Research article

ARHGDIB and AT1R autoantibodies are differentially related to the development and presence of chronic antibody-mediated rejection and fibrosis in kidney allografts

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ABSTRACT

The role of non-HLA autoantibodies in chronic-active antibody-mediated rejection (c-aABMR) of kidney transplants is largely unknown. In this study, the presence and clinical relevance of non-HLA autoantibodies using a recently developed multiplex Luminex-based assay were investigated. Patients with a kidney allograft biopsy at least 6 months after transplantation with a diagnosis of c-aABMR (n = 36) or no rejection (n = 21) were included. Pre-transplantation sera and sera at time of biopsy were tested for the presence of 14 relevant autoantibodies.

A significantly higher signal for autoantibodies against Rho GDP-dissociation inhibitor 2 (ARHGDIB) was detected in recipients with c-aABMR as compared to recipients with no rejection. However, ARHGDIB autoantibodies did not associate with graft survival. Levels of autoantibodies against angiotensin II type 1-receptor (AT1R) and peroxisomal trans-2-enoyl-CoA reductase (PECR) were increased in recipients with interstitial fibrosis in their kidney biopsy. Only the signal for AT1R autoantibody showed a linear relationship with the degree of interstitial fibrosis and was associated with graft survival.

In conclusion, anti-ARHGDIB autoantibodies are increased when c-aABMR is diagnosed but are not associated with graft survival, while higher levels of AT1R autoantibody are specifically associated with the presence of interstitial fibrosis and graft survival.

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1. Introduction

Long-term graft loss of the kidney allograft has changed little in the last decades and improving this outcome is considered an unmet need [1]. Chronic-active antibody mediated rejection (c-aABMR) is recognized as one of the major causes of graft loss in the long term in recipients of a donor kidney [2,3]. The median time to diagnosis is about 6 years with a broad range between 1 year to 15 years after kidney transplantation [4,5]. Histology essentially shows signs of chronic microvascular inflammation (glomerulitis and peritubular capillaritis) leading to endothelial basal membrane duplication and finally interstitial fibrosis and tubular atrophy [6]. The presence of serum anti-donor antibodies is believed to be underlying the pathogenesis. A variable degree of complement activation can be shown as C4d deposition along the capillary walls and binding and activation of Fc-receptor bearing immune cells, notably monocytes and NK cells, results in local production of inflammatory mediators like IL-6 [7,8].

Antibodies against donor tissue may consist of donor-specific HLA-antibodies (DSA) or non-HLA antibodies, which both may be already present before transplantation or arise as de novo antibodies. However, in a substantial percentage of recipients with a diagnosis of c-aABMR no anti-HLA DSA can be detected. Clinical presentation, renal histology and rate of graft loss is not different between recipients with or without detectable anti HLA at time of diagnosis [9,10]. Fluctuations in DSA serum concentrations and antibody absorption by the donor kidney are possible explanations, as well as antibodies directed against non-HLA antigens [11]. Recently, a multiplex luminex technique-based assay was developed for the detection of non-HLA antibodies against target proteins, which were selected based on relevant published studies [12]. In a large Dutch retrospective cohort study it was shown that presence of antibodies against ARGHDIB prior to kidney transplantation was an independent risk factor for long-term kidney allograft survival [13]. In a subsequent study from Senev et al, the

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MFI level of ARHGBIB autoantibodies, particular in combination with DSA, was significantly associated with the risk for graft failure in patients with a histological diagnosis of AMBR but not in the control group of non-ABMR patients [14]. In addition, ARGHDIB antigen and mRNA showed increased expression in the kidney with ABMR.

In the current study serum autoantibody levels at time of diagnosis of c-aABMR and before kidney transplantation were studied in relation to Banff scores of the kidney biopsy, degree of interstitial fibrosis and graft survival.

2. Material and methods

2.1. Study population

Kidney transplant recipients with a for cause kidney biopsy at least 6 months post transplantation within the period June 2015-January 2018 were included in this study (n = 68). No routine HLA DSA screening protocol was present. The cause for biopsy was progressive loss of graft function or proteinuria not otherwise explained. The date of last follow-up was january 2020 yielding a maximum follow-up time of 60 months. Recipients were diagnosed as having either c-aABMR (rejection group) or without any sign of rejection but a variable degree of chronic damage (no rejection group, NR). Biopsies with another diagnosis (e.g. glomerulonephritis, BK nephropathy, diabetic nephropathy or TCMR) were excluded. Clinical and laboratory data were collected at time of biopsy. Kidney biopsies were scored according to the Banff '17 criteria by an experienced renal pathologist. Immunohistochemical staining for C4d was not available in 1 patient. If the first 2 criteria of the Banff were fulfilled than a diagnosis of c-aABMR histology(c-aABMRh) was made, in accordance with recent publications [4,5,14,15]. Chronic damage was assessed along the Banff criteria for tubular atrophy, interstitial fibrosis, arteriolar hyalinosis and vascular fibrous intimal thickening.

