack thereof also offers insight into post-transplant graft monitoring[7]. Early detection of rejection events in conjunction with intervention is therefore the key to the prevention of acute rejection and other subsequent injuries, which can occur within the first few years after transplantation.[8]

1.1 Post-Transplant Immune Response

Within the first few months after organ transplantation, rejection is the most common cause of graft dysfunction (see Figure 1).[9] There are two major immune responses that lead to graft injury, innate alloimmunity including the complement system, and adaptive alloimmunity.[10] Hyperacute rejection occurs within minutes to hours after transplantation

and is primarily due to innate alloimmunity in which preexisting host antibodies activate the complement system and neutrophils are recruited to the graft site. The most common form of rejection, acute rejection, caused by both an innate response and a primary allogeneic response, occurs within the first few days to first 3 months after transplantation.[11,12] Every graft undergoes a degree of ischemic reperfusion injury during transplantation, which activates the innate immune system.[13] This response, including phagocytes, neutrophils, macrophages, dendritic cells, and others, can cause inflammation and oxidative damage. The allogeneic response is mediated by both T cells and antibodies that infiltrate the graft and cause tubulitis and vasculitis, respectively.[6] More specifically, this is mainly driven by the recognition of Major Histocompatibility Complex (MHC) proteins called Human Leukocyte Antigens (HLA) which are expressed on the graft tissue cells.[14,15] MHC molecules can be further classified into either Class I, cell-surface heterodimers, or Class II, MHC-encoded transmembrane proteins. Upon exposure to these foreign antigens in the donor tissue, antigen-specific recipient T cells proliferate and differentiate into effectors (cytotoxic and helper T cells) that eliminate the foreign intruder. Many effector T cells, however, undergo apoptosis as the immune response continues, and the few that survive become memory T cells. [10,11,15,16] The initial response of the effector T cells as well as the memory T cells contribute to B lymphocyte production of antibodies against the foreign tissue.[14,15,17] This can result in allograft injury and loss of function and may lead to acute and chronic rejection, but can also inhibit transplantation tolerance.[11,15] Additionally, innate alloimmunity may cause additional damage. The last form of rejection is chronic rejection, which usually occurs over 90 days post-transplant, and is generally characterized by fibrosis and ischemia.[18–21]

1.2 Post-Transplant Graft Monitoring & Intervention

Generally, immunosuppressive therapies have proven to be very successful at partially or completely reversing these episodes of rejection, aside from cases of hyperacute rejection. Surges, or “pulses,” of high-dose steroids and antilymphocyte reagents have been shown to be effective in reversing the T-cell response, but antilymphocyte antibodies are not as effective with respect to vasculitis.[9] In order to increase the efficacy of such therapies timely intervention is crucial. This can be facilitated through the use of diagnostics that identify early immunological activity and subclinical allograft damage. When considering such biomarkers, one should consider the biomolecules that are critical components of the central dogma of biology. DNA (genome) is transcribed to RNA (transcriptome) and then translated to proteins (proteome).[7] Recent advancements in our ability to comprehensively and accurately quantify different biomolecules in different biospecimens have aided in the identification and validation of potential biomarkers within each of these classes that can be used as indicators of immune response and therefore, graft injury, particularly those injuries pertaining to acute allograft rejection.[22,23]

2.0 Genomic Molecular Diagnostics

DNA acts as the genetic code from which all proteins are derived. The understanding of the human genome has improved significantly in the years since the initiation of the human genome project.[24] Recently, genome-wide association (GWA) studies, which use high

throughput genotyping tools, have been used in the investigation of pathological gene discovery.[25] Moreover, epigenetic alterations to the genome have been shown to include heritable regulation of gene expression that is not directly related to the specific DNA nucleotide sequence. Such molecular modifications to both DNA and chromatin, including the thoroughly studied methylation of DNA, as well as adjustments to the chromatin packaging of DNA by posttranslational histone modifications, have been shown to influence gene expression.[25] Despite recent excitement about our ability and access to whole genome sequencing using next generation sequencing (NGS) in terms of value as a biomarker for graft injury, circulating donor derived cell-free DNA has demonstrated its promise in different organ transplantation.[26,27] As a result, within recent years, the field of molecular diagnostics has seen an increased interest in the use of circulating cell free DNA (cfDNA) as an indicator of cell damage and subsequent lysis. This phenomenon is deemed to be the result of cytotoxic immune responses. With respect to allograft monitoring, donor-derived cell-free DNA (ddcfDNA) has been shown to be measurable in both the blood and urine of transplant recipients.[28,29] This offers the advantage of being a direct indicator of allograft injury, though it lacks the specificity of the cause of said injury. In female recipients that received a solid organ from a male donor, initial thought was to focus on the Y chromosome as an identifier of ddcfDNA.[30,31] *Sigdel et al.* analyzed urinary ddcfDNA as an indicator of kidney transplant injury.[30] In the study, biopsy-matched urine samples from female renal transplant recipients of male donor kidneys were analyzed for chromosome Y. The levels of ddcfDNA were compared to biopsies that were semi-quantitatively scored by a single pathologist. The average urinary ddcfDNA in AR was significantly greater compared to stable recipients or those with chronic allograft injury but no different from recipients with BK virus nephropathy. The group concluded that continuous monitoring of urinary ddcfDNA could be used as a sensitive biomarker of acute injury in the donor organ. This was limited to those that demonstrated the sex-mismatch for ChrY, where the donor was male and the recipient female. The benefit of utilizing urine as the biofluid for analysis offers a low-cost and minimally invasive method of sample collection.

