Non-invasive approaches in the diagnosis of acute rejection in kidney transplant recipients, part II: omics analyses of urine and blood samples

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Abstract

Kidney transplantation (KTx) represents the best available treatment for patients with end-stage renal disease. Still, the full benefits of KTx are regrettably undermined by acute rejection (AR). The diagnosis of AR ultimately relies on transplant needle biopsy. However, such an invasive procedure is associated with a significant risk of complications and is limited by sampling error and interobserver variability. In the present review, we summarize the current literature about non-invasive approaches for the diagnosis of AR in kidney transplant recipients (KTRs), including in vivo imaging, gene-expression profiling and omics analyses of blood and urine samples. Most imaging techniques, such as contrast-enhanced ultrasound and magnetic resonance, exploit the fact that blood flow is significantly lowered in case of AR-induced inflammation. In addition, AR-associated recruitment of activated leucocytes may be detectable by 18F-fluorodeoxyglucose positron emission tomography. In parallel, urine biomarkers, including CXCL9/CXCL10 or a three-gene signature of CD3ε, CXCL10 and 18S RNA levels, have been identified. None of these approaches has yet been adopted in the clinical follow-up of KTRs, but standardization of analysis procedures may help assess reproducibility and comparative diagnostic yield in large, prospective, multicentre trials.

Key words: acute rejection, biomarkers, gene expression, kidney biopsy, proteomics

Introduction

Kidney transplantation (KTx) represents the best available treatment for patients with end-stage renal disease. Each year, 3500 kidney transplants are performed in the EuroTransplant zone (www.eurotransplant.org). Still, the full benefits of KTx are regrettably undermined by acute rejection (AR), which may be cellular or antibody-mediated [1]. AR may affect kidney transplant recipients (KTRs) throughout their lifetime, independent of age.
Transcriptomics

The ‘transcriptome’ corresponds to mRNA produced in toto by a cell or a tissue [19]. Transcriptomic analysis quantifies the expression levels of gene transcripts, thereby identifying actively expressed genes at a given time under a given physiological or pathologic condition [20]. The most commonly used methods for gene expression profiling are microarray and more recently RNA-seq [19, 20].

In urine, transcriptomics detects immune response occurring in case of renal AR by assessing urinary cell levels of mRNA (Table 1). Principal targets are membrane markers of cytotoxic T lymphocytes, which play a central role in the AR process, as well as chemokines and their corresponding receptors [13]. In particular, chemokine receptor CXCR3 and its ligand interferon (IFN)-inducible protein-10 (IP-10, also called CXCL10) have been demonstrated to play a key role in T cell activation and allograft destruction [53]. In 2010, Tatapudi et al. [24] measured the level of urinary transcripts for IP-10 and CXCR3 in 63 urine specimens of 58 KTRs with acute renal dysfunction, including 27 biopsy-proven ARs, and 27 urine specimens from 24 KTRs with stable allograft function. The levels of CXCL10 and CXCR3 mRNA were significantly higher in urinary cells from patients with AR compared with controls, suggesting that CXCL10 and CXCR3 mRNA may represent urine biomarkers of renal AR (for CXCL10: sensitivity 100%, specificity 78% for a cut-off value of 9.11 copies; for CXCR3: sensitivity 63%, specificity 83% for a cut-off value of 11.59 copies). Additional studies of the same group focused on CD103, a cell surface marker of intratubular CD8 cells. The authors found significantly higher CD103 mRNA levels in urinary cells from 30 patients with AR [21]. Additionally, components of the lytic equipment of cytotoxic cells, such as perforin [54] and granzyme B [55], were investigated. Li et al. [29] used 24 urine specimens from 22 KTRs with biopsy-proven AR and 127 urine samples from 63 stable KTRs and found higher levels of perforin and granzyme B mRNA in the urine of patients with documented AR (sensitivity 83%, specificity 83% with the use of a cut-off value of 0.9 fg of perforin mRNA/μg total RNA; sensitivity 79%, specificity 77% with the use of a cut-off value of 0.4 fg of granzyme B mRNA/μg total RNA, for the prediction of AR). Tremendous efforts by the Clinical Trials in Organ Transplantation (CTOT) consortium have strengthened data from previous single-centre studies. In 2013, Suthanthiran et al. [1] prospectively collected urine samples from 485 KTRs from multiple transplant centres at different times following KTxs, including at the time of per-cause transplant biopsy. Levels of mRNA of CD3e, perforin, granzyme B, proteinase inhibitor 9, CD103, IP10, CXCR3, transforming growth factor β1 (TGF-β1) and 18S ribosomal RNA (rRNA) were quantified by PCR. A three-gene signature of CD3e mRNA, CXCL10 mRNA and 18S rRNA levels was defined as the best predictive model of biopsy-proven AR, with an area under the curve (AUC) of 0.85 (sensitivity 79%, specificity 78%). This signature also allowed them to distinguish acute cellular-mediated from antibody-mediated rejection and borderline rejection (AUC 0.78). Retrospectively, the authors noticed that the expression levels of CD3e mRNA, CXCL10 mRNA and 18S rRNA significantly increased during the 20-day period preceding the per-cause biopsy confirming the diagnosis of AR [1]. These observations suggest that this three-gene signature may be a promising tool for monitoring the immune status of KTRs. Indeed, increased levels of urinary mRNA at follow-up may prompt transplant biopsy, thereby allowing faster adjustments of immunosuppressive therapy. The main limitations of this study include the small number of patients with antibody-mediated rejection and the fact that 54 of 298 urine RNA biopsy-matched samples did not
pass quality controls [1]. Urine specimens were classified as passing quality control if the 18S rRNA copy number was $\geq 5 \times 10^7$ copies/μg total RNA isolated from the urine pellet and if the TGF-β1 mRNA copy number was $\geq 100$ copies/μg of total RNA isolated from the urine pellet. If either threshold was not met, the specimen was classified as failing quality control.

