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REVIEW

# Biomarkers in renal transplantation: An updated review

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## Abstract

Genomics, proteomics and molecular biology lead to tremendous advances in all fields of medical sciences. Among these the finding of biomarkers as non invasive indicators of biologic processes represents a useful tool in the field of transplantation. In addition to define the principal characteristics of the biomarkers, this review will examine the biomarker usefulness in the different clinical phases following renal transplantation. Biomarkers of ischemia-reperfusion injury and of delayed graft function are extremely important for an early diagnosis of these complications and for optimizing the treatment. Biomarkers predicting or diagnosing acute rejection either cell-mediated or antibody-mediated allow a risk stratification of the recipient, a prompt diagnosis in an early phase when the histology is still unremarkable. The kidney solid organ response test detects renal transplant recipients at high risk for acute rejection with a very high sensitivity and is also able to make diagnosis of subclinical acute rejection. Other biomarkers are able to detect chronic allograft dysfunction in an early phase and to differentiate the true chronic rejection from other forms of chronic allograft nephropathies no immune related. Finally biomarkers recently discovered identify patients tolerant or almost tolerant. This fact allows to safely reduce or withdrawn the immunosuppressive therapy.

**Key words:** Renal transplantation; Biomarkers; Genomic; Proteomics; Transplant outcome; Molecular signatures



**Core tip:** The uses of biomarkers as a non invasive tool instead of renal biopsy in diagnosing transplant renal complications are entering the clinical practice. Progress in genomics, proteomics and all the "omics" fields has allowed the finding of robust, predictive and useful biomarkers. They are modifying our window on transplantation and are allowing us to predict the renal injury earlier because the pathologic process is evident at molecular level before its histological or clinical manifestations. The future is exciting because new international researches and trials are ongoing in this field.

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## INTRODUCTION

Kidney transplantation represents the optimal therapeutic tool for patients affected by end-stage renal disease (ESRD). Improvements in immunosuppressive therapy have resulted in a decrease in acute rejections (AR) and have significantly increased graft short-term half life[1]. However, late kidney graft loss remains a major problem and challenge in kidney transplantation[2]. To date, renal function after transplantation is primarily evaluated by serum creatinine measurement and core renal biopsy. The latter is considered the gold standard in transplant evaluation. Nonetheless, both approaches have several drawbacks. Serum creatinine levels increase late in injury and are non-specific for the type of injury. Additionally, the serum level of creatinine is not able to predict or evaluate the progression of chronic injury and as a consequence is not specific or predictive. Similarly, renal core biopsy cannot be used to monitor the progression of injury because it is invasive and cannot be performed serially. Additionally, there are problems and possible biases in evaluating the specimen and the procedure is not completely free of complications. Moreover, the predictive power of renal core biopsy is poor. In fact, in the National Institutes of Health (NIH) clinical trial "Steroid-Free *vs* Steroids-based Immunosuppression in pediatric renal transplantation" (SNSO1) protocol, renal biopsies were unable to measure "hidden" tissue injury in clinically stable patients[3,4]. In addition, using protocol biopsies, Naesens *et al*[5] reported that examination of tissue at the molecular level is able to reveal abnormalities in innate and adoptive immune responses long before those abnormalities appear at the histological level. Clearly, the development of noninvasive reliable and predictive biomarkers for early diagnosis and monitoring of any clinical condition after kidney transplantation is essential for tailored and individualized treatment[6-8].

In studying the entire transplantation process, biological markers may be used throughout all phases, starting from the donor and donor kidney retrieval. In this phase, biomarkers may be useful for predicting short-term outcomes, and the incidence and severity of delayed graft function (DGF).

The most studied and used biomarkers are those related to the diagnosis and the identification of different aspects of subacute and acute kidney rejection. In addition, biomarkers able to differentiate true chronic rejection (CR), which is immunologically mediated, from the so-called "chronic allograft dysfunction" (CAD), are important because the treatments are different. Indeed, recently, mining the human urine proteome for monitoring renal transplant injury, Sigdel *et al*[9] found urinary peptides specific for AR, urinary peptides specific for chronic allograft nephropathy (CAN) and urinary peptides specific for BK virus nephropathy (BKVN).

Finally, relevant markers are those associated with tolerance, as these markers might allow for decreasing immunosuppressive treatment, withdrawing or discontinuing any immunosuppressant and monitoring the effects of such measures.

In this review, we describe the principal characteristics of current biomarkers, their power and limitation, the principal sources and their relevance in different clinical settings post renal transplantation.

## RESEARCH METHODOLOGY

For this review, we have analyzed the available papers on biomarkers in renal transplantation. A literature search was performed using PubMed (NCBI/NIH) with the search words renal transplantation, biomarkers, genomic, proteomics, transplant outcome, molecular signatures. Firstly, papers published in the last three years were examined, then we proceeded in a backward way and also studies published previously have been included. Studies currently under way were searched for in "clinical trial.gov" and the European EUDRACT register. Only randomized clinical trials (RCTs) active and enrolling patients have been selected.

## DEFINITION AND PRINCIPAL CHARACTERISTICS OF THE BIOLOGICAL MARKERS

In addition to clinical markers and pathological markers, monitoring of the outcome of a clinical process may be performed using biological markers (biomarkers). A NIH working group recommended the following terms and definitions[10]: A biomarker is a characteristic that is objectively measured and evaluated as an indicator of a normal biological process, pathogenic process or pharmacological response to a therapeutic intervention.

Principal applications of biomarkers are as follows: (1) diagnosis or identification of patients affected by a disease or an abnormal condition; (2) staging of the severity or extent of a disease; (3) prognosis of a disease; and (4) prediction and monitoring of a clinical response to an intervention.

Table 1 clarifies both the definition and the principal characteristics of the biomarkers and the technologies involved[11]. A variety of innovative technologies, ranging from genomics, proteomics, peptidomics, antibodyomics, microbiomics and metabolomics, among others, all referred to as "omics", have emerged in medical fields, to generate new biomarkers[12].

Genomics refers to the study of the genome, and epigenomics is the study of the complete set of epigenetic modifications of the genetic materials of a cell. Transcriptomics is the study of the set of all messenger

**Table 1 Definition and principal characteristics of biomarkers**

| | |
|---|---|
| Biomarker | A characteristic objectively measured as an indicator of a biological process or a response to a pharmacological intervention |
| Proteomics | The systematic analysis of proteins for their identity, quantity and function |
| Genomics | The study of the genome for estimating the risk for an individual to develop a disease |
| Transcriptomics | The study of expression patterns of all gene transcript |
| Metabolomics | The quantitative analysis of all the metabolites of a specific biological sample |

**Table 2 Biomarker candidates in the context of ischemia reperfusion injury and delayed graft function**

| Symbol | Gene description | Cytoband |
|---|---|---|
| *ACTA2* | Actin, alpha 2, smooth muscle, aorta | *10q23.31* |
| *UMOD* | Uromodulin | *16p12.3* |
| *LGALS3* | Lectin, galactoside-binding, soluble, 3 | *14q22.3* |
| *SAT1* | Spermidine/spermine N1-acetyltransferase 1 | *Xp22.11* |
| *HAVCR1* | Hepatitis A virus cellular receptor 1 | *5q33.3* |
| *CXCL1* | Chemokine (C-X-C motif) ligand 1 | *4q13.3* |
| *ANXA2* | Annexin A2 | *15q22.2* |
| *S100A6* | S100 calcium binding protein A6 | *1q21.3* |
| *CYR61* | Cysteine rich angiogenic inducer 61 | *1p22.3* |
| *S100B* | S100 calcium binding protein B | *21q22.3* |
| *AMBP* | Alpha-1-microglobulin/bikunin precursor | *9q32* |
| *LCN2* | Lipocalin 2 | *9q34.11* |
| *C3* | Complement component 3 | *19p13.3* |
| *FABP1* | Fatty acid binding protein 1, liver | *2p11.2* |
| *ATF3* | Activating transcription factor 3 | *1q32.3* |
| *NTN1* | Netrin 1 | *17p13.1* |
| *ENG* | Endoglin | *9q34.11* |
| *GUCY2G* | Guanylate cyclase 2G | *10q25.2* |
| *BID* | BH3 interacting domain death agonist | *22q11.21* |
| *BCL2* | B-Cell CLL/lymphoma 2 | *18q21.33* |
| *BAX* | BCL2 associated X protein | *19q13.33* |
| *PTGS2* | Prostaglandin-endoperoxide synthase 2 | *1q31.1* |
| *ADAMTS1* | ADAM metallopeptidase with thrombospondin type 1 motif 1 | *21q21.3* |
| *CDKN1A* | Cyclin dependent kinase inhibitor 1A | *6p21.2* |

RNA molecules in a population of cells, whereas proteomics is the systematic analysis of proteins with regard to their identity, quantity and function. Metabolomics is the study of all chemical processes involving metabolites.