More recently, *De Vlamincx et al.* developed a method called “genome transplant dynamics” (GTD) that can identify ddcfDNA regardless of sex taking advantage of single-nucleotide polymorphisms (SNPs) distributed across the genome to discriminate donor and recipient DNA molecules.[5] If SNPs occur in the coding region of a gene, they could potentially impact the structure of the mature protein. Even in noncoding regions, such polymorphisms could also influence gene splicing, transcription and translation.[25] They demonstrated a correlation between increased levels of ddcfDNA and acute rejection in a retrospective study of patients with cardiac transplants. The group then conducted a prospective cohort study that tested the utility of ddcfDNA in measuring acute rejection after heart transplantation.[5] They purified and sequenced circulating cell-free DNA from plasma samples collected post-transplant. GTD was shown to have better test characteristics when compared to the AlloMap test, a commercially available gene expression assay for the noninvasive monitoring of rejection after heart transplantation. It is important to note that the performance of the GTD assay depended on multiple variables, including the time after

transplant and the age of the patient. Despite this, the data represented potential for early diagnosis of acute rejection, up to 5 months before detection by biopsy.[5]

In lung transplant recipients, *De Vlamincx et al.* showed that ddcfDNA could be applied as a marker of graft injury to improve upon the performance of transbronchial biopsy, the current gold standard.[27] They accounted for differences in transplant tissue mass between bilateral and single-lung transplants and examined ddcfDNA levels in the plasma of lung transplant recipients diagnosed with acute or chronic rejection. The group found that ddcfDNA was noticeably elevated in samples from patients that presented with rejection. This approach has also been investigated for use in those with liver transplants and shows promise. *Macher et al.* identified the SRY gene on ddcfDNA as a means of quantifying rejection and assessing the health status of liver transplant recipients.[32] Table 1 summarizes the aforementioned post-transplantation genomic biomarkers of acute rejection.

3.0 Transcriptomic Molecular Diagnostics

In addition to the use of DNA as a genetic biomarker, recent investigations into RNA have proven to be fruitful. RNA is the resultant molecule of DNA transcription, as a response to external and internal stimuli which, with or without post-transcriptional modification, can code for proteins. Identifying transcripts as biomarkers of immune response or biological response to allograft injury is a method of identifying allograft injury early, without necessitating significant graft loss prior to identification. Moreover, identifying pathogenesis-based transcripts, which focuses on targets of pathophysiologic processes, not just immune responses or cellular damage, offers an opportunity for diagnostic analyses. For example, antibody-mediated rejection commonly leads to a cluster of genes upregulated in endothelial cells, monocyte, and natural killer cell transcripts. That being said, significant portions of the human genome are transcribed into RNA transcripts without protein-coding potential.[33] On the basis of their size, these so-called non-coding RNAs (ncRNAs) are categorized into long and small subsets. *Lorenzen et al.* have found that long noncoding RNAs (lncRNAs) can be used as indicators of acute rejection of renal transplants.[34] LncRNAs are intracellular noncoding ribonucleotides that assist in the regulation of the genome and proteome. They can be detected in the blood of patients with acute kidney injury. The group tested whether lncRNAs are present in urine and can serve as a new diagnostic of AR. In a validated cohort of patients, compared with controls, RP11-395P13.3-001 and RP11-354P17.15-001 were found to be up-regulated in those presenting with AR. Additionally, they found that lncRNAs are strongly changed in the urine of patients with AR. Therefore, the group concluded that urinary RP11-354P17.15-001 may serve as a novel biomarker of AR in kidney transplant recipients.