| Transcription | Gene | Sensitivity | Specificity | n (AR) | References |
|---------------|------|-------------|-------------|--------|------------|
| Urine CD 103 | NA   | 30          |             | Ding et al. [21] |
| CXCL10 (IP-10) | NA   | 54          |             | Matz et al. [22] |
|               | NA   | 300         |             | Rabant et al. [23] |
|               | 100/78 | 27       |             | Tatapudi et al. [24] |
| CXCR-3        | 63/83 | 27          |             | Tatapudi et al. [24] |
| Fox P3        | 90/73 | 36          |             | Muthukumar et al. [25] |
| Granulysin    | 80/100 | 14       |             | Kotsch et al. [26] |
|               | 96/67 | 31          |             | Seller et al. [27] |
| Granzyme A    | 80/100 | 27       |             | Van Ham et al. [28] |
| Granzyme B    | 79/77 | 22          |             | Li et al. [29] |
|               | 88/79 | 29          |             | Muthukumar et al. [30] |
|               | 60/100 | 31      |             | Seller et al. [27] |
| miR-210       | 52/74 | 62          |             | Lorenzen et al. [31] |
| NKG2D         | 77/81 | 31          |             | Seller et al. [27] |
| Perforin      | 83/83 | 22          |             | Li et al. [29] |
|               | 88/79 | 29          |             | Muthukumar et al. [30] |
| PI-9 (serine proteinase inhibitor-9) | 76/79 | 29              | Muthukumar et al. [30] |
| Tim-3         | NA   | 30          |             | Renesto et al. [32] |
|               | 84/96 | 115         |             | Manfro et al. [33] |
| Combination of mRNA for OX40, OX40L, PD-1 and Fox P3 | 95/92 | 21              | Afnan et al. [34] |
| 3-gene signature: 18S ribosomal mRNA,CD3ε mRNA and CXCL10 mRNA | 79/78 | 47              | Suthanthiran et al. [1] |
| 6-gene signature: CD3ε, CD105, CD14, CD46 and 18S rRNA | NA | 52          |             | Matignon et al. [35] |
| Blood and PBMCs CD40L | NA | 32          |             | Mao et al. [37] |
| CXCL10 (IP-10) | NA | 32          |             | Mao et al. [37] |
| CXCL13        | NA   | 32          |             | Mao et al. [37] |
| Fas ligand    | 91/81 | 11          |             | Vasconcellos et al. [38] |
| Fox P3        | NA   | 28          |             | Wang et al. [39] |
| Granulysin    | NA   | 53          |             | Sabek et al. [42] |
| Granzyme B    | 50/85 | 8           |             | Dugré et al. [41] |
|               | 63/96 | 8           |             | Sabek et al. [42] |
|               | 72/87 | 17          |             | Simon et al. [43] |
|               | 64/85 | 11          |             | Vasconcellos et al. [38] |
| IFN-γ         | 63/85 | 8           |             | Dugré et al. [41] |
| IL-2          | NA   | 6           |             | Lee et al. [44] |
| IL-4          | 50/85 | 8           |             | Dugré et al. [41] |
| IL-5          | 63/92 | 8           |             | Dugré et al. [41] |
| IL-6          | 50/92 | 8           |             | Dugré et al. [41] |
| IL-10         | NA   | 6           |             | Lee et al. [44] |
| IL-15         | NA   | 6           |             | Lee et al. [44] |
| IL-18         | NA   | 8           |             | Striz et al. [45] |
| IFN-γ         | 63/85 | 8           |             | Dugré et al. [41] |
| HLA-DRA       | 83/79 | 8           |             | Sabek et al. [42] |
| mir-142-3p    | 100/65 | 17       |             | Soltaninejad et al. [47] |
| mir-223       | 100/76 | 17       |             | Soltaninejad et al. [47] |
| Notch-1       | NA   | 32          |             | Zheng et al. [48] |
| OX40          | 80/85 | 20          |             | Wang et al. [49] |
| PD-1          | NA   | 19          |             | Wang et al. [50] |
| Perforin      | 50/92 | 8           |             | Dugré et al. [41] |
|               | 63/74 | 8           |             | Sabek et al. [42] |
|               | 88/82 | 17          |             | Simon et al. [43] |
|               | 82/75 | 11          |             | Vasconcellos et al. [38] |
| Tim-3         | 100/87.5 | 24      |             | Luo et al. [52] |
|               | 87/95 | 115         |             | Manfro et al. [33] |

Se, sensitivity; Sp, specificity; n (AR), number of patients with acute rejection; NA, not available.
T regulatory lymphocytes (Tregs) represent a subpopulation of T cells characterized by the expression of the transcription factor Foxp3, which participates in restraining the expansion of effector T cells [56]. Tregs are potentially involved in the promotion of kidney transplant tolerance [57]. Muthukumar et al. [25] reported that the urinary abundance of Foxp3 mRNA, expressed as the ratio of FOXP3 mRNA copies to 18S ribosomal RNA copies, was significantly higher in 36 patients with biopsy-proven AR (3.8 ± 0.5) than in patients with chronic allograft nephropathy (1.3 ± 0.7) or normal histology (1.6 ± 0.4). The optimal cut-off for FOXP3 mRNA reached 3.46 [25]. High levels of FOXP3 mRNA were independently predictive of reversible AR and lower risk of graft failure. These findings are consistent with the hypothesis that Treg cells serve to limit anti-allograft immunity and that the lack of counterregulation by Treg cells during an episode of AR results in unrestrained effector cell activity, impaired allograft function and even graft failure.