Overall, the principal characteristics, challenges and limitations of the biomarkers applied in renal transplantation are as follows: (1) Sensitivity, specificity, positive and negative predictive values and receiver operating characteristics curves (ROC) of biomarkers are essential for assessing their clinical utility; (2) noninvasive candidate biomarkers principally include mRNA transcripts, lymphocyte phenotype markers, chemokines, microRNA (miRNA) and donor-specific antibodies; (3) robust validation studies and assay standardization are needed to identify new biomarkers; and (4) biomarker validations is challenging because of interindividual variations as well as interlaboratory and interplatform variability[13-15].

The main sources of biomarkers in renal transplantation are serum, urine, peripheral blood lymphocytes and tissue.

## BIOMARKERS OF ISCHEMIA-REPERFUSION SYNDROME AND DGF

Ischemia reperfusion injury (IRI) is an unavoidable step occurring after kidney transplantation and may influence both short-term and long-term graft outcomes. Clinically, IRI may be associated with delayed DGF, graft rejection, CR and CAD[16]. The degree of IRI is related to several factors that may occur in the donor, during organ storage and in the recipient[17]. The increasing use of extended criteria donors and the use of organs recovered from non-heart-beating donors (NHBDs) represent an increased risk of severe IRI. Clearly, understanding the factors that potentially lead to severe IRI allow for stratifying the risk to the recipients and for a prompt diagnosis of IRI, enabling the adoption of possible therapeutic measures of prevention and treatment. Identification of biomarkers for IRI may assist in this effort.

Table 2 report a number of biomarkers candidates within the context of IRI and DGF. Such biomarkers have been studied pre or post-transplantation[18].

### *Pre-transplant biomarkers for IRI and DGF*

A number of molecules expressing tubular or vascular damage in the donor organ are associated with the incidence and severity of IRI. In turn, the severity of IRI conditions the incidence of DGF[19,20] and graft survival is strictly related to the incidence of DGF[21].

**Proteomic studies:** Holmen *et al*[22] documented the predictive value of urinary neutrophil gelatinase-associated lipocalin (uNGAL) levels for prolonged DGF. This finding has been confirmed by a study of Reese *et al*[23]. A predictive value of donor uNGAL, urinary kidney injury molecule 1 (uKIM-1) and urinary fatty acid protein binding protein (u-FABP) for DGF was recently documented by a study of Koo *et al*[24].

Other studies documented the association of recipient pretransplant levels of different cytokines as the soluble interleukin 6 receptor (sIL-6R)[25] and the low soluble gp130 with post-transplant DGF.

Recently, Nguyen *et al*[26] measuring tumour necrosis factor receptor 2 (TNFR-2) expressed on circulating T reg cells documented that recipient peripheral blood T reg is a pre-transplant predictor of DGF.

**Genomic studies:** Several studies have investigated the pre-transplant up-regulation of genes possibly associated with IRI and DGF. One of the main limitations in identifying these molecules as a real marker of inflammation and a potential therapeutic target is the lack of causal proof.

In two different studies Schwartz *et al*[27,28] documented that the expression of tubular epithelial cell adhesion molecules was predictive of post-transplant DGF and, similarly, that the lack of up regulation of anti apoptotic genes as B cell lymphoma 2 (*Bcl-2*) and B cell lymphoma extralarge (*Bcl-xl*) in donor kidneys was associated with DGF. More recently, Kaminska *et al*[29] studying the pre-transplant intragraft expression of 29 genes, found that lipocalin-2 (*LCN)* or *NGAL* were related to DGF.

Hauser *et al*[30] and Kainz *et al*[31] studied the expression of 48 genes associated with DGF in pretransplant biopsies and found an up-regulation of genes related to complement and to metabolic and immune pathways. More recently McGuinnes *et al*[32] found that an elevated expression of cyclin-dependent kinase inhibitor 2A (CDKN2A*)* correlated with high DGF incidence.

A recent trial was conducted (ISRCTN78828338) to verify whether steroid pretreatment of the deceased organ donor was able to reduce the incidence of IRI and DGF.

Genomic analysis showed suppressed inflammation and immune response in kidney biopsies from deceased donors who received corticosteroids. Among the proteins encoded by these identified genes, steroids significantly reduced FK506-binding protein 5 (*FKBP5*), ring finger protein 186 (*RNF186*), TSC22 domain family member 3 (*TSC22D3*), Phospholambam (*PLN*), Solute carrier family 25, member 45 (*SLC25A45*), Small G protein signaling modulator 3 (*SGM3*) and Sushi domain-containing protein 3 (*SUSD3*). However, two studies related to the trial[33,34] concluded that such inflammation suppression did not reduce the incidence or duration of post-transplant DGF in allograft recipients; taken together, the studies documented that steroid pretreatment of organ donors did not improve outcomes after kidney or liver transplantation.

### *Post-transplant biomarkers for IRI and DGF*

**Proteomic and genomic studies:** Liangos *et al*[35] conducted a study on patients affected by DGF and documented an association between KIM 1 levels and disease severity.

Several studies have examined the utility of determining serum or urinary levels of NGAL in predicting DGF after renal transplantation.

Experimental and clinical models have documented that urinary biomarkers such as uNGAL, uKIM-1, uIL-18 and u-FABP are specific for acute kidney injury (AKI) and/or IRI[36,37]. Several recipient urinary biomarkers are also reported to be related to graft dysfunction[38-42].

More recently, two studies documented that urinary clusterin and IL-18 allow predicting DGF within 4 h after transplantation[43]. Similarly, NGAL reflects the entity of renal impairment, representing a useful biomarker and an independent risk factor not only for DGF but also for long-term graft dysfunction[44].

A study by Hall *et al*[45,46] showed by multivariate analysis that elevated urinary levels of NGAL or IL-18 were able to predict DGF, with a ROC of 0.82.

Other studies[47,48] documented that high urinary levels of NGAL soon after transplantation are found in patients with AKI, in particular when AKI is due to AR. In a more recent meta-analysis involving 16500 critically ill patients or following cardiac surgery, elevated plasma or urinary levels of NGAL were associated with AKI but not related to rejection[49]. Finally, in a recent review[50], high urinary or serum NGAL levels were found to serve as a predictor of DGF and were associated with reduced graft function at 1 year.

To date several studies have investigated the role of miRNAs as biomarkers of DGF. miRNAs, short endogenous non-coding RNAs that inhibit gene expression, play a fundamental role in DNA and protein biosynthesis. Some studies found that miRNAs contribute to both the induction and progression of chronic kidney disease (CKD)[51]. miRNAs also represent novel therapeutic targets for CKD and for various complications after renal transplantation[52]. A role in the pathogenesis of post-transplant DGF was found for 2 miRNAs: miR-182-5p and mi-21-3p[53]. The same author found high levels of secretory leukocyte peptidase inhibitor (SLPI) in serum and urine proteome of patients affected by AKI post-transplantation as well as a novel miRNA, miR-182-5p[53].