After post-transcriptional modification, the template for protein translation is mRNA. The expression of mRNA, as investigated by *Alakulppi et al.*, has been identified as an early indicator of immunological response in renal transplant recipients.[35] Whole blood samples were collected and the mRNA expression of lymphocyte cytotoxic molecules (granzyme B, perforin, FasL, and granulysin) as well as costimulatory molecules associated with T-cell activation (CD154, ICOS, CTLA4 and PD-) were analyzed with real-time quantitative polymerase chain reaction. According to their results, significantly lower levels of ICOS and

CD154 were expressed in rejection patients compared to controls. Additionally, when rejection patients were contrasted to patients biopsied due to other reasons of graft dysfunction, CD154 levels were found to be lower. The other genes in this study did not offer consistent trends. As a result, the group concluded that whole blood gene expression quantities of costimulatory molecules CD154 and ICOS could reasonably robustly differentiate rejection patients from control patients, the caveat being that the biomarker analysis was not able to differentiate patients with rejection from those with graft dysfunction due to other causes. This highlights one major issue with using mRNA as a biomarker for injury, mRNA analysis provides a snapshot of relative transcript levels, but provides no information on protein levels or what cells are producing the signal.

Another gene of interest, T-cell immunoglobulin mucin domain 3 (TIM-3), was investigated by *Luo et al.* in patients with renal transplants.[36] Donor-specific antigen-triggered T-cells play vital roles in AR.[37,38] Recently, pro-inflammatory TH17 and TH1 cells have been demonstrated to participate in alloimmune reactions.[39,40] TIM-3 is an identifier of TH1 cells as well as other primary T-effector cells, such as cytotoxic T-lymphocyte (CTL), natural killer (NK), and TH17.[41,42] Graft tolerance has been shown to improve with its negative regulation and with regulation of T-helper cell immune responses.[43,44] Therefore, the group concluded that TIM-3 could reflect the immunologic status of an allograft. Using RT-PCR, the group monitored gene expression changes among peripheral blood leukocytes (PBL). They showed that TIM-3 mRNA in PBL exhibited significantly higher expression in AR compared to no AR and stable cohorts. Moreover, the group found that antirejection therapy decreased TIM-3 mRNA expression in all AR patients, which reduced the utility for predicting the prognosis of AR after antirejection treatment due to the great variations of TIM-3 mRNA expression.

A significant number of studies have been performed to assess the gene transcription profile of biospecimens (blood or a biopsy from graft) that have provided a number of surrogate biomarkers as well as revealed underlying molecular mechanisms pertaining to AR.[45–50] In a recent study by *Roedder et al.* the team developed the kSORT module and have demonstrated that a multiple target transcriptomics approach can add increased specificity.[8] The group used logistic regression modeling for ten genes found to be expressed as mRNA (DUSP1, CFLAR, ITGAX, NAMPT, MAPK9, RNF130, IFNGR1, PSEN1, RYBP, NKTR) and were able to accurately predict which samples were from patients that suffered from rejection. In order to better differentiate between AR and no-AR samples, the group used multiple statistical modeling approaches and seven other genes (CEACAM4, EPOR, GZMK, RARA, RHEB, RXRA, SLC25A37) were added to the kSORT module for a total 17 genes. kSORT performance was shown to be independent of time post-transplantation and recipient donor age through multivariate analyses of the sample cohorts. Additionally, the group demonstrated that out of the tested variables, only the kSORT score, the predicted probability of AR, significantly impacted the outcome for AR compared to No-AR.

Khatiri et al. found that irrespective of the source of the tissue sample, there was a recurrent transcriptional response in AR, where 11 genes were overexpressed during allograft rejection.[51] They defined this set of genes (BASP1, CD6, CXCL10, CXCL9, INPP5D, ISG20, LCK, NKG7, PSMB9, RUNX3, and TAP1) as a common rejection module (CRM).

The CRM genes were shown to correlate with the severity of graft injury and had the potential to predict future injury to a graft using protocol biopsies. Based on these findings, *Sigdel et al.* then used qPCR to determine a method of using the CRM genes to quantify transplant injury in AR and predict risk of progressive interstitial fibrosis and tubular atrophy (IFTA). The CRM score (tCRM) was higher in AR samples compared to stable samples. CXCL9 and CXCL10 in AR showed the most promise as indicators of AR and CD6, CXCL9, and LCK were promising in predicting pIFTA.[52] This approach is an example of genome-wide gene expression data mining to evaluate graft immune inflammation. The tCRM score proved to quantify injury in AR and was able to differentiate patients at increased risk of future pIFTA prior to hindered function or histological indication.

