Antibodies against ARHGDIB and ARHGDIB gene expression associate with kidney allograft outcome

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Authorship

AS, MN, MPE and HGO designed the study. EK and HGO produced the non-HLA antibody data. AS, MN, JC, VVS, DK and MPE were involved in data production and data collection. AS and MN performed all data analysis. EL performed the histological analyses. AS and MN wrote the manuscript, and all coauthors revised and approved it.

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Abbreviations:

AMR, antibody-mediated rejection;

AMR_h, histological picture of AMR;

APMAP, Adipocyte plasma membrane-associated protein;

ARHGDI2, Rho GDP-dissociation inhibitor 2;

ARHGEF6, Rho guanine nucleotide exchange factor 6;

AT1R, Angiotensin Type 1 receptor;

C4d, complement split product 4d;

CDC-XM, complement-dependent cytotoxicity cross-matches;

CS, corticosteroids;

DSA, donor-specific anti-HLA antibodies;

ETAR, Endothelin-1 type A receptors;

LMNB1, Lamin B1;

LPLUNC1, BPI fold-containing family B member 1;

MFI, median fluorescence intensity;

MPA, mycophenolic acid;

PECR, Peroxisomal trans-2-Enoyl-CoA reductase;

PLA2R, Phospholipase A2 receptor;

PRKCZ, Protein kinase C zeta type.

TAC, tacrolimus;

Tubb4B, Tubulin beta-4B class IVb

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ABSTRACT

Background. The impact of donor-specific anti-HLA antibodies (DSA) on antibody-mediated rejection (AMR) and kidney allograft failure is well established. However, the relevance of non-HLA antibodies remains unclear.

Methods. We investigated 13 pretransplant non-HLA antibodies and their association with histology of AMR (AMR_h) and kidney allograft failure. We included single kidney recipients (N=203) with AMR_h according to the Banff 2017 classification, and matched AMR_h-free controls (N=219). Non-HLA antibodies were assessed using multiplex Luminex assay.

Results. Of the selected non-HLA antibodies (against agrin, APMAP, ARHGDIB, ARHGEF6, AT1R, ETAR, LMNB1, LPLUNC1, PECR, PLA2R, PRKCZ, TUBB4b, vimentin), only antibodies against Rho GDP-dissociation inhibitor 2 (ARHGDIB) (adjusted MFI [aMFI]≥1000), a minor histocompatibility antigen, associated with graft failure, in univariate and multivariate models (HR=2.7;95%CI,1.3-5.4;p=0.007). There was a 19.5-fold (95%CI,6.0-63.9;p<0.0001) increased risk of graft failure in patients positive for both DSA and anti-ARHGDIB antibodies (aMFI≥1000) versus patients negative for both DSA and anti-ARHGDIB antibodies, compared to a 4.4-fold (95%CI,2.4-8.2,p<0.0001) increased risk in patients with only DSA, and a 4.1-fold (95%CI,1.4-11.7;p=0.009) increased risk in patients with only anti-ARHGDIB antibodies above 2000 aMFI. AMR_h associated with increased intrarenal expression of the ARHGDIB gene. In the absence of AMR_h and DSA, anti-ARHGDIB antibodies were not clearly associated with graft failure.

Conclusions. The presence of pretransplant anti-ARHGDIB antibodies has an additive effect in patients with DSA on the risk of graft failure via AMR_h. Other investigated non-HLA antibodies, including antibodies against AT1R, did not contribute to risk stratification and could not explain the histology of AMR in the absence of DSA.