Finally, microRNAs (miRNAs) are small, non-coding RNA molecules implicated in the post-transcriptional regulation of gene expression [58, 59]. Lorenzen et al. [51] compared the urinary profile of miRNAs of stable KTRs (n = 19) and KTRs with biopsy-proven AR (n = 62) and identified one miRNA as a potential biomarker for AR: miR-210. Low levels of miR-210 were independently associated with poorer kidney function at 1 year post-KTx. Accumulating evidence underlines a critical function for miRNAs in the modulation of innate and adaptive immune responses. Anglicheau et al. [50] identified a set of miRNAs highly dysregulated in renal biopsy samples and peripheral blood mononuclear cells (PBMCs) of patients with AR. As opposed to circulating plasma or serum miRNAs, dysregulated urinary miRNAs might be a better estimate of local intrainrenal changes. Circulating miRNAs, in contrast, might be released by a variety of renal and extrarenal tissues. The fact that miR-210 decreases specifically with the development of AR and increases to control levels after successful anti-rejection therapy provides evidence that miR-210 may serve as a novel biomarker of AR.

In blood, gene transcripts have also been considered as potential sources for biomarkers of AR (Table 1). Transcriptomics of PBMCs seems to be more comprehensive since it most likely reflects the immune cells infiltrating the allograft at the time of AR [13]. An interesting recent multicentre prospective study performed by Kuriar et al. [17] highlighted that global gene-expression profiling of PBMCs by DNA microarrays can be achieved to distinguish KTRs with normal renal function and biopsy histology (n = 46) from those with AR (n = 63) and those with ADNR (n = 39). Genome-wide profiling was executed on whole blood samples and collected at the same time as kidney biopsies. Multiple three-way classifier tools determined the 200 highest-value probe sets, with sensitivity ranging from 82 to 100% and specificity ranging from 76 to 95%. The authors acknowledged that their study design did not actually allow them to associate these gene signatures as predictive markers, but only as a part of a serial blood monitoring protocol. A prospective serial monitoring study is ongoing to validate these three-way classifiers [17].

In parallel, Dugré et al. [41] studied gene-expression profiling in PBMCs of 61 KTRs, including 8 with biopsy-proven AR. The authors detected higher levels of IL-4, IL-5, IL-6, IFN-γ, perforin and granzyme B mRNA in patients with AR. Interestingly, up-regulation of at least two of these markers is detectable in 75% of patients with AR, but only 25% of patients with ADNR. Furthermore, Vasconcellos et al. [38] analysed cytotoxic lymphocyte gene expression in 25 patients: up-regulation of any of two genes among perforin, granzyme B and Fas ligand had excellent positive predictive value (100%) and negative predictive value (NPV; 95%) of biopsy-proven AR [38]. Wang et al. [49] compared the level of costimulatory molecules OX40 and OX40L mRNA in PBMCs from KTRs with biopsy-confirmed AR (n = 20) and KTRs with normal renal function and histology (n = 20). OX40 is mainly expressed on T lymphocytes while OX40L is transiently expressed on antigen-presenting cells. Their interaction is critical for antigen-specific T cell expansion and survival [61]. The authors found a significant increase in OX40 mRNA and a non-significant increase in OX40L mRNA in PBMCs in AR [49, 62]. Similarly, in the Assessment of Acute Rejection in Renal Transplantation (AART) study, a 17-gene set (kSORT) in blood samples allowed identification of patients at high risk for AR [63].

Various studies have focused on cytokine gene expression [64], considering the fact that they are crucial mediators in renal AR. Lee et al. [44] analysed mRNA cytokine transcripts in sequential blood samples of six KTRs. The authors observed an increased expression of genes encoding IL-2, IL-4 and IL-15 and a decreased expression of the IL-10 gene in pre-rejection samples compared with controls (post-KTx samples). In another study of 51 patients among whom 32 had biopsy-proven AR, Mao et al. [37] concluded that gene expressions of chemokines C and CXCL13 were highly up-regulated in PBMCs in case of renal AR, with, intriguingly, even higher levels in AR-resistant patients (n = 10, poor response to anti-rejection therapy) compared with AR-sensitive patients (n = 22, good response to anti-rejection therapy) [37]. However, there was no difference in CXCL10 levels between AR and ATN.

Finally, the expression of miRNA in the serum and PBMCs of KTRs with AR has also been investigated. In a recent study performed on 17 patients with AR and 18 patients with normal allografts, Soltaninejad et al. [47] showed a differential expression pattern of microRNAs in PBMCs of KTRs with T cell–mediated AR, with a significant increase of miR-142-3p and miR-223. This was not confirmed in serum samples by Betts et al. [65].

Proteomics

Proteomics refer to the detection and functional investigation of proteins present in a cell, tissue, organ or organism at a definite moment [66]. This method can be applied to establish protein identity and/or to characterize protein–protein interactions [66]. Acquiring proteomic data is complex and can be achieved using a wide range of procedures, such as protein electrophoresis, enzyme-linked immunosorbent assay (ELISA) or mass spectrometry.