Unlike lncRNAs and mRNAs, microRNAs are a class of non-coding RNAs that play key roles in the regulation of gene expression. They are short, single-stranded RNA molecules. *Farid et al.* have found that both miR-122 and miR-148a are upregulated in recipients experiencing hepatic acute allograft rejection.[53] In blood samples from renal allograft recipients, *Anglicheau et al.* used a TaqMan low-density array human microRNA panel that contained 365 mature human miRNAs and identified miR-142-5p, -155, and -223 as being over-expressed in patients with acute rejection.[54] Table 2 provides an overview of the recent advancements in transcriptomics as related to monitoring of acute rejection post-transplantation of solid-organs.

4.0 Proteomic Analysis Methods

Several approaches of protein measurement and profiling exist. We describe the three most popular approaches here.

(1) Shotgun Proteomics based on liquid chromatography mass spectrometry (LC-MS)

The most popular approach is shotgun proteomics that takes advantage of ability of analyzing peptides as a result of proteolytic cleavage and back tracking source of such peptides to build a complete protein list identified in a biosample. LC-MS leverages the separation capabilities of liquid chromatography (LC) with the ability to perform mass analysis by mass spectrometry (MS). Variations of LC-MS exist including bottom-up proteomics LC-MS where the samples are first digested with protease followed by denaturation prior to LC-MS. One caveat of the bottom-up approach is that it relies on the assumption that proteins are completely digested into peptides that can be detected in a reliable and reproducible manner.[55,56] Additionally, quantification of a specific protein can only be achieved if the peptides are proteolytic peptides, uniquely derived from a particular protein. [56] In order to provide better resolution between peptides of different mass, better separation techniques are required. Moreover, LC-MS can be performed in conjunction with MS (LC-MS/MS) to perform analysis of complex samples where peptide masses may overlap.[57]

(2) CE-MS Based Proteomics

Capillary Electrophoresis Mass Spectrometry (CE-MS) combines the ability of capillary electrophoresis (CE) to provide high separation efficiency with MS. It can be performed at high speeds and requires a small volume of sample. [58] The primary ionization method of CE-MS is electron spray ionization but matrix-assisted laser desorption/ionization has also been used. [59] Combining CE and MS relies on interfaces, which often include supplemental fluids, to facilitate the efficient transfer of analytes from the electrophoretic capillary to the mass spectrometer without reducing separation efficiency. [59] One major caveat of CE-MS is that supplemental fluids can reduce sensitivity. The development of electron-spray ionization interfaces that do not require supplemental fluids works to increase sensitivity.

(3) SELDI Based Proteomics

A variation of matrix-assisted laser desorption/ionization (MALDI) is surface-enhanced laser desorption/ionization (SELDI) which is a soft ionization method in MS. Unlike in MALDI where the sample is mixed with a matrix material and applied to metal plate before irradiation, in SELDI, proteins of interest become bound to a surface and can be used to detect proteins in tissue, blood, urine, and other samples. After ionization, time-of-flight (TOF) MS is primarily used to identify the mass-to-charge ratio and velocity of each ion that is created from the proteins of interest. A major advantage of the SELDI process is a chromatographic separation step. Separation in SELDI is based on retention, therefore any sample impurities that interfere with analytical measurements, such as salts, detergents, and buffers, are removed before analysis with MS. Only the proteins that are bound to the surface are analyzed, which increases the sensitivity of analysis by increasing the probability of detecting analytes of lower concentrations. This platform has revolutionized our ability to identify and quantify proteins and peptides to better understand pathobiological processes and potential biomarkers.

5.0 Proteomic Molecular Diagnostics

Proteins are produced due to a wide variety of immune responses. Long term immune activation is characterized by the prevalence of memory T cells and donor-specific antibodies (DSA). These can be anti-HLA (human leukocyte antigen) and non-HLA (nHLA).[60] According to *Sidgel et al.* there has been a shift of focus on detecting HLA class II antibodies in blood to urine-based protein detection.[61] Within renal transplants, there are twelve proteins that were up-regulated in AR, nine of which are highly specific for AR because of their significant differences from all other transplant categories. Using an independent sample set, fibrinogen beta (FGB), fibrinogen gamma (FGG), and HLA DRB1 were identified to be present in increased levels in AR through ELISA. Adding increased specificity, the fibrinogen proteins further differentiated AR from BK virus nephritis (FGB, FGG). The group validated four proteins due to their relevance in AR: FGB, FGG, small ubiquitin-related modifier 2 (SUMO2), and MHCII protein (HLADRB1). They used commercially available ELISA assays and an independent urine sample set to validate the significance of three proteins, FGB, FGG, and HLADRB1. A recent comprehensive study by *Sidgel et al.* used unbiased proteomics (iTRAQ and label-free LC-MS) on urine samples

collected from 396 kidney transplant patients and reported a panel of 35 proteins to segregate AR, CAN and BKVN from stable graft.[62]