In summary, miRNAs have a potential role as new biomarkers in all phases of kidney transplantation, even though most of the studies concerning IRI thus far have been conducted on mice[54].

Overall the use of biomarkers, though relevant, has several limitations in the field of IRI. First most studies have been conducted on mice, and their translation to humans is questionable. Second, a proof of cause is lacking, and the only study performed with regard to reducing markers of inflammation failed to report a reduction in IRI incidence and severity. Third, a gold standard for comparison, such as renal biopsy, is lacking.

## BIOMARKERS FOR ACUTE REJECTION

For acute rejection also pretransplant biomarkers have been described.

### *Pre-transplant biomarkers for acute rejection*

The most investigated pre-transplant serum biomarker has been the soluble form of CD30 (sCD30). sCD30 is a glycoprotein expressed on human $CD4^+CD8^+$ T cells that secretes Th2-type cytokines[55]. sCD30 reflects those recipients who will generate an alloimmune response against a grafted kidney. Weimer *et al*[56] documented that sCD30 was a predictor of a poor graft outcome. Other studies highlighted that more often such poor outcome was related to a higher incidence of AR[57-61].

Other studies[62,63] found that recipients with increased levels of C-X-C motif chemokine ligand 10 (CXCL10), an interferon induced chemokine associated with Th1 immune response have higher incidence transplant failure due to a higher AR incidence. Similar findings have been reported for C-X-C motif chemokine ligand 9 (CXCL9)[64].

Using systematic application of interferon-gamma (IFN-gamma) enzyme linked immunospot (ELISPOT) assay, different studies documented that the pretransplant frequency of donor specific IFN-gamma-producing cells correlates with AR among recipients of cadaveric kidney allograft[65-68].

### *Post-transplant biomarkers for acute rejection*

Based on the studies of Naesens *et al*[5] and Sigdel *et al*[9], including genomic and proteomic studies, there are two important points concerning acute and CR, both from genomic and proteomic studies. First, genomic studies have confirmed that smoldering tissue immune activation increases over-time after transplantation and drives progressive CAN independently from AR episodes. Second, the same genomic studies reported that molecular injury in CAN and AR is similar. There is a "so-called" threshold effect for AR, and in the clinical phase of AR, the molecular injury is the same as that found in CAN, though at a higher level. These results were confirmed by urinary proteomic studies. It is therefore important to determine a sensitive and robust biomarker for differentiating AR from other forms of CAD.

Several unbiased plasma and urine proteomic studies have revealed molecules associated with AR. Cohen Freue *et al*[69] found that 7 proteins were up-regulated in the plasma of patients with acute rejection, including connectin (TTN), lipopolysaccharide-binding protein (LBP), peptidase inhibitor 16 (PI16), complement factor D (CFD), mannose-binding lectin (MBL2), recombinant SERPINA10 protein (SERPINA 10) and beta 2 microglobulin (B2M). Using urine samples, Sigdel[70] found proteins related to major histocompatibility complex (MHC) antigens and the complement cascade. Proteins such as uromodulin, serpin peptidase inhibitor, clade F member 1 (SERPINF1) and CD44 were further validated by enzyme-linked immunosorbent assay (ELISA) and Wu *et al*[71] reported 66 proteins in plasma associated with AR, including nuclear factor kappa B (NF-κB), signal transducer and activator of transcription 1 (STAT1) and STAT3. In addition, Loftheim *et al*[72] reported growth-related proteins as Insulin-like growth factor-binding protein (IGFBP7), Vasorin, epidermal growth factor (EGF) and Galactin-3 binding protein (Gal-3BP) to be up-regulated in urine during AR.

Finally, in a recent study, Sigdel *et al*[73] identified and validated by ELISA three urine proteins: Fibrinogen beta (FGB), fibrinogen gamma (FGG) and HLADRB1 during AR. Proteins related to BKVN and CAN were also identified in the same study. All these studies are listed in Table 3.

Other selected studies of biomarkers specific for AR were recently reported by Lo *et al*[7]. Granzyme B (GZMB), perforin (PRF1) and Fas Ligand (FASLG) mRNA are elevated in peripheral blood and tissue[74]. GZMB and PRF1 mRNA are also elevated in the urine of patients with AR[75]. By investigating mRNAs in urinary cells, elevated levels of gene signature of tumor necrosis factor (TNF) receptor superfamily member 4 (*TNFRSF4),* TNF ligand superfamily member 4 (*TNFSF4),* and programmed cell death protein 1 (*PDCD1*) were found in another study[76]. The multicenter CTOT 04 trial reported a urinary three- gene signature of 18S ribosomal RNA of CD3ε mRNA, interferon inducible protein 10 (CXCL10) mRNA and 18S rRNA in patients with biopsy-confirmed acute cellular rejection[77]. CTOT-01 study[78] also revealed elevated levels of urinary CXCL9 mRNA as the best predictor of AR and the authors of this study[78] concluded that low urinary CXCL9 could be used as a biomarker to identify transplant recipients at low risk for immunological events[79]. The findings of the CTOT-01 study represent important news in the field of biomarkers and immunological events in transplantation. Nonetheless, the following open questions remain: (1) whether urinary CXCL9 can be used to decrease indication rates for performing renal biopsy; (2) whether CXCL9 is an adequate tool to distinguish between rejection and injury not immunologically related; and (3) whether the absence of urinary CXCL9 might help to identify the subset of patients whose immunosuppression may be reduced without risks. In a Canadian study[80], the urinary CXCR3 chemokine receptor was shown to be the most promising candidate for detecting subclinical inflammation. This receptor decreases after successful treatment and has a predictive value for detecting subsequent CAN.

In a recent review of urine proteomics[81] , several urine biomarkers were correlated with allograft injury, including CXCL9, CXCL10, C-C motif chemokine ligand 2 (CCL2), NGAL, IL-18, cystatin C, KIM1, T-cell immunoglobulin and mucine domains-containing protein 3 (TIM3). The review also highlighted the aforementioned findings of the CTOT-01 study[78]. In a very recent study[82], four new proteins were found to be related to AR: Alpha-1-antitrypsin (A1AT), alpha 2 antiplasmin (A2AP), serum amyloid A (SAA) and apolipoprotein CIII (APOC3).

miRNAs play critical roles in the modulation of innate and adaptive immune responses. Sui *et al*[83] found 20

**Table 3 Unbiased proteomic studies for acute rejection**

| Ref. | Biomarker candidate | Sample type | Sample numbers | Outcome |
|---|---|---|---|---|
| Freue *et al*[69] | TTN, LBP, CFD, MBL2, SERPINA10, AFM, KNG1, LCAT, SHBG | Plasma | 32 | AR |
| Sigdel *et al*[70] | UMOD, PEDF, CD44 | Urine | 60 | AR |
| Wu *et al*[71] | NF-κB, STAT1, STAT3 and 63 other proteins | Plasma | 13 | AR |
| Loftheim *et al*[72] | IGFBP7, VASN, EGF, LG3BP | Urine | 12 | AR |
| Sigdel *et al*[73] | HLA-DRB1, FGB, FGA, KRT14, HIST1H4B, ACTB, KRT7, DPP4 | Urine | 154 | AR |

AR: Acute rejection; TTN: Titin; LBP: Lipid binding protein; MBL2: Mannose binding lectin 2; SERPINA 10: Protein Z-dependent protease inhibitor; AFM: Atomic force microscopy; KNG1: Kininogen1 protein; LCAT: Lecithin–cholesterol acyltransferase; SHBG: Sex hormon binding protein; UMOD: Uromodulin; PEDF: Pigment epithelium derived factor; NFκB: Nuclear factor kappa B; STAT1: Signal transducer and activator of transcription 1; STAT3: Signal transducer and activator of transcription 3; IGFBP7: Insulin like growth factor binding protein 7; VASN: Vasorin; EGF: Epidermal growth factor; LG3BP: Galectin-3-binding protein; FGB: Fibrinogen beta chain precursor; FGA: Fibrinogen alpha chain precursor; KRT14: Keratin14; HIST1H4B: Histone cluster 1 H4 family member b; ACTB: Actin beta; KRT7: Keratin 7; DPP4: Dipeptidil-peptidasi 4.