In urine, several biomarkers have been identified in renal AR (Table 2). These include cytokines and their binding receptors, extracellular matrix proteins and renal tubular cell components, such as CXCL9, CXCL10, NGAL, KIM-1, IL-1R and IL-20 [13]. Currently, the most promising biomarkers are IFN-γ-induced protein 10 kDa (IP-10, also known as CXCL10) and monokine induced by IFN-γ (MIG, also known as CXCL9). CXCL9 and CXCL10 are implicated in the recruitment of activated T cells to the site of inflammation, thereby promoting tissue infiltration and inflammation [88]. In a study of 75 KTRs, Schaub et al. [74] demonstrated that urinary CXCL9 and CXCL10 levels were significantly higher in subclinical tubulitis Ia/b than in subclinical borderline tubulitis and normal tubular histology, which indicates a correlation between chemokine levels and the extent of subclinical tubulitis. In contrast, Jackson et al. [73] performed a cross-sectional urinalysis of 110 adult and 46 pediatric KTRs across multiple diagnoses, including inflammatory and non-inflammatory conditions. The authors found that urine CXCL9 and CXCL10 were equivalently elevated, without statistically significant distinction, in both adults and children with acute kidney injury (AKI) and BK virus
infection. These observations suggest that urine CXCL9 and CXCL10 actually detects inflammation in kidney allografts, but do not point towards a specific cause [73, 89]. Another prospective, multicentre observational study of 280 KTRs designed by Hricik et al. [71] compared the diagnostic and predictive utility of non-invasive biomarkers for transplant outcomes. The investigators found that urinary levels of CXCL9 were significantly higher in patients with greater than Banff 1a AR, with an elevation detectable up to 30 days before per-cause biopsy. The authors suggested that low urinary CXCL9 in KTRs presenting with acute renal dysfunction could be used to rule out AR with a NPV of >92%. In a recent review paper, Hirt-Minkowski et al. [15] concluded that urinary CXCR3 chemokines may help detect subclinical rejection since their levels increase before clinical manifestations of AR.

Neutrophil gelatinase–associated lipocalin (NGAL) has also been assessed as an indicator of AKI in KTRs [64]. Heyne et al. [78] measured urinary NGAL in 182 KTRs on maintenance immunosuppression with stable allograft function (n = 138), AR (n = 9) or AKI from other causes (n = 44). In this cohort, levels of urinary NGAL (with a cut-off at 100 ng/mL) were able to discriminate AR from ADNR, with an AUC of 0.98 (sensitivity 100%, specificity 93%).

In blood, the identification of biomarkers appears even more challenging, considering the ratio between the abundance of plasma proteins and the putative low concentration of the proteins of interest [13, 90]. Blood proteome is largely composite and complex since it reflects the secretion and absorption of proteins from every tissue in the body, which therefore requires highly resolving fractionation methods [91]. Preliminary results in a study conducted by Cibrik et al. [92] using cohorts of healthy subjects, stable KTRs and KTRs with biopsy-proven AR suggest that a specific pattern of protein expression may help distinguish KTRs with AR. By Luminex, Xu et al. [87] retrospectively compared the levels of 95 cytokines/chemokines and their soluble receptors in the serum of 526 patients with versus without AR. They detected different expression patterns in 26 proteins in pre-AR patients compared with stable controls. The combination of IL-1 receptor antagonist, IL-20 and sCD40L showed the most

### Table 2. Proteomics in the non-invasive diagnosis of renal acute rejection

| Proteomics | Protein | Se/Sp (%) | n (AR) | References |
|------------|---------|-----------|--------|------------|
| Urine      | ANXA11  | NA        | 10     | Srivastava et al. [67] |
|            | β2-microglobulin | 83.3/80 | 30     | Oetting et al. [68] |
|            | β-Defensin-1/α1-antichymotrypsin | NA | 42     | O’Riordan et al. [69] |
|            | C4d     | NA        | 26     | Lederer et al. [70] |
|            | CXCL9   | 83/84     | 53     | Hricik et al. [71] |
|            | CXCL9:Cr | 86.4/91.3 | 28     | Hu et al. [72] |
|            | CXCL10 (IP-10) | 86.4/91.3 | 25     | Jackson et al. [73] |
|            |          | 86/64     | 22     | Schaub et al. [74] |
|            |          | 93/89     | 15     | Hauser et al. [75] |
|            |          | 81.2/34.5 | 300    | Rabant et al. [23] |
| Fractalkine| TNF-α   | NA        | 10     | Srivastava et al. [67] |
|            | sVCAM   | NA        | 26     | Lederer et al. [70] |
|            | 9 urine proteins (HLA class II protein HLA-DRB1, KRT14, HIST1H4B, FGG, ACTB, FGB, FGA, KRT7, DPP4) | 90 (cut-off = 30 ng/mL)/91 (cut-off >130 ng/mL) | 9 | Heyne et al. [78] |
| Blood      | CXCL10 (IP-10) | 73.3/68 | 15     | Zhang et al. [80] |
|            | CXCR3   | 80/76     | 15     | Zhang et al. [80] |
|            | CD30    | 70/71.7   | 23     | Nafar et al. [81] |
|            | Fractalkine | 88/100 | 25     | Pelzl et al. [82] |
|            | IL-2    | 70/73.6   | 10     | Shooshtarizadeh et al. [83] |
|            | IL-4    | 70/73.6   | 10     | Shooshtarizadeh et al. [83] |
|            | IL-6    | 70/73.6   | 10     | Shooshtarizadeh et al. [83] |
|            | M-CSF   | 80/NA     | 25     | Le Meur et al. [85] |
|            | 18 plasma proteins (titin, lipopolysaccharide-binding protein, peptidase inhibitor 16, complement factor D, etc.) | 80/90a | 27 | Freue et al. [86] |
| Combination: IL-1r antagonist, IL-20 and sCD40L | 91/96 | NA | Xu et al. [87] |

Se, sensitivity; Sp, specificity; n (AR), number of patients with acute rejection; NA, not available.

*aClassification of BCAR based on a four-protein ELISA classifier: CFD, LCAT, SHBG and F9.*
discrimination for AR (sensitivity 91%, specificity 96%). Furthermore, this signature was able to distinguish patients with AR from those with non-immunological delayed graft function (DGF). Freue et al. [86] used isobaric tag for relative and absolute quantification (iTRAQ) technology to identify proteomic signatures in plasma during early AR in a case–control discovery cohort of 305 patients, including 27 cases of AR. A panel of 18 plasma proteins discriminating AR biopsy was identified and included titin, lipopolysaccharide-binding protein, proteinase inhibitor 16, complement factor D, mannose-binding lectin, protein Z-dependent protease, β2-microglobulin, kinogingen-1, afamin, serine protease inhibitor, phosphatidylcholine-sterol acyltransferase and sex hormone-binding globulin [86].