Allograft failure was defined by the need for starting dialysis treatment or a kidney re-transplantation. The local medical ethical committee approved the study (MEC-2015-222) and humans involved in this study were treated in accordance with the Declaration of Helsinki and the clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the 'Declaration of Istanbul on Organ Trafficking and Transplant Tourism'

2.2. Characterization of anti-HLA antibodies

All patients were transplanted ABO-blood group compatible with a CDC negative crossmatch. All sera at time of diagnosis of c-aABMR(h) were screened for the presence of donor-specific antibodies against HLA (DSA) using the Lifecodes Lifescreen Deluxe (LMX) kit, according to the manufacturer's manual (Immunocor Transplant Diagnostics Inc. Stamford, CT, USA). Samples were considered positive for either HLA class I (HLA-A or HLA-B or HLA-C) or HLA class II (HLA-DQ or HLA-DR) antibodies were further analyzed with a Luminex Single Antigen assay, using LABscreen HLA class I and class II antigen beads (One Lambda Canoga Park, GA, USA). All tested sera were heat-treated for 30 min at 56 Celsius to avoid interference of complement in the Luminex assay.

An in house assay was developed for detection of a selection of relevant non-HLA target proteins (n = 14), as has been described in detail previously [12,13,16]. Target proteins were: agrin, adipocyte plasma membrane-associated protein (APMAP), Rho GDP-dissociation inhibitor 2 (ARHGDIB), Rho guanine nucleotide exchange factor 6 (ARHGEF6), endorepellin, Lamin B1 (LNMB1), BPI fold-containing family B member 1 (LPLUNC1), protein kinase C zeta type (PRKCZ), tubulin beta-4B (Tubb4B), Angiotensin II type 1-receptor (AT1R) and endothelin type A receptor (ETAR), vimentin, peroxisomal trans-2-enoyl-CoA reductase (PECR) and phospholipase A2 receptor (PLA2R).

AT1R and PECR were coupled directly with carboxylated Mag-Plex Microspheres (Luminex Corp, Austin, TX). The other 12 of the 14 proteins with a HaloTag to optimize antibody binding. The HaloTag proteins were coupled via a HaloTag Amine (O4) Ligand (Promega, Madison, WI) to the carboxylated MagPlex microspheres. Transferrin (directly- or HaloTag-coupled) served as a negative control, since it is ubiquitously present and no autoantibodies against transferrin have been reported. Sera (1:25 dilution) were incubated overnight with the microsphere mix. Next, R-phycoerythrin-conjugated goat-anti human antibody was added. After 30 min of incubation, wash buffer was added and samples were measured on a Luminex 200 flow analyzer (Luminex Corp).

Transferrin (directly- or HaloTag-coupled) served as a negative control. The data were expressed as mean fluorescence intensity MFI and signal to background ratio (STBR) using the MFI for transferrin as the background signal.

2.3. Statistical analysis

Normally distributed data are expressed as mean +/− SD, non-normally distributed data as median with 25th and 75th percentiles. A p-value of <0.05 was considered statistically significant. Differences between groups of non-normally distributed data were analyzed by the Mann-Whitney test and the Wilcoxon signed-rank test for paired data. Correlations between clinical parameters (eGFR (CKD-EPI formula) at diagnosis, time after transplantation and presence of DSA and non-DSA anti-HLA antibodies) and signals of autoantibodies of interest were investigated. The signal intensity of autoantibodies and Banff scores of chronic damage were analyzed in a dichotomous fashion (present or absent) and by exploring the relation with the Banff score using ANOVA linear trend analysis.

Death-censored graft survival was assessed by Kaplan-Meier analysis with log-rank statistics for difference between strata. Univariate Cox regression analysis was performed to identify an association between the intensity of the autoantibody signals and graft survival after biopsy-proven diagnosis. Crude MFI values normalized by log transformation and/or STBR ratios were used as variables. Statistical analysis was performed with software IBM SPSS statistics 21.

3. Results

3.1. Patient characteristics

The clinical and demographical characteristics of the patients/recipients are shown in Table1. The percentage of recipients with a living donor kidney was about 70%. The c-aABMRh group versus the no rejection (NR) group did not differ with regard to recipient age at transplantation, donor age and the time of biopsy after transplantation. Median follow-up after diagnosis was shorter in the c-aABMRh group because of a higher rate of graft failure.

The c-aABMRh group more often had a positive PRA (>4%) at time of transplantation, but similar median number of HLA mismatches as compared to the NR group. eGFR at time of biopsy was similar for both groups but the c-aABMRh group had a significantly higher level of proteinuria.