**Table 4 Selected promising molecules and pathways evaluated as biomarkers in acute rejection[7]**

| Biomarker | Sample (assay method) | Patients/ samples | Rejection/no rejection | Sensitivity/ specificity (%) | PPV/NPV(%) | AUC |
|---|---|---|---|---|---|---|
| Granzyme B, perforin and FasL[74] | PBL (PCR) | 25/31 | 11/20 | 100/95 | 100/95 | NA |
| FOXP3[88] | PBL, urine (PCR) | 65/78 | 20/58 | 94-100/ 95/100 | 94-100/ 95/100 | 0.95-1.00 |
| Granzyme B, perforin[75] | Urine (PCR) | 85/151 | 24/127 | 79-83/77-83 | NA | NA |
| OX40, OX40L, PD-1 and FOXP3[76] | Urine (PCR) | 46/46 | 21/25 | 95/92 | NA | 0.98 |
| CD3ε,CXCL10, 18S rRNA[77] | Urine (PCR) | 485/4300 | 43/1,70 | 79/78 (71/72) | NA | 0.85 (0.74) |
| TIM-3[81] | PBL, urine (PCR) | 115/160 | 65/95 | 87-100/95-100 | 87-100/93-100 | 0.96-1.00 |
| CXCL9, CXCL10[78] | Urine (multiplex bead assay) | 156/156 | 25/131 | 80-86/76-80 | NA | 0.83-0.87 |
| CXCL9 mRNA and protein[79] | PBL, urine (PCR, ELISA, SELDI-TOF-MS | 280/2770 | 37/113 | 66.7-85.2/ 79.6/80.7 | 61.5/67.6/83-92 | 0.78-0.85 |
| miR-142-5p<br>miR-155<br>miR-223[83] | Biopsy sample (PCR) | 32/33 | 12/21 | 92-100/90-95 | NA | 0.96-0.99 |
| miR-210[85] | Urine (PCR) | 81/88 | 68/20 | 52/74 | NA | 0.7 |
| IFNγ-producing memory T cells[89] | PBL (ELISPOT) | 23/23 | 12/10 | 80/83 | NA | 0.8 |

All the studies include a validation set. PPV: Positive predictive value; NPV: Negative predictive value; AUC: Area under the curve; PBL: Peripheral blood lymphocytes; PCR: Polymerase chain reaction; NA: Not available; PD-1: Programmed death 1; CXCL10: Interferon-inducible cytokine IP-10; 18S rRNA: 18S ribosomal RNA; TIM-3: T-cell immunoglobulin and mucin-domain containing-3; CXCL9: C-X-C motif chemokine 9; ELISA: Enzyme-linked immunosorbent assay; SELDI-TOF-MS: Surface-enhanced laser desorption/ionization time-of-flight MS; miRNA: microRNA; IFNγ: Interferon gamma; ELISPOT: Enzyme-linked immunoSpot.

miRNAs in AR samples, 8 of which were up-regulated and 12 down-regulated. These findings were confirmed in another study by Anglicheau *et al*[84]. Lorenzen *et al*[85] demonstrated a specific role for urinary miR-210, decreasing during AR but normalizing after successful treatment.

Studies of miRNA in peripheral blood cells (PBCs) are also emerging. For example, Betts *et al*[86] in a small study found miR-223 and miRNA 10a to be significantly reduced during AR. In another study Grigoryev *et al*[87] found that inhibition of miR-155 and miR-221 is associated with T cell proliferation, whereas miR-142-3p is associated with tolerant kidney allograft recipients.

Other studies have documented that the level of forkhead box P3 (FOXP3) mRNA in urinary cells is higher in patients with biopsy-confirmed AR[88]. In the same study, the association between low FOXP3 mRNA and high serum creatinine predicted a poor allograft outcome.

T lymphocytes are also being studied as markers of AR. ELISPOT is the best tool for evaluating T lymphocyte phenotypes, and more reliable results are obtained by studies detecting the quantity of IFNγ-producing T cells after stimulation with donor antigens[89]. The Reprogramming the Immune System for Establishment of Tolerance (RISET) consortium has also demonstrated the value of the IFNγ assay[90]. All these studies are reported in Table 4.

Finally, donor-derived cell-free DNA (ddcfDNA) may be detected in the recipient's blood and urine[91]. Indeed, García Moreira *et al*[92] documented an increase in ddcfDNA during AR.

However, the specificity of this finding is questionable because Sigdel *et al*[93] found that ddcfDNA in urine was also present in AR and in BKVN. Additionally, urinary ddcfDNA may be present in cases of pyelonephritis[94]. Thus, the usefulness of ddcfDNA in detecting AR remains questionable.

**Genomic studies for acute rejection:** With the evolution of array technologies, new insight is surfacing and

**Table 5 Seventeen genes involved in the study kidney solid organ response test**

| Symbol | Gene name | Cytoband |
|---|---|---|
| Genes derived from the NIH SNSO1 study | | |
| *DUSP1* | Dual-specificity phosphatase 1 | *5q35.1* |
| *NAMPT* | Nicotinamide phosphoribosyltransferase | *7q22.3* |
| *PSEN1* | Presenilin 1 | *14q24.2* |
| *MAPK9* | Mitogen-activated protein kinase 9 | *5q35.3* |
| *NKTR* | Natural killer cell triggering receptor | *3p22.1* |
| *CFLAR* | CASP8 and FADD like apoptosis regulator gene | *2q33.1* |
| *IFNGR1* | Ligand binding chain of the gamma interferon receptor gene | *6q23.3* |
| *ITGAX* | Integrin alphaXchain protein encoding gene | *16p11.2* |
| *RNF130* | Ring finger motif encoding gene | *5q35.3* |
| *RYBP* | RING1 and YY1 binding protein encoding gene | *3p13* |
| Genes added to improve the accuracy of kSORT | | |
| *CEACAM4* | Carcinoembryonic antigen related cell adhesion molecule 4 | *19q13.2* |
| *EPOR* | Erythropoietin receptor encoding gene | *19p13.2* |
| *GZMK* | Granzyme K encoding gene | *5q11.2* |
| *RARA* | Retinoic acid receptor encoding gene | *17q21.2* |
| *RHEB* | Ras homolog enriched in brain encoding gene | *7q36.1* |
| *RXRA* | Retinoic X receptor alpha encoding gene | *9q34.2* |
| *SLC25A37* | Solute carrier family 25 number 37 encoding gene | *8p21.2* |

The 17 gene set was selected in 143 samples for acute rejection classification and predicted AR up to 3 mo prior to detection by the current gold standard (biopsy). kSORT: Kidney solid organ response test; SNSO1: Steroid-Free *vs* Steroid-Based Immunosuppression in Pediatric Renal (Kidney) Transplantation.

genomic studies are being applied to detect AR[95].

In the CTOT-04 study, Suthanthiran *et al*[77] found an AR diagnostic three gene signature: CD3ε, IP-10 and 185r RNAs[78].

Flechner *et al*[96] in a small study reported that several genes in peripheral blood lymphocytes (PBLs) and in kidney biopsies are able to characterize patients with AR. These genes are related to immune inflammation, transcription factors, cell growth and DNA metabolism.