Finally, a prospective study including 77 KTRs was performed by Shooshhtarizadeh et al. [93] using serum samples collected 24 h before KTx and analysed for CD30 by ELISA. CD30 is a co-stimulatory molecule, notably expressed by a subgroup of activated T cells, with pleiotropic functions. The authors found a significant correlation between pre-transplant serum levels of CD30 and AR (PPV = 29.1%, NPV = 94.3%) [83]. Similarly, Trailing et al. [94] observed a significant decrease in the level of sCD30 measured by ELISA 4 days after KTx in non-rejecting patients, in strong contrast to rejecting patients. Nafar et al. [81] compared the pre-transplant and post-transplant serum levels of CD30 in 203 KTRs and found that post-transplant sCD30 was higher in the AR group than in controls (cut-off value at 41 U/mL).

**Metabolomics**

The term ‘metabolomics’ refers to ‘the comprehensive characterization of small molecules in biological systems which provides an overview of the metabolic status and global biochemical events associated with a cellular or biological system’ [www.metabolomicssociety.org]. Such global profiling appears particularly useful to identify novel prognosis and diagnosis markers. In nephrology, metabolomics has been applied to study drug-induced AKI and ischaemia–reperfusion injury [95, 96].

In urine, Blydt-Hansen et al. [12] retrospectively used quantitative mass spectrometry (MS) to assay samples (n = 277) from 57 paediatric KTRs with surveillance or per-cause kidney biopsies (Table 3). Samples without cellular-mediated AR (n = 183) were compared with borderline tubulitis (n = 54) and cellular-mediated AR (n = 30). This pilot study established sensitive and specific correlations of urine MS metabolome with cellular-mediated AR. Most important, urinary metabolites contributing to the discriminant score for cellular-mediated AR included proline, produced by activated macrophages, and kynurenine (Kyn), implicated in the Th1 immune response. Significant limits of this non-prospective study need to be acknowledged, including (i) the lack of timed samples immediately before or after transplant biopsy, (ii) the late profile post-KTx of most AR episodes, (iii) the limitation to paediatric KTRs and (iv) the lack of documentation in antibody-mediated AR. In adult KTRs, an MS-based metabolite signature of the ratio of 3-sialyllactose to xanthosine in urine supernatants was able to discriminate cellular-mediated AR from non-rejection in 1516 urine samples from the multicentre CTOT-04 study [97]. It should be emphasized, however, that this study only focused on patients with biopsy-confirmed AR and patients with normal histology, and did not systematically evaluate the diagnostic performance in ‘real-life’ patients with allograft dysfunction due to any cause, including antibody-mediated AR, ADNR or BK virus nephropathy. Furthermore, urine samples were cell-free supernatants collected after centrifugation, which significantly influence metabolomics results [100]. Nuclear magnetic resonance (NMR)-based metabolomics of the urine has never been applied to urine from KTRs with AR. Compared with MS, NMR-based metabolomics has the benefits of being non-destructive, quantitative, highly reproducible and less time consuming, with minimal sample preparation [101]. This technique is particularly adapted to analyse biofluids such as urine [102].

In blood, Zhao et al. [98] investigated metabolic changes linked to AR in KTRs with (n = 11) and without (n = 16) AR by applying a non-targeted liquid chromatography (LC)-MS approach (Table 3). The investigators detected discriminative metabolites of AR, including creatinine, kynurenine, uric acid, polyunsaturated fatty acid, phosphatidylycerolines, sphingomyelins and lysophosphatidylcholines. More specifically, the serum level of tryptophan (Trp) was decreased in the non-AR group, whereas Kyn was increased. The increase in the Kyn:Trp ratio may be caused by increased activity of indoleamine 2,3-dioxygenase, which may be graft protective. Another study designed by Mao et al. [99] using gas chromatography–MS analysed serum metabolome in 22 KTRs with AR versus 15 stable KTRs and highlighted a metabolomic pattern of rejection. The levels of 17 metabolites, including amino acids, carbohydrates, carboxylic acids and lipids, as well as lactate, urea and myo-inositol, were significantly higher in the AR group than in controls, whereas the levels of alanine, lysine, leucine, aminomalonic acid and tetradecanoic acid were lower in the AR group.

**Conclusions**

Renal AR remains one of the leading causes of reversible acute dysfunction in KTRs and is an early predictor of subsequent
graft failure [3, 4]. The diagnosis and classification of AR ultimately rely on transplant needle biopsy. However, the rapid development of innovative imaging techniques and biofluid analysis by omics may help non-invasively detect AR, thereby hastening and improving KTR management. Furthermore, non-invasively discriminating AR from ADNR would help avoid needless and risky transplant biopsies. On the basis of the current literature, pioneering imaging approaches, including MRI and 18F-FDG-PET/CT [16, 103], and urine biomarkers, including CXCL9, CXCL10 or a three-gene signature of CD3e, CXCL10 and 18S RNA levels, appear most promising. Nevertheless, none of these approaches has been adopted yet in the clinical follow-up of KTRs. This may be partly explained by methodological limitations, cost and biological plausibility [104, 105]. Standardization and validation of analysis procedures are urgently required to assess reproducibility in prospective multicentric trials. Furthermore, additional studies should focus on the comparative diagnostic yield of imaging versus omics methods, as well as on the benefits of combining both approaches.

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Conflict of interest statement

The authors have no conflicts of interest to report. This manuscript has not been previously published elsewhere, in whole or in part.