Non-HLA antibodies (nHLA antibodies) that target donor (non-HLA) antigens are reported to be factors in transplant injury. A study by *Terasaki et al.*[63] among recipient of cadaveric organ 38% graft loss were related to non-HLA factors whereas only 18% were attributed to HLA related factors and 43% to non-immunologic factors. Recently, there has been increased interest in investigation of the role of nHLA antibodies in organ transplantation. It is not always easy to explain the association of nHLA antibodies with different diseases, however they may serve as excellent surrogate biomarkers for transplant injury and rejection. A number of studies have reported an association of different nHLA antibodies in different injury type including acute rejection.[64] More recently, nHLA antibodies have also shown to be involved in hyperacute rejection, most of which are proposed to target the vascular endothelium and which are called anti-endothelial cell antibodies (AECAs).[64] In one study, *Jackson et al.* looked at living-donor kidney transplant patients, and through flow cytometry and solid-phase bead immunoassays on donor-derived endothelial cell precursors (ECPs) found that a cohort of the patients tested positive for donor-reactive IgG AECAs. This finding correlated with a higher incidence of cellular rejection during the early post-transplant period.[65] Similar findings were found in heart transplant recipients by *Ismail et al.* Additionally, other nHLA antibodies, MHC class I-related chain A and B (MICA and MICB) have also been proposed to be associated with AR, as studied by *Narayan et al.* in a case study of a highly sensitized renal transplant recipients that demonstrated that donor-specific anti-MICA antibodies may be associated with acute AMR.[66] Further studies must be conducted to better understand the specific correlation between these classes of proteins and the severity of AR. Besides MICA and AECAs, additional nHLA proteins have been investigated. Notable proteins include protein kinase C, found to be associated with renal transplant AR by *Sutherland et al.*[64] In lung transplantation, the role of IgM antibodies to nHLAs has been assessed by *Smith et al.* in hyperacute rejection and AR.[64] *Sumitran-Holgersson et al.* studied the correlation between liver sinusoidal endothelial cells (LSECs) and rejection.[64] Lastly, in BMT, the role of PSMA4 in rejection and tolerance was investigated by *Porcheray et al.*[64] The largest challenge presented by nHLAs is that many of the responses in AR could be patient-specific making it difficult to discover a common list of pathogenic nHLA antibodies without using a large cohort of patients.[67]

Besides HLA and nHLA antibodies, other proteins have been investigated as markers of acute rejection. Studies have shown that high pre- or post-transplant serum levels of soluble CD30 (sCD30) may be associated with AR.[68,69] *Grenzi et al.* investigated whether post-transplant sCD30 levels are associated with subclinical rejection, whether CD30+ cells were present within the graft, and measured the expression of immune response genes in peripheral blood mononuclear cells. *Grenzi et al. and Amirzargar et al.* showed that high sCD30 levels are independent predictors of graft dysfunction and may contribute to patient selection protocols by indicating the state of activation of the immune system. [70,71] Moreover, *Grenzi et al.* noted a correlation between sCD30 levels and CD30 gene expression in peripheral blood mononuclear cells. According to *Germani et al.* studies have identified certain antibodies expressed as immunological responses can also be indicators of inflammation.[72] The expression of CD28 and CD38 was analyzed on CD3+, CD4+ and

CD8+ cells in liver transplanted patients in one study that showed that the mean frequencies of CD28 and CD38-expressing T cells were higher in patients with AR.

Another biomarker recently studied in kidney transplantation is neutrophil gelatinase associated lipocalin (NGAL).[73] NGAL is secreted by renal epithelial cells, neutrophils, and renal proximal tubules associated with renal immunological conditions. *Mamatov et al.* aimed to investigate the ability of quantifying NGAL to predict rejection in donor-specific antibody (DSA)-positive and DSA-negative patients. They found that an increase in NGAL level does not always correlate with renal injury because NGAL is also produced in other circumstances. Therefore, NGAL cannot be used alone to diagnose rejection, but it can be used as an initial warning signal. If the NGAL level is high, subsequent study of DSA and sub-clinical rejection should be performed.

In heart transplantation, *Ahn et al.* developed a high-sensitivity assay for cardiac troponin I (hs-cTnI) which they claim can predict acute rejection episodes after transplant when compared with grade of rejection in endomyocardial biopsy.[74] Cardiac troponin I is a sensitive and specific indicator of damage to the heart muscle (myocardium). *Ahn et al.* performed a multivariable mixed model analysis in relation to the presence of acute rejection within two months after transplant and noted that hs-cTnI levels were significantly higher in AR. The group concluded that an increased hs-cTnI ratio index was significantly related to rejection episodes. Serial monitoring of hs-cTnI and comparing it to baseline values without rejection may provide an opportunity for diagnostic use post heart transplant.[75–79] *Ahn* also demonstrated that it was possible to detect more distinct differences between normal and pathologic samples using the Abbott Laboratories' ARCHITECT STAT high-sensitivity cTnI assay. This suggests that biomarkers previously identified as not demonstrating significant differences in levels between pathologic and healthy patient populations could be revisited utilizing more sensitive instruments which may be able to establish distinctions between patient populations.