Introduction

Antibody-mediated rejection (AMR) is an important cause of late kidney allograft failure.¹⁻³ Donor-specific anti-HLA antibodies (DSA) play the leading role in the graft damaging process associated with AMR.^{2,4} However, in more than half of recipients with histological signs of AMR, DSA or even nondonor specific anti-HLA-antibodies are never detected.⁵⁻⁸ For this reason, there is an active interest in the contribution of non-HLA antibodies to AMR and graft failure.⁹

The potential involvement of non-HLA antibodies in antibody-mediated injury has been suggested by studies in HLA identical siblings and patients with biopsy-proven rejection without DSA.¹⁰ The first focus for potential non-HLA targets are endothelial cells. Presence of antibodies against non-HLA antigens expressed on endothelial cells (AECAs) has been associated with increased acute and hyperacute rejection rates and with poor graft function in recipients without DSA.¹¹⁻¹³ However, these findings for AECAs were not replicated in all studies.^{14,15}

In the past decade, a large number of antibodies against different non-HLA antigens have been identified and linked with increased rejection rates and worse graft outcome.¹⁶⁻²¹ However, as most of these antibodies are autoantibodies, which can also be found in healthy individuals, no firm conclusions about their pathogenicity can be made. In addition, it remains unclear which thresholds for positivity of these autoantibodies are relevant in clinical practice.

The aim of this study was to investigate the clinical relevance of pretransplant antibodies against 13 selected non-HLA antigens, using a newly developed multiplex Luminex assay, and to evaluate whether these antibodies associate with the histology of AMR especially in the absence of DSA and graft failure.

Materials and Methods

Study population and data collection

From the consecutive adult recipients of a kidney transplant at the University Hospitals Leuven between March 2004 and February 2013 (N=1137), we retrospectively selected cases with at least 1 biopsy with histology of antibody-mediated rejection (AMR_h; N=208) and matched control cases without AMR_h (N=219).⁵ All transplantations were performed with negative complement-dependent cytotoxicity cross-matches (CDC-XM). No patient received preconditioning desensitization. The clinical data were prospectively collected during routine clinical follow-up. This study is approved by the Ethics Committee of the University Hospitals-Leuven (S53364).

Histological scoring and biopsies

All posttransplant renal allograft biopsies performed in the cohort, until time of data extraction on November 2017, were included. Protocol-specified biopsies were performed in addition to the clinically indicated biopsies (“indication biopsies”). Timing and scoring of the biopsies were described in detail previously.⁵ Diagnosis of the rejection phenotypes, antibody-mediated rejection (AMR) and T-cell mediated rejection (TCMR) was based on the criteria as defined by the Banff 2017 consensus.^{22,23}

Detection of circulating non-HLA antibodies

Pretransplant non-HLA antibodies were detected on day 0 serum samples by using multiplex non-HLA antibody assay. The protein targets for this non-HLA assay were selected based on a literature search for proteins directly or indirectly associated with kidney disease or with kidney transplantation. More details for non-HLA antibody selection, the development and validation of this assay were described in detail previously.^{24,25} In this study we analyzed the microspheres (beads) with directly coupled non-HLA (agrin, APMAP, ARHGDIB, ARHGEF6, AT1R, ETAR, LMNB1, LPLUNC1, PECR, PLA2R, PRKCZ, TUBB4b and vimentin) proteins. The tests were

performed according to the previously published protocol,^{24,25} and the individual (absolute) MFI values were obtained by a Luminex 200 flow analyzer (Luminex Corp). As we observed relatively high background signals for some sera samples, we calculated the background-adjusted (aMFI) values for each target bead by subtracting the absolute MFI value of the empty bead from the absolute MFI value of the bead coupled with protein. In all multivariate analyses only the background-adjusted MFI values were used.