References

1. Suthanthiran M, Schwartz JE, Ding R et al. Urinary-cell mRNA profile and acute cellular rejection in kidney allografts. N Engl J Med 2013; 369: 20–31
2. Williams WW, Taheri D, Tolkowski-Rubin N et al. Clinical role of the renal transplant biopsy. Nat Rev Nephrol 2012; 8: 110–121
3. Rush D, Nickerson P, Gough J et al. Beneficial effects of treatment of early subclinical rejection: a randomized study. J Am Soc Nephrol 1998; 9: 2129–2134
4. Loupy A, Vernerey D, Tinel C et al. Subclinical rejection phenotypes at 1 year post-transplant and outcome of kidney allografts. J Am Soc Nephrol 2015; 26: 1721–1731
5. Gigliotti P, Lofaro D, Leone F et al. Early subclinical rejection treated with low dose i.v. steroids is not associated to graft survival impairment: 13-years’ experience at a single center. J Nephrol 2016; 29: 443–449
6. Thomas ME, Blaine C, Dawny A et al. The definition of acute kidney injury and its use in practice. Kidney Int 2015; 87: 62–73
7. Haas M, Sis B, Racusen LC et al. Banff 2013 meeting report: inclusion of C4d-negative antibody-mediated rejection and antibody-associated arterial lesions. Am J Transplant 2014; 14: 272–283
8. Furness PN, Taub N. International variation in the interpretation of renal transplant biopsies: report of the CERTPAP Project. Kidney Int 2001; 60: 1998–2012
9. Azancot MA, Moreso F, Salcedo M et al. The reproducibility and predictive value on outcome of renal biopsies from expanded criteria donors. Kidney Int 2014; 85: 1161–1168
10. Einollahi B, Bakhtiari P, Simforoosh N et al. Renal allograft accumulation of technetium-99m sulfur colloid as a predictor of graft rejection. Transplant Proc 2005; 37: 2973–2975
11. Khalifa F, Beach ME, El-Ghar MA et al. Dynamic contrast-enhanced MRI-based early detection of acute renal transplant rejection. IEEE Trans Med Imaging 2013; 32: 1910–1927
12. Blydt-Hansen TD, Sharma A, Gibson IW et al. Urinary metabolomics for noninvasive detection of borderline and acute T cell-mediated rejection in children after kidney transplantation. Am J Transplant 2014; 14: 2339–2349
13. Ong S, Mannon RB. Genomic and proteomic fingerprints of acute rejection in peripheral blood and urine. Transplant Rev 2015; 29: 60–67
14. Pawelski H, Schnöckel U, Kentrup D et al. SPECT- and PET-based approaches for noninvasive diagnosis of acute renal allograft rejection. Biomed Res Int 2014; 2014: 674785
15. Hirt-Minkowski P, De Serres SA, Ho J. Developing renal allograft surveillance strategies – urinary biomarkers of cellular rejection. Can J Kidney Heal Dis 2015; 2: 28
16. Lovinfosse P, Weekers L, Bonvoisin C et al. Fluorodeoxyglucose F(18) positron emission tomography coupled with computed tomography in suspected acute renal allograft rejection. Am J Transplant 2016; 16: 310–316
17. Kurian SM, Williams AN, Gelbart T et al. Molecular classifiers for acute kidney transplant rejection in peripheral blood by whole genome gene expression profiling. Am J Transplant 2014; 14: 1164–1172
18. Banham GD, Clatworthy MR, B-cell biomarkers in transplantation—from genes to therapy. Tissue Antigens 2015; 85: 82–92
19. McGettigan PA. Transcriptomics in the RNA-seq era. Curr Opin Chem Biol 2013; 17: 4–11
20. Wang Z, Gerstein M, Snyder M. RNA-seq: a revolutionary tool for transcriptomics. Nat Rev Genet 2009; 10: 57–63
21. Ding R, Li B, Muthukumar T et al. CD103 mRNA levels in urine and CXCL10 might predict immunological quiescence in clinically and histologically stable kidney recipients. Am J Transplant 2013; 13: 1307–1312
22. Matz M, Beyer J, Wunsch D et al. Early post-transplant urinary IP-10 expression after kidney transplantation is predictive of short- and long-term graft function. Kidney Int 2006; 69: 1683–1690
23. Rabant M, Amrouche L, Morin L et al. Early low urinary CXCL9 and CXCL10 might predict immunological quiescence in clinically and histologically stable kidney recipients. Am J Transplant 2016; 16: 1868–1891
24. Tatapudi RR, Muthukumar T, Dadhania D et al. Noninvasive detection of renal allograft inflammation by measurements of mRNA for IP-10 and CXCR3 in urine. Kidney Int 2004; 65: 2390–2397
25. Muthukumar T, Dadhania D, Ding R et al. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. N Engl J Med 2005; 353: 2342–2351
26. Kotsch K, Mashreghi MF, Bold G et al. Enhanced granulysin mRNA expression in urinary sediment in early and delayed acute renal allograft rejection. Transplantation 2004; 77: 1866–1875
43. Simon T, Opelz G, Wiesel M et al. Serial peripheral blood perforin and granzyme B gene expression measurements for prediction of acute rejection in kidney graft recipients. *Am J Transplant* 2003; 3: 1121–1127

44. Lee B, Oh CK, Kim MS et al. Cytokine gene expression in peripheral blood mononuclear cells during acute renal allograft rejection. *Transplant Proc* 2012; 44: 236–240

45. Striz I, Krasna E, Eliska K et al. Interleukin 18 (IL-18) upregulation in acute rejection of kidney allograft. *Immunol Lett* 2005; 99: 30–35

46. Simon T, Opelz G, Wiesel M et al. Serial peripheral blood interleukin-18 and perforin gene expression measurements for prediction of acute kidney graft rejection. *Transplantation* 2004; 77: 1589–1595