3.2. ARHGDIB autoantibodies are significantly increased in cases with c-aABMRh at time of diagnosis

The unadjusted MFI and STBR at time of diagnostic kidney biopsy are shown for all autoantibodies in Table 2. Of note is that

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Table 1
Clinical and demographical characteristics.

	No rejection	c-aABMR	p-value
Male/female recipient	11/10	25/11	>0.1
Age recipient at time transplantation median years (IQR*)	49 (35–64)	44 (31–61)	>0.1
Time to biopsy median months (IQR)	48 (32–91)	42 (28–74)	>0.1
Follow-up after biopsy median months (IQR)	42 (28–52)	29 (23–41)	0.02
Graft survival censored for death at 3 year follow up	88%	75%	0.01
PRA > 4% at time of transplantation	18%	50%	0.03
HLA mismatches median	4	3	>0.1
DSA present (%)**	–	19 (53%)	–
Non-DSA anti-HLA antibodies present (%)	–	20 (56%)	–
Re-transplantation (%)	20%	30%	>0.1
Living kidney donor	80%	78%	>0.1
Age donor years Median, (IQR)	53 (45–60)	50 (45–60)	>0.1
eGFR at time of biopsy	32 (26–40)	34 (27–45)	>0.1
Urine protein/creatinine ratio (mg/mmol)	27 (9–52)	80 (24–132)	0.003

* IQR: interquartile range.
** % of total cases within group.

most MFI values were in the low range and only the median MFI and STBR for ARHGDIB autoantibodies were significantly higher in the c-aABMRh group (Table 2 and Fig. 1). The median signal of ARHGDIB autoantibodies was significantly higher at time of biopsy versus pre-transplantation in the c-aABMRh group (MFI 912 vs 694, p = 0.03 and STBR 2.08 vs 1.17, p = 0.04), but not in the NR group (Fig. 1 and Supplemental Fig. 1). The median MFI for ARHG-DIB antibodies was similar between both groups at time of kidney transplantation.

Previous reports showed a relation between a high MFI for ARHGDIB and increased risk for respectively graft loss and ABMR, using the same autoantibody assay [14]. Using a cut off for the MFI for ARGHDIB above 1000 (corresponding to the median MFI level, Fig. 1) or below or above the median STBR (median = 2, data not shown) did not reveal a difference in graft survival in the c-aABMRh group. In addition, we could not find any association between MFI for ARHGDIB (or any other auto-antibody) and the presence of DSA or non-DSA HLA antibodies (data not shown).

PECR (Fig. 2A, Supplemental Fig. 1) and AT1R (Fig. 2B, Supplemental Fig. 1) autoantibodies increased significantly over time but with no apparent differences between both the NR and c-aABMRh groups. The other autoantibodies showed a remarkable stable median signal, comparing time at transplantation with time at biopsy, 40–50 months post-transplantation (Supplement Fig. 2). Autoantibodies to every target protein tested, did not correlate significantly with any other clinical or demographical parameter as shown in Table 1. In addition, MFIs of auto-antibodies did not correlate with presence of vascular inflammation in the c-aABMR biopsies.

3.3. PECR and AT1R autoantibodies associate with interstitial fibrosis and graft survival

The signal strength of the autoantibody panel was related to the Banff scores for chronic damage. Only the PECR and AT1R autoantibodies showed an association with the score for interstitial fibrosis with no significant difference between c-aABMRh and NR. Recipients with any degree of interstitial fibrosis in the kidney biopsy revealed a higher median MFI for PECR and AT1R as compared to patients without interstitial fibrosis in their biopsies

Table 2
Non-HLA antibody mean fluorescence intensity and signal-to-background ratio at time of diagnostic kidney biopsy.