The NIH SNSO1 randomized study collected human blood and graft biopsies from 367 patients from 12 United States pediatric transplant programs. The genes revealed by microarray were subsequently validated by quantitative polymerase chain reaction (qPCR). A five-gene set [dual specifity phosphatase 1 (*DUSP1*), nicotinamide phosphoribosyltransferase (*PBEF1*), presenil 1 gene (*PSEN1*), mitogen-activated protein kinase 9 gene (*MAPK9*) and natural killer cell-triggering receptor gene (*NKTR*)] was able to identify patients affected by AR with high accuracy (ROC AUC = 0.955), though the addition of five other genes known to be involved in AR did not improve the accuracy[97,98]. Kurian *et al*[99] reported 200 genes possibly related to AR, with ROC values ranging from 76% to 95%. However, the number of patients enrolled was rather small, and the findings need to be verified.

The assessment of AR in renal transplantation (the AART study) involved 436 adult/pediatric renal transplant patients from eight transplant centers in the United States, Spain and Mexico, and the kidney solid organ response test (kSORT) was used to detect renal transplant patients at high risk for AR in the AART study[100]. A 43 rejection-gene set related to AR was identified by genome microarray analysis of biopsies and blood from patients enrolled in the study[97,101].

Ten of these genes were also found in the NIH SNSO1 study[97]. Utilizing different statistical methods for improve accuracy in diagnosing AR, seven additional genes were added in the kSORT study. All these genes are shown in Table 5.

The kSORT results using a 17-gene set had very high sensitivity (AUC = 0.944), and these results were validated in several ways, such as in adult *vs* pediatric recipients, in samples collected from different sites and in samples across different ages and settings.

Overall, kSORT performance was similar among different cohorts (training set, validation set, cross-validation set (Table 6).

kSORT was also able to predict subclinical acute rejection (scAR) alone or in combination with the IFNγ ELISPOT. In the evaluation of subclinical acute rejection prediction study (ESCAPE)[102], both techniques were applied in renal transplant patients with protocol biopsies at 6 mo. The kSORT assay documented high accuracy in predicting both sub clinical antibody-mediated rejection (scABMR) and sub clinical T cell-mediated rejection (scTCMR). ELISPOT was also predictive for scTCMR but less specific in diagnosing scABMR. The predictive probabilities for diagnosing both scABMR and scTCMR were higher when combining the assays, with an AUC > 0.85.

A different approach for identifying acute rejection genes is to employ meta-analysis of eight independent datasets from four different organs (heart, kidney, liver and lung allograft), and a common rejection module (CRM) consisting of 11 genes significantly over-expressed in AR was thus identified[103]. These genes are presented in Table 7.

**Table 6 Performance of kidney solid organ response test in the acute rejection in renal transplantation AART143, AART124, and AART100 cohorts**

| | kSORT predictions | | | | | |
|---|---|---|---|---|---|---|
| | AART143 (training set) | | AART124 (validation set) | | AART100 (cross-validation set) | |
| | AR | No AR | AR | No AR | AR | No AR |
| Real results | | | | | | 3 |
| AR | 39 | 8 | 21 | 2 | 36 | 43 |
| No AR | 9 | 87 | 1 | 100 | 3 | |
| Sensitivity (95%CI) | 82.98% (69.19%-92.35%) | | 91.30% (71.96%-98.38%) | | 92.31% (79.13%-98.38%) | |
| Specificity (95%CI) | 90.63% (82.95%-95.62%) | | 99.01% (94.61%-99.97%) | | 93.48% (82.1%-96.63%) | |
| PPV (95%CI) | 81.25% (68.06%-89.81%) | | 95.46% (78.20%-99.19%) | | 93.21% (79.68%-97.35%) | |
| NPV (95%CI) | 91.58% (84.25%-95.67%) | | 98.04% (93.13%-99.46%) | | 93.48% (82.45%-97.76%) | |
| AUC (95%CI) | 0.94 (0.91-0.98) | | 0.95 (0.88-1.00) | | 0.92 (0.86-0.98) | |

kSORT: Kidney solid organ response test; AART: Assessment of acute rejection in renal transplantation; AR: Acute rejection; PPV: Positive predictive value; NPV: Negative predictive value; AUC: Area under the curve.

**Table 7 Eleven genes overexpressed in the common rejection module[103]**

| Symbol | Gene name | Cytoband |
|---|---|---|
| *BASP1* | Brain abundant membrane attached signal protein 1 | *5p15.1* |
| *CD6* | CD6 molecule | *11q12.2* |
| *CXCL10* | C-X-C Motif chemokine ligand 10 | *4q21.1* |
| *CXCL9* | C-X-C Motif chemokine ligand 9 | *4q21.1* |
| *INPP5D* | Inositol polyphosphate-5-phosphatase D | *2q37.1* |
| *ISG20* | Interferon stimulated exonuclease gene 20 | *15q26.1* |
| *LCK* | LCK protooncogene, SRC family tyrosine kinase | *1p35.2* |
| *NKG7* | Natural killer cell granule protein 7 | *19q13.41* |
| *PSMB9* | Proteasome subunit beta 9 | *6p21.32* |
| *RUNX3* | Runt related transcription factor 3 | *1p36.11* |
| *TAP1* | Transporter 1, ATP binding cassette subfamily B member | *6p21.32* |

These genes were overexpressed in acute rejection across all transplanted organs and could diagnose acute rejection with high specificity and sensitivity.

In a study on the kidney, the 11-gene qPCR CRM score (tCRM) was found to be significantly increased in AR, with the greatest significance for CXCL9 and CXCL10[104]. Additionally, the tCRM score correlated with the extent of AR lesions and was predictive of CAD. In the already mentioned paper by Li *et al*[97], 8 genes were found by qPCR to be overexpressed in AR (*CFLAR*, $P = 0.0016$; *DUSP1*, $P = 0.0013$; *IFNGR1*, $P = 0.0062$; *ITGAX*, $P = 0.0011$; *PBEF1*, $P = 0.00008$; *PSEN1*, $P = 0.00007$; *RNF130*, $P = 0.0459$; and *RYBP*, $P = 0012$) and 2 genes were underexpressed (*MAPK9*, $P = 0.0006$; *NKTR*, $P = 0016$).

More recently[105], PCR measurement of the above gene set was evaluated in the urine of transplanted patients with acute allograft dysfunction; only 5/11 genes were highly significant at the time of rejection, and in a validation cohort, the urine common rejection module (uCRM) score for AR had an AUC of 0.961. However, in another study, the uCRM score was found to be elevated in other kidney injuries, such as acute tubular necrosis (ATN) and BKVN.

In summary, the suspicion of AR in kidney transplantation may be assessed by both proteomic and genomic biomarkers. Principal limitations appear to be the specificity of the biomarkers, as many of them are common with CAN and other forms of chronic nephropathies such as the related condition BKVN.

In the last years, genomic analyses are becoming more specific, and relevant progress has been made by kSORT applied to AART study. Unifying databases derived from studies on acute rejection of other organs such as the liver, lung and heart have allowed for realization of a common rejection module from which new genes specific for kidney rejection can be found.