47. Soltaninejad E, Nicknam MH, Nafar M et al. Differential expression of microRNAs in renal transplant patients with acute T-cell mediated rejection. *Transpl Immunol* 2015; 33: 1–6

48. Zheng K, Sun X, Wu W et al. A new index for acute rejection after renal transplant: Notch receptor-1. *Exp Clin Transplant* 2012; 10: 433–438

49. Wang Y-L, Fu Y-X, Zhu Z-J et al. OX40 mRNA in peripheral blood as a biomarker of acute renal allograft rejection. *Chin Med J (Engl)* 2012; 125: 5786–5790

50. Wang Y-W, Wang Z, Shi B-Y. Programmed death 1 mRNA in peripheral blood as biomarker of acute renal allograft rejection. *Chin Med J (Engl)* 2011; 124: 674–678

51. Shin G-T, Kim S-J, Lee T-S et al. Gene expression of perforin by peripheral blood lymphocytes as a marker of acute rejection. *Nephron Clin Pract* 2005; 100: c63–c70

52. Luo Y, Shi B, Qian Y et al. Sequential monitoring of TIM-3 gene expression in peripheral blood for diagnostic and prognostic evaluation of acute rejection in renal graft recipients. *Transplant Proc* 2011; 43: 3669–3674

53. Hancock WW, Lu B, Gao W et al. Requirement of the chemokine receptor CXCR3 for acute renal allograft rejection. *J Exp Med* 2000; 192: 1515–1520

54. Liu CC, Walsh CM, Young JD. Perforin: structure and function. *Immunol Today* 1995; 16: 194–201

55. Atkinson EA, Barry M, Darmon AJ et al. Cytotoxic T lymphocyte-assisted suicide: caspase 3 activation is primarily the result of the direct action of granzyme B. *J Biol Chem* 1998; 273: 21261–21266

56. Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. *Eur J Immunol* 2010; 40: 1830–1835

57. Shaley I, Selznier N, Shyu W et al. Role of regulatory T cells in the promotion of transplant tolerance. *Liver Transpl* 2012; 18: 761–770

58. Buckingham S, Buckingham S. The major world of microRNAs. *Nature* 2003; 1–3

59. Harris A, Kramer SM, Martinez OM. MicroRNAs as immune regulators: implications for transplantation. *Am J Transplant* 2010; 10: 713–719

60. Anglicheau D, Sharma VK, Ding R et al. MicroRNA expression profiles predictive of human renal allograft status. *Proc Natl Acad Sci USA* 2009; 106: 5330–5335

61. Gough MJ, Weinberg AD. OX40 (CD134) and OX40L. *Adv Exp Med Biol* 2009; 647: 94–107

62. Li L, Khush K, Hsieh S-C et al. Identification of common blood gene signatures for the diagnosis of renal and cardiac acute allograft rejection. *PLoS One* 2013; 8: e82153

63. Roedder S, Sigdel T, Salomonis N et al. The kSORT assay to detect renal transplant patients at high risk for acute rejection: results of the multicenter AART study. *PLoS Med* 2014; 11: e1001759