	no rejection (N = 21)	c-aABMR (N = 36)	p-value
Agrin			
MFI*, median (IQR)	368 (213–521)	385 (271–487)	0.7
STBR**	0.95 (0.86–1.15)	1.03 (0.91–1.29)	0.4
APMAB			
MFI	347 (285–530)	409 (336–586)	0.2
STBR	1.02 (0.96–1.19)	1.17 (0.96–1.62)	0.08
ARGHDIB			
MFI	694 (369–1363)	912 (642–2422)	0.03
STBR	1.17 (0.87–1.74)	2.08 (1.31–3.99)	0.04
ARHGEF			
MFI	375 (272–562)	418 (298–595)	0.6
STBR	1.17 (0.97–1.34)	1.17 (0.97–2.45)	0.7
AT1R			
MFI	1967 (1271–3867)	2044 (1400–4458)	0.7
STBR	8.7 (6.6–14.6)	10.0 (6.9–24.3)	0.3
Endorepellin			
MFI	361 (259–502)	398 (291–3532)	0.4
STBR	1.05 (0.93–1.16)	1.05 (0.92–1.49)	0.4
ETAR			
MFI	402 (269–401)	374 (279–577)	0.5
STBR	1.13 (0.95–1.46)	1.14 (0.91–1.30)	0.5
LMNB1			
MFI	421 (289–589)	435 (305–609)	0.4
STBR	1.12 (0.99–1.33)	1.19 (1.05–1.37)	0.4
LPLUNC1			
MFI	392 (278–579)	422 (296–607)	0.5
STBR	1.15 (1.02–1.22)	1.12 (1.00–1.44)	0.5
PECR			
MFI	851 (584–1878)	626 (480–1040)	0.3
STBR	3.34 (2.52–5.45)	3.23 (2.26–4.61)	0.8
PLA2R1			
MFI	439 (279–539)	402 (290–528)	0.9
STBR	1.05 (0.92–1.29)	1.02 (0.92–1.27)	0.8
PRKCZ			
MFI	475 (268–617)	458 (376–668)	0.7
STBR	1.24 (1.12–1.43)	1.28 (1.16–1.60)	0.2
Tubb4B			
MFI	373 (284–559)	465 (333–658)	0.13
STBR	1.16 (1.01–1.25)	1.36 (1.06–1.68)	0.2
Vimentin			
MFI	393 (239–620)	374 (280–526)	0.8
STBR	1.07 (0.91–1.13)	1.00 (0.86–1.30)	0.8

* IQR: interquartile range, MFI: mean fluorescence signal, STBR:signal to background ratio.

(Fig. 2). Of note, the pre-kidney transplantation levels were already increased in patients who developed interstitial fibrosis later on in their kidney biopsies, but remained low in patients without interstitial fibrosis. Only the MFI of AT1R autoantibodies showed a significant association with the degree of interstitial fibrosis, with a linear progression in median levels of MFI (p = 0.02, Fig. 3). There was no correlation between MFI levels for PECR and AT1R (data not shown).

The median MFI level for PECR and AT1R was used to divide recipients in low versus high autoantibody levels. Only high versus low MFI of AT1R showed a significant difference in graft survival in c-aABMRh (Fig. 4). Graft survival censored for death at 3 years was 95% in the AT1R low-group and 70% in the AT1R high-group. By Cox regression, a higher hazard ratio for graft failure after diagnosis of c-aABMRh was found in relation to signal strength of ARHG-DIB autoantibodies at time of biopsy (HR 1.57, p = 0.05 for log2 normalized MFI and HR 1.04, p = 0.014 for STBRs). Although hazard ratios in the same range as time of biopsy were found for pre-kidney transplant values of ARHGDIB autoantibodies, this association did not reach statistical significance (data not shown).

The incidence of graft loss in the NR group was to low (n = 4) for meaningful analysis but 3 out of 4 graft losses occurred in the AT1R high group.

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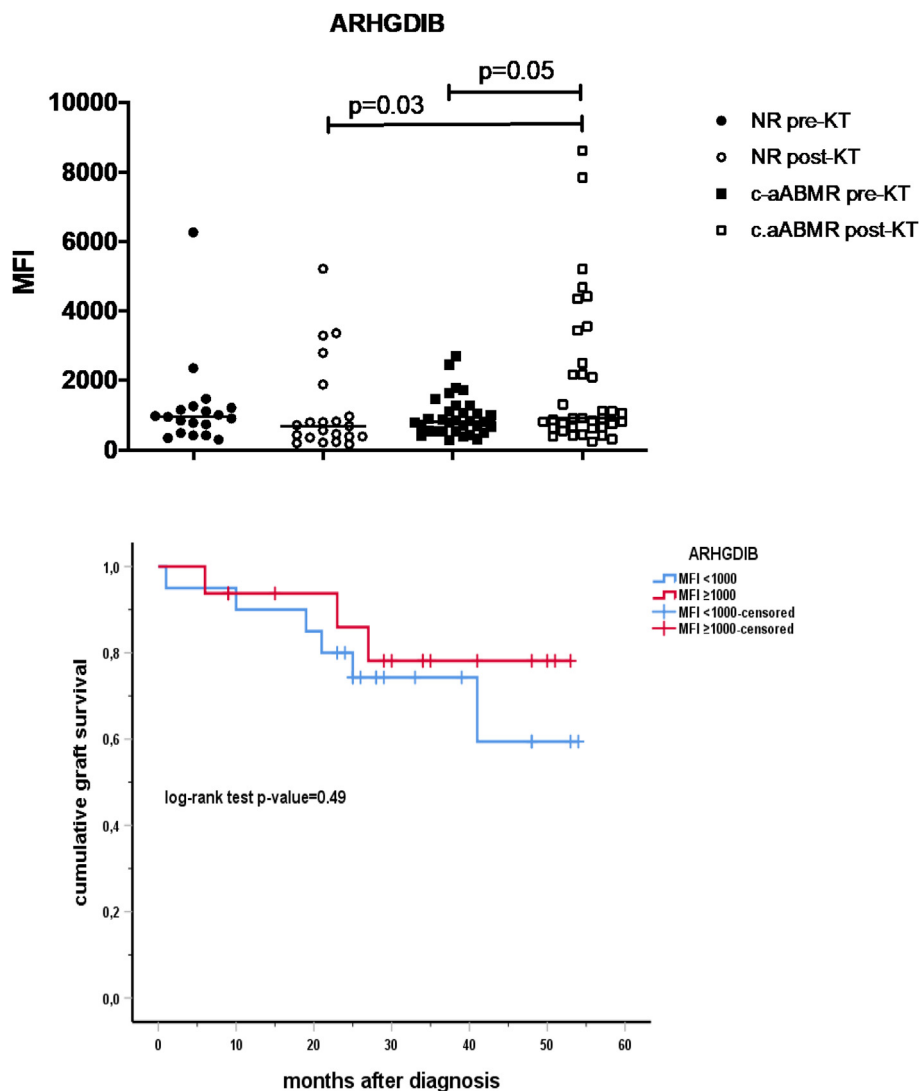
Fig. 1. Mean fluorescence intensity (MFI) for ARHGDIB autoantibodies for cases with a diagnosis of c-aABMR or no rejection (NR) before kidney transplantation (pre-KT) or at time of kidney biopsy (post-KT). The dotted horizontal line is the median MFI for the negative control (transferrin microbeads). Only p-values < 0.05 for comparison between groups are shown. The lower panel shows graft survival after the biopsy-proven diagnosis of c-aABMR according to the level of ARHGDIB autoantibodies (above or below median MFI).