## BIOMARKERS FOR CAD

The term CAD has replaced the term CAN because the latter has been used too broadly, preventing identification of true CR and other aetiologies of chronic dysfunction, such as drugs and viruses, not related to immunological causes. Two main concerns are associated with the identification of non-invasive biomarkers of CAD. First several proteomic and genomic studies[7,9] have found that the molecular mechanisms responsible for acute and CR may be extremely similar and that differentiation should be principally based on the so-called "threshold effect". As a consequence, identification of biomarkers

**Table 8 Analysis of pooled urine proteins collected from patients with acute rejection, BK virus nephropathy, and chronic allograft nephropathy when compared to STA urine with the criteria of > 1.5 fold change of each transplant injury phenotype (acute rejection, BK virus nephropathy, and chronic allograft nephropathy), compared to STA pooled urine and with a *P*-value of ≤ 0.05[131]**

| Increased in AR | Increased in BKVN | Increased in CAN |
|---|---|---|
| HLA-DRB1, FGB, FGA, FGG, KRT14, HIST1H4B, KRT7, DPP4 | KRT18, SUMO2, STMN1, CFHR2, KRT8, KRT19, RPL18, KRT75, FAM3C, HIST1H2BA | CALR, FAM151A, SERPINA2P, FAM3C, DAG1, KITLG, LUM, FABP4, AGT, LRG1 |

AR: Acute rejection; BKVN: BK virus nephropathy; CAN: Chronic allograft nephropathy; FGB: Fibrinogen beta chain; FGA: Fibrinogen alpha chain; FGG: Fibrinogen gamma chain; KRT14: Keratin 14; HIST1H4B: Histone cluster 1 H4 family member b; KRT7: Keratin 7; DPP4: Dipeptidyl peptidase 4; KRT18: Keratin 18; SUMO2: Small ubiquitin-like modifier 2; STMN1: Stathmin1; CFHR2: Complement factor H related 2; KRT8: Keratin 8; KRT19: Keratin 19; RPL18: Ribosomal protein L18; KRT75: Keratin 75; FAM3C: Family with sequence similarity 3 member C; HIST1H2BA: Histone cluster 1 H2B family member a; CALR: Calreticulin; FAM151A: Family with sequence similarity 151 member A; SERPINA2P: Serpin family A member 2; FAM3C: Family with sequence similarity 3 member C; DAG1: Dystroglycan 1; KITLG: KIT ligand; LUM: Lumican; FABP4: Fatty acid binding protein 4; AGT: Angiotensinogen; LRG1: Leucine rich alpha-2-glycoprotein 1.

responsible for CAD should be performed with extreme caution and with careful dosing of the suspected molecules. Second, the causes of CAD may be quite different, and the aim of these studies should also take into account differentiation of the molecules or genes responsible for different aetiologies.

Non-invasive biomarkers of CAD are essentially based on proteomics and genomics.

### *Proteomic studies for CAD*

In a review published in 2012, Bohra *et al*[11] discussed the main proteomic and metabolomic studies aimed at identifying biomarkers of CAD. Additionally, Johnston *et al*[106] reported β2 microglobulin as a urinary biomarker for CAD. In a large study by Kurian *et al*[107], 302 proteins in peripheral blood were identified as responsible for mild CAD and 509 for severe CAD, and Quintana *et al*[108] found uromodulin and kininogen in urine to be useful biomarkers for CAD. Based on a two-dimensional differential gel electrophoresis of urine, Bañon Maneus *et al*[109] found 21 proteins associated with CAD, including A1AT, α-1 β glycoprotein (A1BG), angiotensinogen (AGT), anti-TNF alpha antibody light chain, β2 microglobulin (B2M), brevin, heparan sulfate proteoglycan (HSPG), leucine-rich α 2-glycoprotein 1 (LRG1) and transferrin.

In a more recent study, Nakorchevsky *et al*[110] in a large-scale proteogenomic analysis of tissue biopsies found more than 1000 proteins associated with mild to-severe CAD.

Jahnukainen *et al*[111] in a proteomic analysis of urine in kidney transplant patients with BKVN applied surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) analysis to distinguish protein profile characteristics of BKVN but were unable to identify different proteins. More recently, Sigdel *et al*[73] found BKVN selective proteins to be associated with contractile fibers, with gene expression regulation, with glycolysis and with response to viruses. In this study the top 10 most significant urine proteins for AR, BKVN and CAN are shown (Table 8).

Recent studies on calcineurin inhibitor toxicity documented altered expression of 38 proteins *in vitro* after incubation with cyclosporine (CyA)[112], and in a clinical setting, urine N-acetylβ-D-glucosaminidase (NAG) was found to be specific for CyA-related toxicity[113].

The discovery and use of mRNAs has shed new light on CAD and on the unique form of CAD called interstitial fibrosis/tubular atrophy (IF/TA).

One recent study reported the miRNA characteristics of patients affected by IF/TA[114], in particular five miRNAs (miR142-3p, miR-32, miR204, miR-107 and miR-211) were differentially expressed in tissue biopsy samples. These miRNAs were further confirmed in the urine of patients affected by CAD. In a follow-up study by the same group[115], a selected panel of miRNAs, miR99a, miR-140-3p, mi 200b and miR-200, monitored at different time points after transplantation were found to be differentially expressed in urine according to graft outcome and useful markers in graft monitoring. In a recent study, Zununi Vahed *et al*[116] observed that urinary miRNAs exibit different behaviors in patients affected by IF/TA according to whether they received a living or cadaveric donor kidney.

In another recent study on renal biopsies of patients affected by IF/TA, miR-142-5p and miR-142-3p were significantly up-regulated, whereas miR-211 was significantly down-regulated[117]. As the same results were observed in PBCs from the same patients, the authors suggested that PBCs might be used in a non-invasive approach for monitoring kidney graft function.

Finally, evaluating miRNA profiles in transplanted patients, Iwasaki *et al*[118] found that miR-486-5p was significantly over-expressed in these patients who produced donor-specific antibodies (DSA) and exhibited biopsy-proven chronic antibody-mediated rejection (CAMR).

### *Genomic studies for CAD*

Mas *et al*[119] used microarrays to evaluate renal tissue from patients affected by CAD with IF/TA and found up-regulation of genes related to fibrosis, extracellular matrix deposition and the immune response, as provided in Table 9. Markers of genes such as transforming growth factor beta (TGF-β), epidermal growth factor receptor (EGFR), and AGT were similarly found to be elevated in

**Table 9 Genes higher (fold change higher than 6.00) expressed in renal tissue of patients affected by interstitial fibrosis/tubular atrophy[119]**

| Symbol | Gene name | Cytoband |
|---|---|---|
| *IGHA1* | Immunoglobulin heavy constant alpha 1 | *14q32.33* |
| *IGHG1* | Immunoglobulin heavy constant gamma 1 | *14q32.33* |
| *CCR2* | Chemokine C-C motif receptor 2 | *3p21.31* |
| *DFFB* | DNA fragmentation factor 40 Da beta subunit | *1p36.32* |
| *CD44* | CD44 antigen | *11p13* |
| *IFNA1* | Interferon alpha 1 | *9p21.3* |
| *GZMK* | Granzyme K | *5q11.2* |
| *MMP9* | Matrix metallopeptidase 9 | *20q13.12* |
| *TNFRSF17* | Tumor necrosis factor receptor superfamily, member 17 | *16p13.13* |
| *CXCR4* | Chemokine C-X-C motif receptor 4 | *2q22.1* |

urine samples.

In the multicenter CTOT-04 trial, in addition to validating the three-gene signature of CD3ε mRNA, CXCL10-mRNA and 18S rRNA, which is predictive of acute rejection, Lee *et al*[120], examined urinary mRNA by PCR and reported a 4-gene signature of mRNAs for vimentin, NKCC2, E-cadherin and 18S rRNA that was diagnostic of IF/TA.

The above-mentioned tCRM[104] is a computational gene expression score for predicting immune injury in renal allograft. A subset of 7 genes [CD6 molecule (*CD6*), inositol polyphosphate-5-phosphatase D (*INPP5D*), interferon-stimulated exonuclease hene 20 (*ISG20*), natural killer cell granule protein 7 (*NKG7*), proteasome subunit beta 9 (*PSMB9*), runt-related transcription factor 3 (*RUNX3*) and transporter 1, ATP-binding cassette subfamily B member (*TAP1*)] had higher predictive value for patients developing IF/TA over time.