64. Alachkar N. Serum and urinary biomarkers in acute kidney transplant rejection. *Nephrol Ther* 2012; 8: 13–19
65. Betts G, Shankar S, Sherston S et al. Examination of serum miRNA levels in kidney transplant recipients with acute rejection. *Transplantation* 2014; 97: e28–e30.
66. Chandramouli K, Qian P-Y. Proteomics: challenges, techniques and possibilities to overcome biological sample complexity. *Hum Genomics Proteomics* 2009; 2009; pii: 239204.
67. Srivastava M, Eidelman O, Torosyan Y et al. Elevated expression levels of ANXA11, integrins β3 and α3, and TNF-α contribute to a candidate proteomic signature in urine for kidney allograft rejection. *Proteomics Clin Appl* 2011; 5: 311–321.
68. Oetting WS, Rogers TB, Krick TP et al. Urinary beta2-microglobulin is associated with acute renal allograft rejection. *Am J Kidney Dis* 2006; 47: 898–904.
69. O’Riordan E, Orlowa TN, Podust VN et al. Characterization of urinary peptide biomarkers of acute rejection in renal allografts. *Am J Transplant* 2007; 7: 930–940.
70. Lederer SR, Friedrich N, Regenbogen C et al. Non-invasive monitoring of renal transplant recipients: urinary excretion of soluble adhesion molecules and of the complement-split product C4d. *Nephron Clin Pract* 2003; 94: c19–c26.
71. Hricik DE, Nickerson P, Formica RN et al. Multi-center validation of urinary CXL9 as a risk-stratifying biomarker for kidney transplant injury. *Am J Transplant* 2013; 13: 2634–2644.
72. Hu H, Aizenstein BD, Puchalski A et al. Elevation of CXCR3-binding chemokines in urine indicates acute renal-allograft dysfunction. *Am J Transplant* 2004; 4: 432–437.
73. Jackson JA, Kim EJ, Begley S et al. Urinary chemokines CXCL9 and CXCL10 are noninvasive markers of renal allograft rejection and BK viral infection. *Am J Transplant* 2011; 11: 2228–2234.
74. Schaub S, Nickerson P, Rush D et al. Urinary CXCL9 and CXCL10 levels correlate with the extent of subclinical tubulitis. *Am J Transplant* 2009; 9: 1347–1353.
75. Hauser JA, Spiegler S, Kiss E et al. Prediction of acute renal allograft rejection by urinary monokine induced by IFN-gamma (MIG). *J Am Soc Nephrol* 2005; 16: 1849–1858.
76. Blydt-Hansen TD, Gibson IW, Gao A et al. Elevated urinary CXCL10-to-creatinine ratio is associated with subclinical and clinical rejection in pediatric renal transplantation. *Transplantation* 2015; 99: 797–804.
77. Peng W, Chen J, Jiang Y et al. Urinary fractalkine is a marker of acute rejection. *Kidney Int* 2008; 74: 1454–1460.
78. Heyne N, Kemmner S, Schneider C et al. Urinary neutrophil gelatinase-associated lipocalin accurately detects acute allograft rejection among other causes of acute kidney injury in renal allograft recipients. *Transplant J* 2012; 93: 1252–1257.
79. Sigdel TK, Salomonis N, Nicora CD et al. The identification of novel potential injury mechanisms and candidate biomarkers in renal allograft rejection by quantitative proteomics. *Mol Cell Proteomics* 2014; 13: 621–631.
80. Zhang Q, Liu Y-F, Su Z-X et al. Serum fractalkine and interferon-gamma inducible protein-10 concentrations are early detection markers for acute renal allograft rejection. *Transplant Proc* 2014; 46: 1420–1425.
81. Nafar M, Farrokhhi F, Vaezi M et al. Pre-transplant and post-transplant soluble CD30 for prediction and diagnosis of acute kidney allograft rejection. *Int Urol Nephrol* 2009; 41: 687–693.
82. Pelzl S, Opelz G, Daniel V et al. Evaluation of posttransplantation soluble CD30 for diagnosis of acute renal allograft rejection. *Transplantation* 2003; 75: 421–423.
83. Shooostarizadeh T, Mohammadali A, Ossareh S et al. Relation between pretransplant serum levels of soluble CD30 and acute rejection during the first 6 months after a kidney transplant. *Exp Clin Transplant* 2013; 11: 229–233.
84. Kutukcuier N, Clark K, Rigg KM et al. The value of posttransplant monitoring of interleukin (IL)-2, IL-3, IL-4, IL-6, and soluble CD23 in the plasma of renal allograft recipients. *Transplantation* 1995; 59: 333–340.
85. Le Meur Y, Leprivey-Longevet V, Mons S et al. Serum levels of macrophage-colony stimulating factor (M-CSF): a marker of kidney allograft rejection. *Nephrol Dial Transplant* 2004; 19: 1862–1865.
86. Freue GVC, Sasaki M, Meredith A et al. Proteomic signatures in plasma during early acute renal allograft rejection. *Mol Cell Proteomics* 2010; 9: 1954–1967.
87. Xu X, Huang H, Cai M et al. Combination of IL-1 receptor antagonist, IL-20 and CD40 ligand for the prediction of acute cellular renal allograft rejection. *J Clin Immunol* 2013; 33: 280–287.
88. Panzer U, Reinkinger RR, Steinmetz OM et al. CXCR3 and CCR5 positive T-cell recruitment in acute human renal allograft rejection. *Transplantation* 2004; 78: 1341–1350.
89. Rabant M, Amrouche L, Lebreton X et al. Urinary C-X-C motif chemokine 10 independently improves the noninvasive diagnosis of antibody-mediated kidney allograft rejection. *J Am Soc Nephrol* 2015; 26: 2840–2851.
90. Gwinner W, Metzger J, Husi H et al. Proteomics for rejection diagnosis in renal transplant patients: where are we now? *World J Transplant* 2016; 6: 28–41.
91. Yoshol H, Brenden N, Müller D et al. Evaluation of biomarker discovery approaches to detect protein biomarkers of acute renal allograft rejection. *J Proteome Res* 2005; 4: 1192–1199.
92. Cibrik DM, Warner RL, Kommareddi M et al. Identification of a protein signature in renal allograft rejection. *Proteomics Clin Appl* 2013; 7: 839–849.
93. Horie R, Watanabe T. CD30: expression and function in health and disease. *Semin Immunol* 1998; 10: 457–470.
94. Traill NC. [Estimation of soluble serum CD30 in the diagnosis of early renal allograft dysfunction]. *Klin Khir* 2009; 10: 44–46.
95. Weiss RH, Kim K. Metabolomics in the study of kidney diseases. *Nat Rev Nephrol* 2011; 8: 22–33.
96. Weekers L, de Tullio P, Bovy C et al. Activation of the calcium-sensing receptor before renal ischemia/reperfusion exacerbates kidney injury. *Am J Transpl* 2015; 7: 128–138.
97. Suhre K, Schwartz JE, Sharma VK et al. Urine metabolite profiles predictive of human kidney allograft status. *J Am Soc Nephrol* 2016; 27: 626–636.
98. Zhao X, Chen J, Ye L et al. Serum metabolomics study of the acute graft rejection in human renal transplantation based on liquid chromatography-mass spectrometry. *J Proteome Res* 2014; 13: 2659–2667.
99. Mao Y-Y, Bai J-Q, Chen J-H et al. A pilot study of GC/MS-based serum metabolic profiling of acute rejection in renal transplantation. *Transpl Immunol* 2008; 19: 74–80.
100. Ammerlaan W, Trezzi J-P, Mathay C et al. Method validation for preparing urine samples for downstream proteomic and metabolomic applications. *Biopreserv Biobank* 2014; 12: 351–357.
acute renal transplant rejection. J Clin Ultrasound 2001; 29: 483–490

103. Liu G, Han F, Xiao W et al. Detection of renal allograft rejection using blood oxygen level-dependent and diffusion weighted magnetic resonance imaging: a retrospective study. BMC Nephrol 2014; 15: 158

104. Bohra R, Klepacki J, Klawitter J et al. Proteomics and metabolomics in renal transplantation—quo vadis? Transpl Int 2013; 26: 225–241

105. Sigdel TK, Gao Y, He J et al. Mining the human urine proteome for monitoring renal transplant injury. Kidney Int 2016; 89: 1244–1252