3.4. Discussion

The results of this study show that using a multiplex autoantibody assay, the MFI of 3 out of 14 selected target proteins showed a relation with either a diagnosis of c-aABMR or interstitial fibrosis and graft survival. Autoantibodies against ARHGDIB were exclusively increased at time of biopsy in the cases diagnosed with c-aABMRh while the MFI remained stable in the cases without rejection. The levels of AT1R and PECR autoantibodies were specifically related to the presence of interstitial fibrosis in the kidney biopsy and AT1R autoantibodies also with graft survival. These associations are of interest and appear to be specific, as MFIs of the autoantibodies to 11 other target proteins remained remarkably stable in both patient groups.

This study shows for the first time an association between ARGHDIB autoantibodies and a diagnosis of c-aABMR. The first publication with the multiplex assay used in this study found that increased MFI for ARGHDIB pre-kidney transplantation was associated with decreased graft survival, independent of the presence of DSA [13]. The chosen cut off for both unadjusted MFI and STBR was high (the top 2.9%) and there was no information about the cause of graft loss. In a completely different cohort of patients, Senev et al. showed that cases of early ABMR had a significantly higher MFI for ARHGDIB at time diagnosis [14]. In addition, a high MFI (defined as > 1000), particular in association with the presence of DSA, was an independent risk factor for kidney graft loss. Of interest, the ARGHDIB protein was expressed in relevant kidney cells (e.g.endothelial cells) [13,14] and intrarenal mRNA levels were sharply increased in cases of ABMR [14].

Our findings constitute the second cohort of cases in which a relation was found between ARGHDIB and humoral rejection, although the number of cases is smaller and includes patients with chronic AMBR long after transplantation. This is a relevant difference as the previous publications in particular showed an early effect on graft survival of ARGHDIB autoantibodies. Time of diagnosis, in particular early acute ABMR versus late c-aABMR, has a dif-