A relevant international study of Genomics of Chronic Allograft Rejection (GoCAR) (Clinical Trials.gov NCT 00611702)[121] aimed to identify genes that correlate with chronic allograft dysfunction index (CADI) scores at 12 mo in patients with a normal biopsy at three months.

A set of 13 genes showed independent predictive value for the development of fibrosis (Table 10). This gene set also has a predictive value higher than that of clinical and pathological variables.

A new approach of the Mount Sinai group[122] is to utilize genomics to identify therapeutic agents for IF/TA. Based on an 85-gene signature from IF/TA molecular datasets in Gene Expression Omnibus and using a computational repurposing analysis, two new drugs, in addition to well-known azathioprine already used for AR and pulmonary fibrosis, appear to be promising: Kamferol, which attenuates TGF-β1, and Esculetin, which inhibits the Wnt/β catenin pathway. Both drugs were effective and safe in preclinical models.

## BIOMARKERS TO PREDICT AND MONITOR TOLERANCE

No more than 100 cases of clinical operational tolerance (COT) have been reported in renal transplantation[123].

A number of consortia have been realized in an attempt to find valid tolerance signatures. The more important consortia are reported in Table 11[124,125].

Thirty-nine genes have been found to be up-regulated in COTs in different sites, in different patient cohorts and using different microarrays; 24 of these genes (69%) are B cell related, with CD79b and prepronociceptin (PNOC) being the more highly expressed[126-128]. Additionally, Danger *et al*[129] documented up-regulation of miR-142-3p in B cells of COT patients.

T reg cells ($CD4^+$, $CD25^+$, Fox $P3^+$) have been extensively studied in operational tolerance, though their role in COT remains unclear[128,130]. A role for natural killer (NK) cells in COTs has also been postulated[128].

In another relevant study, Roedder *et al*[131] highlighted that tolerance biomarkers are dependent on the age of the recipient and may differ according the organ transplanted and that there is a need for further validation studies. The same authors identified different biomarkers according to age and the organ transplanted.

### *Genomic studies for tolerance*

A study on gene expression in peripheral B cells showed an up-regulation of membrane-spanning 4-domains A1 (*MS4A1*) (*CD20*), T-cell leukemia/lymphoma 1A (*TCL1A*), CD79b molecule, immunoglobulin-associated beta (*CD79B*), tolerance-associated gene 1 (*TOAG1*) and Forkhead Box P3 (*FOXP3*) genes. *TOAG1* was also up-regulated intragrafts[132].

In a recent study, a group from Northwestern University in Chicago found an important role for Treg cells. Indeed, in their study on COTs patients *vs* non-tolerant patients, the number of circulating Treg cells was significantly time-dependently higher in tolerant patients[133]. Additionally, in the same study, a role for a different 357 gene signatures of tolerance was found (Table 12).

A principal approach for identifying genes actually involved in COTs derives from comparison of tolerant patients *vs* those with immunosuppression; immunosuppressive treatment in the latter group might influence and generate bias in the gene expression signature. To overcome the problem, a multicenter study[134] reviewed a cohort of 246 kidney transplant recipients (232 with

**Table 10 Thirteen genes associated with chronic allograft dysfunction identified by biopsy transcriptome expression[121]**

| Symbol | Gene description | Cytoband | CADI 12 mo correlation | *P* value |
|---|---|---|---|---|
| *CHCHD10* | Coiled-coil-helix-coiled- coil helix domain containing 10 | *22q11.23* | 0.404 | $2.85 \times 10^{-5}$ |
| *KLHL13* | Kelch-like family member 13 | *Xq23-q24* | 0.369 | $1.49 \times 10^{-4}$ |
| *FJX1* | Four jointed box 1 | *11p13* | 0.367 | $1.60 \times 10^{-4}$ |
| *MET* | Met proto-oncogene | *7q31* | 0.352 | $3.01 \times 10^{-4}$ |
| *SERINC5* | Serine incorporator 5 | *5q14.1* | 0.318 | 0.0012 |
| *RNF149* | Ring finger protein 149 | *2q11.2* | 0.28 | 0.0046 |
| *SPRY4* | Sprouty homolog 4 | *5q31.3* | 0.27 | 0.0062 |
| *TGIF1* | TGF-β induced factor homeobox 1 | *18p11.3* | 0.244 | 0.0140 |
| *KAAG1* | Kidney associated antigen 1 | *6p22.1* | 0.24 | 0.0154 |
| *ST5* | Suppressor of tumorigenicity 5 | *11p15* | 0.232 | 0.0197 |
| *WNT9A* | Wingless-type MMTV integration site family member 9A | *1q42* | 0.212 | 0.0332 |
| *ASB15* | Ankirin repeat and SOCS box-containing 15 | *7q31.31* | -263 | 0.0079 |
| *RXRA* | Retinoid X receptor alpha | *9q34.3* | -0.3 | 0.0023 |

CADI: Chronic allograft dysfunction index.

**Table 11 International research consortia in rejection/tolerance**

| Acronym | Description | Year |
|---|---|---|
| ITN | Immune tolerance network | Since 2002 |
| IOC | Indices of tolerance | 2003-2007 |
| RISET | Reprogramming the immune system for establishment of tolerance | 2005-2010 |
| GAMBIT Study | Genetic analysis and monitoring of biomarkers of immunological tolerance | 2010 |
| The One Study | A unified approach to evaluating cellular immunotherapy in solid organ transplantation | 2011 |
| Bio-DRIM | Personalized minimization or immunosuppression after solid organ transplantation by biomarker driven stratification of patients to improve the long-term outcome and health-economic data of transplantation | 2012 |
| BIOMARGIN | Biomarkers of renal graft injuries in kidney allograft recipients | 2013 |

GAMBIT: Genetic Analysis and Monitoring of Biomarkers of Immunological Tolerance.

immunosuppression, 14 tolerant) using the Genetic Analysis and Monitoring of Biomarkers of Immunological Tolerance method, and the investigators were able to identify a nine gene immunosuppression-independent gene signature (Table 13).

Recently, 21 genes involved in tolerance were identified at the University of California San Francisco (UCSF), in the program kidney spontaneous operational tolerance test (kSPOT). These investigators studied 348 HLA-mismatched renal transplant patients and identified 21 genes involved in COT. These 21 TOL genes were validated, and independent qPCR for the 21 genes was preformed. Additionally, the authors were able to refine and validate a three-gene assay [Kruppel-Like Factor 6 (*KLF6*), Basonuclin 2 (*BNC2*), and Cytochrome P450 Family 1 Subfamily B Member 1 (*CYP1B1*)] to detect the state of operational tolerance, with an AUC 0.95[135]. Interestingly, *BNC2* and *CYP1B1* are both related to tolerance in kidney and liver transplantation[136,137].

In conclusion, a number of studies have searched for a "tolerance signature". However, such an endeavour is difficult because of the small number of COT patients. The search for biomarkers is principally useful for identifying tolerant patients. Among the different studies, that of Newell *et al*[127], which was aimed at finding a gene expression profile for tolerant patients, and the microarray analysis of Sagoo *et al*[128] stand out in this field.

In addition, the reclassification of transplant patients according to immune risk threshold may be achieved using the cited kSORT, tCRM, uCRM and kSPOT. This might help in determining which recipients would benefit from withdrawal or minimization of immunosuppression.

## FUTURE PERSPECTIVES

Several prospective research programs and clinical trials are ongoing using already-known biomarkers or are searching for new ones.

Biomarker-driven personalized immunosuppression (BIO-DrIM) is a European Consortium aimed at the Methodical and Clinical Validation of Biomarkers for guiding immunosuppression[138]. The programs of the Consortium include: (1) The targeting and partial weaning of immunosuppression in long-term liver and kidney transplant patients; and (2) biomarker analysis and data management.