Chemokines and cytokines are released in conjunction with the formation of antibodies and the activation of the complement system. Activated T cells produce and release cytokines and chemokines which, in immune cell recruitment and can function as pro-inflammatory molecules. Chemokine and cytokine genetic polymorphisms influence their protein levels and hence their signaling effects.[80] *Hricik et al.* showed that in renal allografts, urinary CXCR3 chemokines (i.e. CXCL9 and CXCL10) are amongst the most promising markers to help detect subclinical inflammation; increasing up to a month prior to AR identification through biopsy.[81] They have also been shown to decrease in response to anti-rejection therapy and could have prognostic value for development of allograft dysfunction.[81,82] The urinary CXCR3 chemokines can be measured by ELISA, which are commonly found in clinical laboratories. *Huang et al.* and *Hu et al.* suggested that the detection of the ligands to the CXCR3 chemokines (i.e. MIG and IP-10) may be more readily measured and thus constitute a better method for diagnosing AR [83,84] *Huang et al.* found that serum levels of MIG and IP-10 were much higher in patients with T cell-mediated acute rejection compared with stable patients.[83] *Hu et al.* found that urinary IP-10 and MIG were significantly elevated in samples collected from recipients with AR.[84]

Lastly, particular cytokines have been proven to have an important role in graft transplantation. *De Serres et al.* studied six cytokines (IL-1 β , IL-6, TNF- α , IL-4, GM-CSF, and monocyte chemoattractant protein-1) that had been suggested to be indicative of AR in renal transplant recipients. Through logistic regression modeling, the group identified that a single cytokine, IL-6, proved to be the most accurate predictor. [85] Other cytokines including IL-9, IL-23 and IL-17 still need further investigation but show promise.[86,87] Table 3 provides an overview of the recent advancements in proteomics as related to monitoring of acute rejection post-transplantation of solid-organs.

6.0 Key Search Terms

We searched PubMed and Google Scholar using the following terms: “molecular diagnostics”; “organ transplantation”; “graft monitoring”; “organ transplant monitoring”; “biomarkers for organ transplant monitoring”; “biomarkers for graft monitoring”; “molecular diagnostics for graft injury”; “indicators of graft injury”; “indicators of organ transplant injury”; “indicators of immune response in graft organ”; “indicators of immune response in organ transplantation”; “personalized post-transplant care”; “personalized management of organ transplant”; “personalized management of graft organ”; “DNA for monitoring graft injury”; “DNA for monitoring organ transplantation”; “RNA for monitoring graft injury”; “RNA for monitoring organ transplantation”; “Proteomics for monitoring graft injury”; “Proteomics for monitoring organ transplantation”; “Cytokines for monitoring graft injury”; “Cytokines for monitoring organ transplantation.” Full-text publications relevant to the field were selected and relevant articles from reference lists and relevant review articles were also included.

7.0 Expert Commentary

There is a need for effective and reliable tools/assays to identify subclinical injury and acute rejection in solid organ transplantation to facilitate timely intervention. Research in recent years has highlighted an emphasis on pursuing noninvasive methods of monitoring graft function, particularly through the assessment of various biofluids (human urine, serum, plasma) as well as biomarkers from different stages of genetic relevance, see Figure 2. The genome offers the potential due to its relative stability and improvements in PCR and microarray technologies. Unfortunately, much of the work that has been completed has focused on the products of tissue damage rather than upstream of that approach. Nucleases and proteases offer challenges for transcriptome and proteome analysis but these biomarkers also offer great potential due to their direct relationship with immune responses. Within the realm of purely genomic identifiers, beyond genome-wide analysis, more targeted approaches have yielded a potential for early indicators of AR. Circulating donor-derived cell-free DNA offer a promising indicator of cellular damage. Unfortunately, this alone does not give insight into the cause of such damage, but can be used in conjunction with other diagnostic methodologies for more robust analyses. Within transcriptomics, value has been demonstrated in quantifying indicators of immunological responses which could in-turn, as with proteomics, provide for early detection of alloreactive states.