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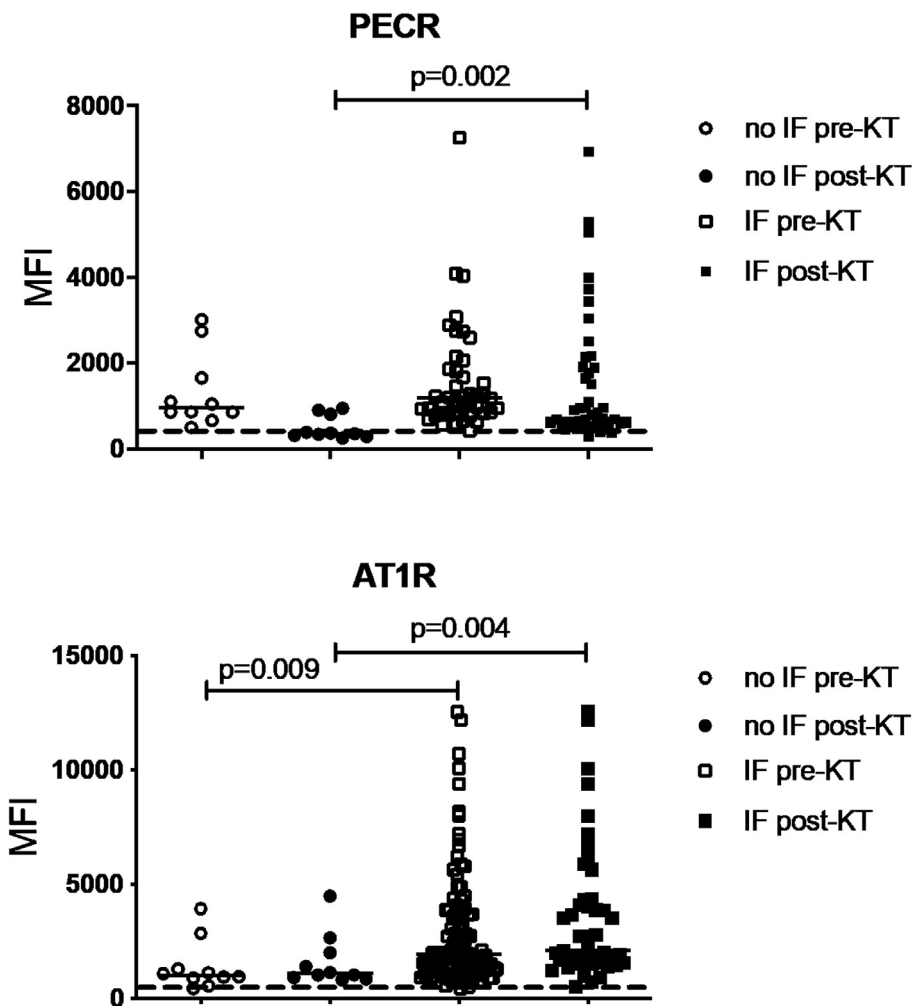
Fig. 2. The mean fluorescence intensity (MFI) for autoantibodies against PECR (A) and AT1R (B) before kidney transplantation (pre-KT) or at time of kidney biopsy (post-KT) in relation to the presence or absence of interstitial fibrosis (IF) in the kidney biopsy. The dotted horizontal line is the median MFI for the negative control (transferrin microbeads). Only p-values < 0.05 for comparison between groups are shown.

ferent relation with response to therapy and the presence of DSA as a risk factor for decreased graft survival [17,18]. For instance, in cases of c-aABMR the presence of DSA is not associated with graft survival [9,10]. Therefore, the lack of association of autoantibodies for ARGHDIB and graft survival after c-aABMR as found in this study is not necessarily in contradiction with the results of the other studies. A possible scenario could be that pre-transplant ARGHDIB autoantibodies aggravate an inflammatory response when the intracellular located protein in the damaged endothelial cells is exposed during acute ABMR. ARHGDIB may activate the humoral immune response as it is recognized as a minor alloantigen [19]. In contrast, c-aABMR is a relatively slow immunological process evolving over a number of years and this may lead to a mild increased exposure of ARHGDIB to the immune system and subsequent increase of autoantibody titers. The increase in titers, as reflected in the mild increase in MFI for ARHGDIB observed in this study, is then a result of the ongoing chronic inflammation but does not foster the process itself and hence does not contribute to graft loss. Alternatively, increasing ARHGDIB auto-antibody levels could potentially be involved in leading to chronic ABMR, which once present, is a risk factor for allograft loss.

A novel finding was the relation between MFI levels for PECR and AT1R and interstitial fibrosis in the kidney biopsy. This association was relatively weak for PECR autoantibodies and did not translate in a clinical relevant end-point like graft survival. This is important, as argued before in a previous publication, autoantibodies can be detected for many proteins and a sensible cut-off needs to be defined, preferably based on clinical significance [16].

Of interest is that several studies have shown a relation between AT1R autoantibodies and graft loss but not in relation to the degree of fibrosis as shown in this study [20–23].

The level of AT1R autoantibodies was significantly lower and close to the MFI of the negative control in recipients with no interstitial fibrosis in their renal biopsies. In addition, the MFI increased significantly from pre-transplant to time of biopsy in the cases with interstitial fibrosis but remained low in the cases with now interstitial fibrosis. Therefore, a high MFI for AT1R autoantibodies appeared to have a strong negative predictive value for the outcome "no interstitial fibrosis" in the allograft kidney even when c-aABMR is the underlying pathology. In combination with the association of MFI levels and graft survival, these findings argue