Detection of circulating anti-HLA antibodies and HLA genotyping

The pretransplant and posttransplant follow-up of anti-HLA antibodies was systematically monitored in 1 histocompatibility laboratory (HILA – Belgian Red Cross Flanders). All sera were routinely screened using a LIFECODES LifeScreen Deluxe (LMX) kit (Immucor Inc., Norcross, GA) and in case of positive screening, the donor-specificity was assessed using LIFECODES Single Antigen Bead (LSA) kits (Immucor). In order to determine true donor specificity, donor DNA samples were genotyped at high-resolution for the HLA -A, -B, -C, -DRB1, -DRB3, -DRB4, -DRB5, -DQA1, -DQB1, -DPA1, and -DPB1 loci. Assignment of the presence of HLA-DSA was described in detail previously.²⁶

Biopsy sample collection, preparation and microarray procedure

In a subset of the cases included in this study, microarray gene expression analyses were performed on posttransplant kidney biopsies. Two-needle cores were obtained at each kidney allograft biopsy. One was used for histology, at least half of the other one was immediately stored for RNA expression analysis. RNA extraction and gene expression analysis were recently described by our group.²⁷

Statistical Analysis

The 2-sample t-test and Mann-Whitney U test were used for continuous variables, Pearson's chi-square or Fisher's Exact test when appropriate for categorical variables and the Wilcoxon test for the comparison of medians. Kidney allograft survival was plotted using the Kaplan-Meier method and groups were compared using the Log-rank test. Univariate and multivariate Cox models were applied to quantify the hazard ratios for allograft failure. Where appropriate, we applied the Benjamini-Hochberg procedure for multiple testing with a false discovery rate of 0.10. In the case of patient death with a functioning graft, we censored graft survival at time of death. Gray's test was used for comparison of the cumulative incidence of histological phenotypes (AMR_h, TCMR, AMR_h or TCMR, cAMR_h and IFTA) between the different groups. All tests were two-sided. We used SAS (version 9.4, SAS Institute Inc., Cary, NC) for the statistical analyses and GraphPad Prism (version 8.1.1, GraphPad Software, San Diego, CA) for graphical presentation.

Results

Study population characteristics

Among 935 transplantations with at least 1 posttransplant biopsy and anti-HLA antibody follow-up, we identified 208 cases with at least 1 biopsy fulfilling the first 2 Banff criteria for AMR diagnosis (the histological picture of AMR, AMR_h), as recently described by our group.⁵ All cases with available day0 serum samples (N=203) were compared with matched controls (N=219) that never developed AMR_h (NoAMR_h) during the biopsy follow-up. In total, 1805 posttransplant biopsies were performed in this cohort.

Patient characteristics are provided in Table 1. Median follow-up time posttransplant was 7.7 (IQR 4.8) years. Recipient age, recipient gender, recipient ethnicity, mean donor age, donor gender, donor type, mean cold ischemia time and immunosuppression regime were comparable between AMR_h and NoAMR_h cases. At the time of transplantation, patients with AMR_h received more induction

therapy (52% vs. 38%, $p=0.003$) and experienced more delayed graft function (24% vs. 13%, $p=0.008$) compared to NoAMR_h patients. Also repeat transplantation (27% vs. 10%, $p<0.0001$) and pretransplant HLA antibodies (54% vs. 23%, $p<0.0001$) were more prevalent in the AMR_h cases than in the controls. At the time of the first AMR_h biopsy, 84/203 (41%) patients were DSA-positive (DSA_{pos}AMR_h), while in 119/203 cases (59%) DSA were never detected (DSA_{neg}AMR_h group). Except for donor age and HLA mismatch, the main pretransplant demographic characteristics between DSA_{neg}AMR_h and NoAMR_h patients were comparable (Table 1).

