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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 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

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and gender [2]. Furthermore, subclinical AR affects 10–30% of KTRs within the first year after KTx and is an early predictor of subsequent graft failure [3–5]. Subclinical AR has been defined as 'the documentation by light histology of unexpected evidence of AR in a stable patient'. Such a significant incidence of subclinical AR has encouraged some transplant centres to perform 'surveillance' transplant biopsies between 3 and 12 months after-KTx. Since current immunosuppressive drugs efficiently treat AR, diagnosing AR early is crucial. Regarding the particular case of subclinical AR, there is still no consensus as to whether it should be treated or not. Although there are centres that treat subclinical AR, there are many others that do protocol biopsies but do not compulsively treat those patients, because of the lack of strong evidence about the risk/benefit balance of increased immunosuppression. In a 10-year observational prospective cohort study of 1001 consecutive non-selected KTRs who underwent ABO-compatible, complement-dependent cytotoxicity-negative crossmatch KTx and who underwent screening biopsies at 1 year, treatment of subclinical T cell–mediated AR may result in similar long-term graft survival as in patients without rejection. In contrast, subclinical antibody-mediated AR detected at the 1-year screening biopsy carries a negative prognostic value independent of initial donor-specific antibody status, previous immunologic events, current estimated glomerular filtration rate (eGFR) and proteinuria [4].

In clinical practice, the detection of AR mostly relies on periodic assessments of serum creatinine (SCr), an insensitive measure of renal injury [6]. Ultimately, an AR diagnosis requires transplant needle biopsy. Examining kidney samples by light microscopy provides well-characterized and gold-standard criteria for renal AR, as summarized in the conventional Banff classification [2, 7]. However, such an invasive procedure may cause graft bleeding or arteriovenous fistula. In addition, distinct reports highlighted sampling error and interobserver variability [8, 9]. Moreover, repeated biopsies to evaluate renal graft status pose challenges, including practicability and cost. Therefore, alternative, less invasive but as sensitive modalities are currently under investigation to reinforce our armamentarium in AR diagnosis [1, 10–16]. Likewise, it would be useful to non-invasively predict non-rejection in KTRs with acute renal dysfunction and suspected AR to avoid needless transplant biopsy. The term 'acute dysfunction with no rejection' (ADNR) has been recently proposed by Kurian *et al.* [17] to reflect such a condition in which AR is suspected on the basis of clinical and biological judgments but not confirmed by histology. The mechanisms and long-term consequences of ADNR remain unknown.

In the present review, we summarize the current literature about non-invasive approaches for the diagnosis of AR in KTRs, including gene-expression profiling and omics analyses of blood and urine samples. A large number of studies have looked for biomarkers in the field of renal AR. In particular, the development of omics technologies, including transcriptomics, proteomics and metabolomics, that respectively quantify the abundance of mRNA, proteins and metabolites present in cells, tissue extracts or biofluids, has opened up new opportunities in the non-invasive diagnosis of renal AR. Most studies have focused on T cell–mediated AR, with less information about antibody-mediated AR. Banham and Clatworthy [18] have recently detailed the emerging literature about putative B cell biomarkers. Of note, the benefits and limitations of imaging methods, such as magnetic resonance imaging (MRI) and ^{18}F-fluorodeoxyglucose positron emission computed tomography (^{18}F-FDG-PET/CT), in the workup of renal AR are reviewed in a complementary Part I article.

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 pathological 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 KTx, including at the time of per-cause transplant biopsy. Levels of mRNA of CD3ε, 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 CD3ε 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 CD3ε 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

Table 1. Transcriptomics in the non-invasive diagnosis of renal acute rejection

Transcriptomics	Gene	Se/Sp (%)	*n* (aRx)	References
Urine	CD 103	59/75	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	Seiler *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	Seiler *et al.* [27]
	miR-210	52/74	62	Lorenzen *et al.* [31]
	NKG2D	77/81	31	Seiler *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	Afaneh *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	92/90	25	Shoker *et al.* [36]
	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	Sarwal *et al.* [40]
	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]
			6	Lee *et al.* [44]
	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	NA	Striz *et al.* [45]
		64/92	17	Simon *et al* [46]
	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]
		NA	7	Shin *et al.* [51]
		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.

pass quality controls [1]. Urine specimens were classified as passing quality control if the 18S rRNA copy number was $\geq 5 \times 10^7$/μg total RNA isolated from the urine pellet and if the TGF-β1 mRNA copy number was ≥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.

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.* [31] 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.* [60] 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 intrarenal 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 Kurian *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 OX4O 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/Ib 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

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]
		86/80	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]
	CXCL10 (IP-10)	86.4/91.3	28	Hu *et al.* [72]
	CXCL10:Cr	80/76	25	Jackson *et al.* [73]
		68/90	22	Schaub *et al.* [74]
		77/60	35	Blydt-Hansen *et al.* [76]
		81.6/50.8	300	Rabant *et al.* [23]
	Fractalkine	74.4/75	67	Peng *et al.* [77]
	Integrin α3	NA	10	Srivastava *et al.* [67]
	Integrin β3	NA	10	Srivastava *et al.* [67]
	NGAL	90 (cut-off = 30 ng/mL)/91 (cut-off >130 ng/mL)	9	Heyne *et al.* [78]
	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)	NA	74	Sigdel *et al.* [79]
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]
		88/100	25	Pelzl *et al.* [82]
		70/73.6	10	Shooshtarizadeh *et al.* [83]
	Fractalkine	73.3/65	15	Zhang *et al.* [80]
	IL-2	NA	7	Kutukculer *et al.* [84]
	IL-4	NA	7	Kutukculer *et al.* [84]
	IL-6	NA	7	Kutukculer *et al.* [84]
	M-CSF	80/NA	25	Le Meur *et al.* [85]
	18 plasma proteins (titin, lipopolysaccharide-binding protein, peptidase inhibitor 16, complement factor D, etc.)	80/90[a]	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.
[a]Classification of BCAR based on a four-protein ELISA classifier: CFD, LCAT, SHBG and F9.

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-Minkowki *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 accurate

Table 3. Metabolomics in the non-invasive diagnosis of renal acute rejection

Metabolomics	Metabolite	Se/Sp (%)	*n* (aRX)	References
Urine	Kynurenine	83/83	183	Blydt-Hansen *et al.* [12]
	Proline	83/83	183	Blydt-Hansen *et al.* [12]
	mRNA signature + 1.1164*log(3-sialyllactose/xanthosine) kynurenine	82/87	242	Suhre *et al.* [97]
Blood	Creatinine, kynurenine, uric acid, polyunsaturated fatty acid, phosphatidylcholines, sphingomyelins, lysophosphatidylcholines and more specifically Kyn/trp	NA	11	Zhao *et al.* [98]
	Levels of 17 metabolites, including amino acids, carbohydrates, carboxylic acids, lipids, lactate, urea and myo-inositol	NA	22	Mao *et al.* [99]
	Levels of alanine, lysine, leucine, aminomalonic acid and tetradecanoic acid	NA	22	Mao *et al.* [99]

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

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, peptidase inhibitor 16, complement factor D, mannose-binding lectin, protein Z-dependent protease, β2-microglobulin, kininogen-1, afamin, serine protease inhibitor, phosphatidylcholine-sterol acyltransferase and sex hormone–binding globulin [86].

Finally, a prospective study including 77 KTRs was performed by Shooshtarizadeh *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, Trailin *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, phosphatidylcholines, 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 ^{18}F-FDG-PET/CT [16, 103], and urine biomarkers, including CXCL9, CXCL10 or a three-gene signature of CD3ε, 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.

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