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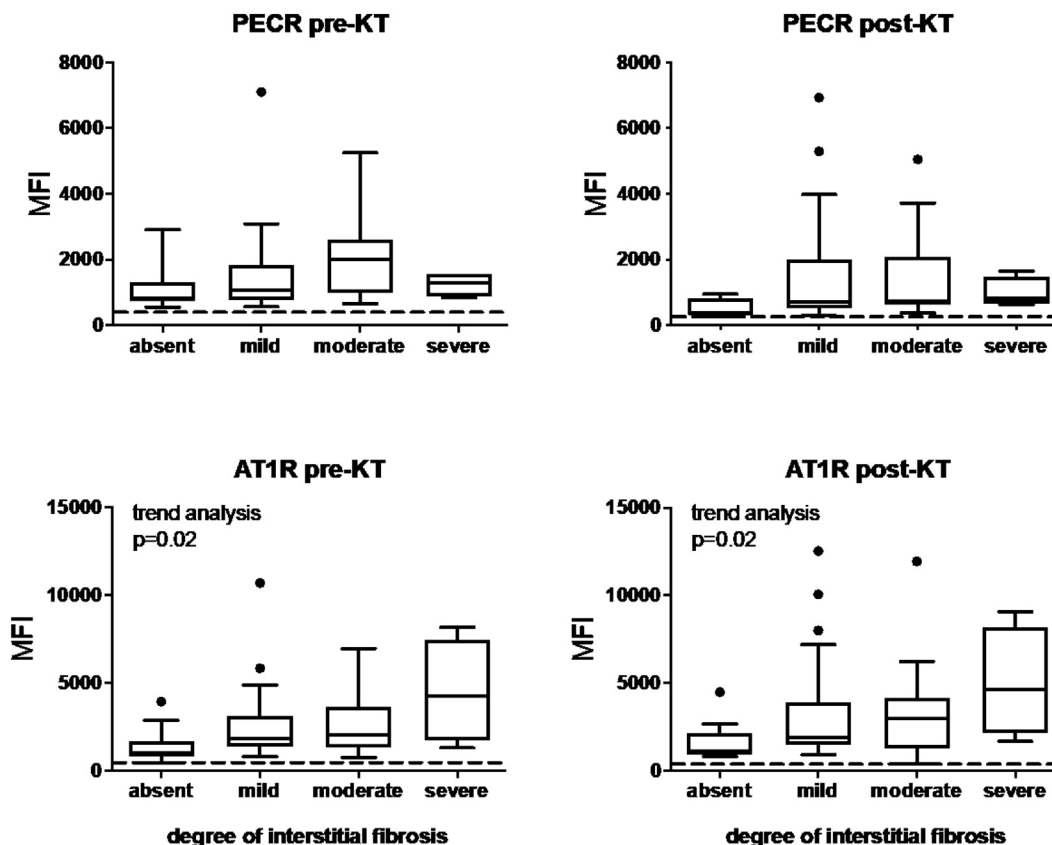
Fig. 3. Tukey box plots of the MFI values for PECR and AT1R in groups of cases ranked according to the degree of interstitial fibrosis (IF) in the kidney allograft biopsy at time of diagnosis. MFI values before kidney transplantation (pre-KT) or at time of kidney biopsy (post-KT) are shown separately. According to the Banff criteria the degree of IF is scored as absent (n = 4), mild (<25%, n = 25), moderate (25–50% IF, n = 16) and severe (>50% IF, n = 4).

for a pathogenic role of AT1R antibodies in the initiation and progression of interstitial fibrosis. AT1R is broadly expressed in virtually all cells in the renal tissue including vascular endothelial cells, vascular smooth muscle cells but also inflammatory cells. AT1R-antibodies exert an allosteric, agonistic effect on the receptor and produce a sustained activation. This may lead to graft injury by persistent vasoconstriction and triggering inflammation, fibrosis, thrombosis and vascular remodeling [24]. The pathophysiological chain of events is likely clinically important as a number of studies have indicated an increased risk for kidney graft loss in the presence of increased titers of pre-transplantation AT1R-antibodies [25].

Of note, the MFI values for AT1R in the kidney transplant recipients fall in the range of the MFI in healthy individuals [12]. Therefore, it seems likely that these autoantibodies only are pathogenic when other factors are active that initiate and promote interstitial damage. Most likely, in c-aABMR the initial damage to the kidney is caused by anti-donor specific antibodies such as anti-HLA antibodies which cause activation of and damage to the microvasculature, followed by tubular atrophy and interstitial fibrosis. In other cases there may be a damage-initiating role for prolonged calcineurin inhibitor use.

Along the same line of reasoning for ARHGDIB autoantibodies, this will also be a relatively slow immunological process, but in contrast to the ARHDIB target the AT1R protein is constitutively expressed on the cell surface of e.g. renal tubular and endothelial cells [26] and cell death is not a prerequisite for antigen exposure.