Different specimens have their own significance in carrying potential biomarkers. A tissue specimen from the transplanted organ is probably the most informative bio-specimen to capture biological events which are occurring at the time of sampling. However, compared to urine for kidney transplantation, bronchoalveolar lavage (BAL) for lung transplantation, and blood samples for all solid organ transplants, sampling of tissue is an invasive procedure associated with complications. Though it is reasonable to use biopsies collected for pathological evaluations for biomarker discovery processes, the use of biofluids (urine, serum, or plasma), BAL, and circulating blood cells (PBMCs or whole blood) offer better options in terms of the convenience of sampling. In addition, analysis of PBMCs provides an opportunity to look into biomarkers that are enriched in specific cells. A method to analyze immune cell subsets in complex tissues has also been reported. [88] The choice also depends on the biomarkers of interest. For example, serum and plasma samples are the best specimen for assaying antibodies. [89] Circulating-cell free DNA, which are an indication of cell death/apoptosis, are best assessed in the serum/plasma or in the urine. [26,90] Biomarkers that are associated with infiltrating cells are best assessed in the cells that are derived from the graft. Such cells are either analyzed in the graft or pelleted from urine and used for gene expression analysis to identify potential biomarkers. [52,91]

Two general approaches of biomarker assessment exist. In the first approach, an unbiased high throughput assay is used to profile biomolecules (gene, proteins, antibodies, metabolites) on a global scale. This unbiased discovery, if done with sufficient sample size, is powerful in that it helps identify molecules that were not previously reported, but since no literature exists in interpreting such observations many more subsequent studies are necessary to test the hypotheses generated from the initial study. The weakness of such unbiased high throughput studies is that if not followed up, the observations will never be validated or result in any meaningful conclusions. By contrast, a second approach focuses on indicators of pathophysiology. In this approach, molecules that are implicated in certain pathophysiology are targeted and evaluated for their value as surrogate biomarkers. The study of donor-derived cfDNA in the blood of heart transplant patients and the analysis of endothelial cell antibodies as surrogate biomarkers of increased rejection are such examples where the analysis is focused on the biological realm relevant to the pathophysiology rather than a comprehensive look into the global changes at the molecular level. [26,67]

There is much potential in the discoveries as of late, but before they can be fully realized, further validation in larger samples sets must be conducted. Additionally, though much work has been completed to identify molecular indicators of transplant organ injury, further work must be completed in order to differentiate between different causes of injury to better inform therapeutic decisions.

8.0 Five Year View

The fast pace of technological advancements to assist with molecular profiling methods and rapidly growing publicly available datasets will help validate true biomarkers for AR in organ transplantation. The clinical use of such biomarkers in diagnosis or acute rejection and the monitoring of graft status will help customize immunosuppressive therapy and interventions. This has the potential to improve survival rates of the transplanted organ. The

use of such sensitive biomarkers will put less burden on the patients in terms of pain and cost of healthcare after organ transplantation. In recent years we have seen advances in the fields of genomics, transcriptomics, and proteomics. There has been a marked increase in research within transcriptomics and groups are looking to further understand gene expression profiles as potential indicators of immunological responses. Moreover, research in the coming years will focus heavily on highly sensitive methods of detection such that significant differences in levels of biomarkers can be used for diagnostic measures. Additionally, research has demonstrated that looking at multiple biomarkers as diagnostic arrays provides better insight into diagnoses, as demonstrated by the predictive value of kSORT and CRM modules discussed in this review. Advances in high throughput urine proteomics and high throughput PCR can facilitate noninvasive methods of multiplexing and processing multiple gene targets simultaneously. Such advancements will allow for earlier detection of potential rejection episodes and thus studies involving subsequent early intervention with targeted therapies will need to be performed to determine the impact on allograft outcomes.

9.0 Key Issues

- There is a need for sensitive indicators of subclinical immune injury in order to better inform therapeutic treatments to increase solid organ transplant efficacy.
- Advancements in genomics, particular in both areas of epigenetic alterations, genome-wide association studies, as well as particular biomarkers such as cell free DNA have proven to be ripe for potential cultivation as indicators of increased risk for rejection.
- Using GTD one can identify ddcfDNA through the analysis of SNPs on the target molecules.
- Within transcriptomics, both noncoding and coding transcripts have been shown to correlate with activated immune response, this includes lncRNAs, mRNA, miRNA.
- Genetic expression of multiple targets, such as in the CRM and kSORT models, in induced immunological states can provide specificity when performing multivariate analyses of graft injury.
- A wide variety of proteomic targets can stratify different stages of immune response, or particular immunological domains.
- Newly identified relationships between allograft injury and the levels of chemokines, cytokines, HLA class II proteins, and nHLA related proteins in various biofluids offer noninvasive methods of immune analysis.
- The major hurdle that must be overcome to improve molecular diagnostics for use in personalized medicine is to determine methods of differentiating between different causes of injury to better inform therapeutic decisions.