The biomarker platforms of BIODrIM are as follows: (1) An ELISPOT platform for detecting donor-reactive memory/effector T cells[139]; (2) a real-time RT-PCR platform to identify molecular tolerance signatures[140]; and (3) a multiparameter flowcytometry platform to characterize circulating immune cell subsets[141].

The BIODrIM consortium is designing two clinical trials in solid organ transplantation using biomarkers for decision making.

**Table 12 Immune/inflammatory molecules among the 357 gene signatures of tolerance**

| Categories | Diseases or functions annotation | Molecules | No. of molecules |
|---|---|---|---|
| Cell-to-cell signaling and interaction, cellular function and maintenance, hematological system development and function, inflammatory response | Phagocytosis of leukocyte cell lines | FGR, MRC1, TLR4 | 3 |
| Cell-to-cell signaling and interaction, hematological system development and function, immune cell trafficking, inflammatory response, tissue development | Binding of neutrophils | FGR, LSP1, TLR4 | 3 |
| Antimicrobial response, inflammatory response | Antibacterial response | CARD9, FGR, LYST, NLRC4, TLR4 | 5 |
| Cell-to-cell signaling and interaction, hematological system development and function, inflammatory response | Binding of professional phagocytic cells | FGR, LSP1, NOTCH2, TLR4 | 4 |
| Inflammatory response | Immune response of cells | CARD9, CLEC7A, ETS2, FGR, MRC1, SCARF1, MYO7A, TLR4 | 8 |
| Antimicrobial response, inflammatory response | Antimicrobial response | CARD9, CLEC7A, FGR, LYST, NLRC4, TLR4 | 6 |
| Inflammatory response | Innate immune response | CARD9, CLEC7A, TLR4, TRIM59 | 4 |
| Cellular function and maintenance, inflammatory response | Phagocytosis | CLEC7A, ETS2, FGR, MRC1, MYO7A, TLR4, TPCN2 | 7 |
| Cell-to-cell signaling and interaction, cellular growth and proliferation, hematological system development and function, inflammatory response | Stimulation of phagocytes | IL4R, TLR4 | 2 |
| Antimicrobial response, humoral immune response, inflammatory response | Antifungal response | CARD9, CLEC7A | 2 |
| Cell-to-cell signaling and interaction, cellular function and maintenance, inflammatory response | Phagocytosis of cells | CLEC7A, ETS2, FGR, MRC1,MYO7A, TLR4 | 6 |

These genes potentially predict those patients that can be successfully weaned off immunosuppression[133]. FGR: Tyrosine-protein kinase Fgr; MRC1: Mannose receptor, C type 1; TLR4: Toll-like receptor 4; FGR: Tyrosine-protein kinase Fgr; LSP1: Lymphocyte-specific protein 1; CARD9: Caspase recruitment domain family member 9; LYST: Lysosomal-trafficking regulator; NLRC4: NLR family CARD domain-containing protein 4; NOTCH2: Neurogenic locus notch homolog protein 2; CLEC7A: C-type lectin domain family 7 member A; ETS2: Protein C-ets-2; SCARF1: Scavenger receptor class F member 1; MYO7A: Unconventional myosin-VIIa; TRIM59: Tripartite motif-containing protein 59; TPCN2: Two pore calcium channel protein 2; IL4R: Interleukin 4 receptor.

**Table 13 Immunosuppression-independent gene signatures predicting tolerance[134]**

| Symbol | Gene name | Molecular function | Biological processes |
|---|---|---|---|
| *ATXN3* ↓ | Ataxin 3 | Ubiquitin-specific protease activity | Protein metabolism |
| *BCLA1* ↓ | BCL2-related protein A1 | Receptor signaling complex scaffold activity | Apoptosis |
| *EEF1A1* ↓ | Eukaryotic translation elongation factor 1 alpha 1 | Transcription regulator activity | Regulation of cell cycle |
| *GEMIN7* ↑ | Gem associated protein 9 | Ribonucleoprotein | Regulation of nucleobase, nucleosides, nucleotide and nucleic acid metabolism |
| *IGLC1* ↑ | Immunoglobulin lambda constant 1 | Antigen binding | Immune response |
| *MS4A4A* ↑ | Membrane-spanning 4-domains, subfamily A, member 4A | - - - | - - - |
| *NFκBIA* ↑ | Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha | Transcription regulator activity | Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism |
| *RAB40C* ↑ | RAB40C, member of RAS oncogene family | GTPase activity | Cell communication, signal transduction |
| *TNFAIP3* ↓ | Tumor necrosis factor, alpha-induced protein 3 | Transcription regulator activity | Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism |

↓Immunosuppression-free gene expression downregulated in tolerant patients; ↑Immunosuppression-free gene expression upregulated in tolerant patients; BCL2: B-cell lymphoma 2.

The trial LIST[138] will apply molecular signatures to guide immunosuppression in liver transplant patients.

The kidney transplant trial design of BIODrIM is Cellimin, a prospective multicenter randomized trial utilizing IFNγ ELISPOT to stratify kidney transplant recipients into high/low responders. Only low-responder patients will be randomized to receive either standard immunosuppression or low-dose immunosuppression. The trial will evaluate the donor specific cellular alloresponse for immunosuppression minimization (EudraCT-Number: 2013-005041-37)[142].

Another European research program is "Biomarkers of Renal Graft Injuries in kidney allograft recipients" (BIOMARGIN)[143], which has the aims to: (1) select and validate blood or urine biomarkers at different-omics levels related to allograft lesions; and (2) select

and validate biomarkers as early predictors of CAD. The research will allow for selecting the best candidate biomarkers and biomarker signatures. In addition, the work will evaluate the sensitivity, selectivity, false positive value and false negative value of biomarkers. Finally, one goal of the study is to select biomarker signature predictors of three-year graft outcomes.

By using the aforementioned biomarkers of kSORT, the TITRATE trial has the aim of testing immunosuppression Threshold in Renal Allografts to improve the estimated glomerular filtration rate (eGFR). Overall, the main outcomes of the trial are the rate and severity of acute rejection and the CADI score at one year based on protocol biopsy. Evaluation of eGFR is also a principal endpoint. The study is ongoing in Mexico and at UCSF[144].

Another Clinical Trial, NIH UO1 trial TASK, employs the biomarkers of kSORT, uCRM, and tCRM. The TASK trial has the aim of evaluating Treg adoptive therapy for subclinical inflammation in kidney transplantation by comparing the results of three patients' cohorts according to surrogate markers of the immune response[145].

The Precision Medicine Offers Belatacept Monotherapy study[146] is being conducted at four centers in the United States, Spain, France and Mexico. The trial has the aim of determining the safety and feasibility of converting kidney transplant recipients to Belatacept monotherapy. In addition, the trial has the goal of evaluating the percentage of patients who can be converted to a Belatacept regimen of once every 8 wk. The patients enrolled in the trial will have a quiescent immunologic profile evaluated by kSORT, uCRM and tCRM. Only those with elevated kSPOT will be tested for the once every 8-wk administration.

The epithelial-to-mesenchymal transition (EMT) is a process in which fibrosis is generated due to the transformation from the epithelial to mesenchymal phenotype. The process is induced and facilitated by several molecular signatures, among which TGF beta, EGF, insulin like growth factor 2 and fibroblast growth factor 2 (FGF2) are prominent[147]. An interesting ongoing trial is Prediction of Chronic Allograft Nephropathy (Prefigur)[148]. By using non-invasive biomarkers and evaluating urinary cells in the first year post-transplantation, the investigators are developing a non-invasive approach for predicting fibrosis as a substitute of allograft biopsy, *via* longitudinal assessment of the mRNA expression level of genes implicated in EMT fibrogenesis.

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