Although the results of this study need to be confirmed in a large cohort, the data are intriguing and indicate that autoantibodies may be involved in the pathogenesis of interstitial fibrosis. This is in cases of c-aABMR highly relevant as the degree of interstitial fibrosis in the renal biopsy has consistently been found to be the only histological score predicting progression to graft failure [9,15,27].

There are several limitations of the present study which should be recognized. The number of cases is relatively small and the study design does not include serial kidney biopsies over time with serum sampling. The latter could have given more insight into the possible cause and effect relation between the appearance of ARGHDIB and AT1R antibodies and intragraft pathology. Nevertheless, the findings are in support of results of previous studies which indicated a pathogenic role of these autoantibodies in graft injury in other conditions than c-aABMR.

In conclusion, ARHGDIB antibodies are specifically increased over time after transplantation in cases of c-aABMR but do not affect graft survival. Instead AT1R autoantibodies are associated with interstitial fibrosis and of clinical relevance for graft survival.

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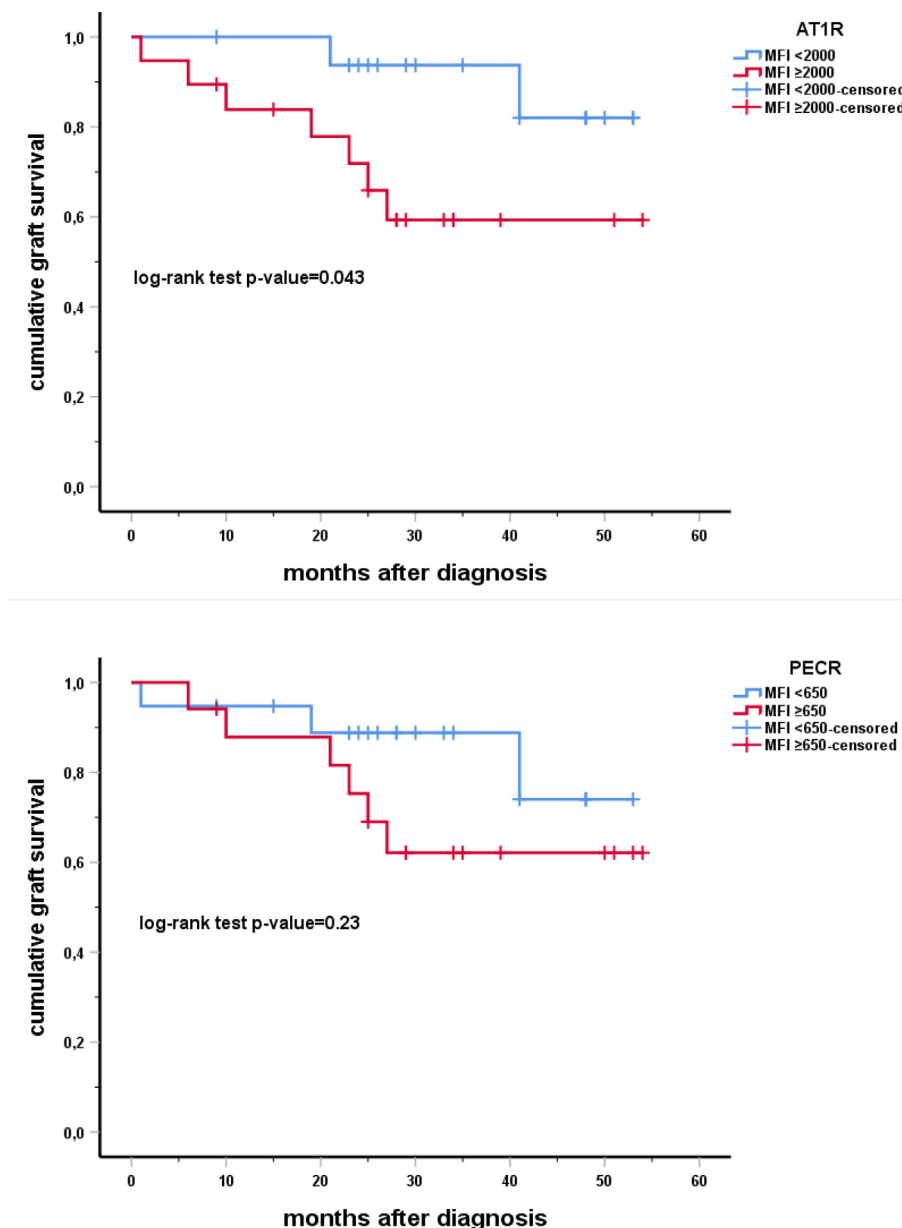
Fig. 4. Graft survival after diagnosis of c-aABMR for cases with high (n = 19) or low (n = 17) MFI for AT1R (A) or high (n = 17) or low (n = 19) MFI for PECR (B) autoantibodies based on the median MFI.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2020.12.003.

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