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Reference annotations

* Of interest

** Of considerable interest

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Figure 1.

Timeline of rejection event. Hyperacute, acute, and chronic rejection are classified by the type of injury exhibited. Each form is characterized by different immune activity. During Hyperacute Rejection (HR), normally occurring minutes to hours post-transplant, neutrophil recruitment and preexisting host antibodies activate the complement system of the immune system. During Acute Rejection (AR), normally occurring 6–90 days post-transplant, T cells have differentiated due to the activation of the cell-mediated immune response. Activated helper T cells signal B cells to produce antibodies against HLA of the graft. The T-cells cause the graft cells to lyse or produce cytokines that recruit other inflammatory cells, which may induce cytotoxicity and apoptosis, eventually causing necrosis of allograft tissue. In Chronic Rejection (CR), normally occurring after 90 days post-transplant, proliferation of smooth muscle cells and production of collagen by fibroblasts cause accelerated graft arteriosclerosis and results in fibrosis which can cause ischemia and cell death. Throughout these immune processes, there are biomarkers that can be found in three realms: the genome, the transcriptome, and the proteome.

Figure 2.

Overview of recent advancements in molecular diagnostics of acute rejection. Each realm of biology, (Epi-)Genomics, transcriptomics, and proteomics offer unique biomarkers of immunological activity that can be correlated to the acute rejection of allografts.

Table 1

Post-Transplantation DNA Biomarkers of Acute Rejection

Organ	Sample	Biomarker	Methodology	References
Kidney	Urine	Donor-derived cell free DNA (ddcfDNA)	qPCR	<i>Sigdel, et al.</i> [30]
Heart, Lung	Blood (plasma)	Donor-derived cell free DNA (ddcfDNA)	qPCR	<i>De Vlamincx et al.</i> [5,27]

Table 2

Post-Transplantation Transcriptomics Biomarkers of Acute Rejection

Organ	Sample	Biomarker	Methodology	References
Kidney	Urine	Long noncoding RNAs (lncRNAs): RP11-354P17.15-001	RT-qPCR	<i>Lorenzen et al.</i> [34]
Heart, Lung	Whole Blood	mRNA: CD154 and ICOS	RT-qPCR	<i>Alakulppi et al.</i> [35]
Kidney	Peripheral Blood	mRNA: TIM-3	RT-PCR	<i>Luo et al.</i> [36]
Kidney	Blood	17 genes: CFLAR, DUSP1, IFNGR1, ITGAX, MAPK9, NAMPT, NKTR, PSEN1, RNF130, RYBP, CEACAM4, EPOR, GZMK, RARA, RHEB, RXRA, SLC25A37	qPCR	<i>Roedder et al.</i> [8]
Kidney, Heart, Lung, Liver	Biopsy Tissue	11 genes: BASP1, CD6, CXCL10, CXCL9, INPP5D, ISG20, LCK, NKG7, PSMB9, RUNX3, and TAP1	qPCR	<i>Khatri et al.</i> [51]
Liver	Blood (Plasma)	miR-122 and miR-148a	Taqman-qPCR	<i>Farid et al.</i> [53]
Kidney	Blood	miR-142-5p, -155, and -223	Taqman-qPCR	<i>Anglicheau et al.</i> [54]

Table 3

Post-Transplantation Protein & Cytokine Biomarkers of Acute Rejection

Organ	Sample	Biomarker	Methodology	References
Kidney	Urine	HLA Class II Proteins: FGB, FGG, HLA DRB1	ELISA	<i>Sigdel, et al.</i> [61]
Kidney	Blood	nHLAs: AECAs, MICA, and MICB	Flow Cytometry and Immunoassays	<i>Jackson et al.</i> [65] <i>Narayan et al.</i> [66]
Kidney	Blood	CD30, sCD30	ELISA	<i>Amirzargar et al.</i> [70] <i>Grenzi we al.</i> [71]
Kidney	Peripheral Blood	Neutrophil Gelatinase Associated Lipocalin (NGAL)	ELISA	<i>Mamatov et al.</i> [73]
Heart	Blood	Cardiac Troponin I (cTnI)	High Sensitivity Assay	<i>Ahn et al.</i> [74]
Kidney	Urine	CXCR3: CXCL9 and CXCL10; IP-10, MIG	ELISA	<i>Hricik et al.</i> [81] <i>Huang et al.</i> [83] <i>Hu et al.</i> [84]
Kidney	Blood (plasma)	IL-6	ELISA	<i>De Serres et</i> [85]