Non-HLA antibodies and allograft failure

The absolute and adjusted MFI values of all non-HLA targets included in this study are depicted in Figure S1 (SDC, <http://links.lww.com/TP/B826>), together with the positive and negative control beads. In univariate Cox proportional hazard analysis with the aMFI values and after applying the Benjamini-Hochberg procedure for multiple testing, antibodies against Rho GDP-dissociation inhibitor 2 (ARHGDIB) (Hazard Ratio [HR] 1.28 per 1000 MFI; 95%CI, 1.08-1.52; $p=0.004$) and against Tubulin beta-4B class IVb (TUBB4b) (HR=1.35 per 1000 MFI; 95%CI, 1.09-1.68; $p=0.007$) were associated with posttransplant graft failure (Table 2). To exclude any possible unspecific reaction with the target beads, we performed additional univariate analysis with different MFI thresholds (Table S1, SDC, <http://links.lww.com/TP/B826>) and with calculated ratios with the MFI of the negative control (Table S2, SDC, <http://links.lww.com/TP/B826>). These analyses illustrated that only anti-ARHGDIB antibodies were robustly associated with graft failure. No consistent relation with allograft outcome was observed for the other non-HLA antibodies.

In subsequent multivariate Cox models with aMFI value, only anti-ARHGDIB antibodies associated with graft failure, adjusted for other cofounders including pretransplant DSA. Anti-TUBB4b antibodies lost their association with graft failure in this analysis (Table S3, SDC, <http://links.lww.com/TP/B826>). Figure 1 depicts the Kaplan-Meier survival analysis of patients with and without anti-ARHGDIB antibodies with different aMFI thresholds. Patients with anti-ARHGDIB aMFI $\geq$ 1000 had a significantly higher risk of graft failure compared to patients without anti-ARHGDIB antibodies (HR=2.65; 95%CI, 1.30-5.40; p=0.007, Figure 1B, Table S3, SDC, <http://links.lww.com/TP/B826>).

Non-HLA antibodies and allograft rejection

When we compared the medians of aMFI values between the AMR_h cases and controls for all 13 non-HLA antibodies and after applying the Benjamini-Hochberg procedure for multiple testing, we did not find any antibody to be more prevalent in AMR_h patients compared to NoAMR_h controls (Figure S2A, SDC, <http://links.lww.com/TP/B826>). Next, we compared the medians of aMFI values between DSA_{neg}AMR_h cases and NoAMR_h controls, and we found the median of anti-PECR antibodies to be higher in DSA_{neg}AMR_h cases (454, IQR=938 vs. 235, IQR=446; p=0.004) (Figure S2B SDC, <http://links.lww.com/TP/B826>). To investigate the possible role in AMR_h, we analyzed different thresholds for positivity of anti-PECR antibodies. With the lowest threshold of aMFI $\geq$ 500, we found 44.5% of the DSA negative patients with AMR_h and 26.9% of the patients that never developed AMR_h to have anti-PECR antibodies at the time of transplantation (Table S4, SDC, <http://links.lww.com/TP/B826>). Finally, we did not find any antibody to be predictive for the development of AMR_h within the first 5 years after transplantation (Figure 2).

As ARHGDIB antibodies were the only associated with graft failure, we investigated the impact of these antibodies with 4 different thresholds for positivity on allograft rejection phenotypes. We found no difference in the incidence of AMR_h, TCMR, any rejection (AMR_h or TCMR), cAMR (biopsy with the first 2 Banff criteria for chronic AMR) or IFTA (score 2 and 3) in patients with vs. without anti-ARHGDIB antibodies (Gray's test, all $p>0.05$) (Figure S3, SDC, <http://links.lww.com/TP/B826>).

Allograft survival stratified according to anti-ARHGDIB antibodies and DSA status

Next, we grouped the patients according to DSA and anti-ARHGDIB antibodies with the 4 different aMFI thresholds. In Kaplan-Meier survival analysis stratified by the presence of anti-ARHGDIB antibodies and DSA, patients with only DSA or patients with both anti-ARHGDIB and DSA antibodies had worse survival rates compared to patients negative for both antibodies (log-rank test, $p<0.0001$) (Figure 3). Patients positive for anti-ARHGDIB antibodies with aMFI above 1500 or 2000 had worse survival rates (61.5% and 47.6%) compared to DSA-negative and anti-ARHGDIB negative patients (86.1%) (log-rank test, $p=0.03$ and $p=0.002$, respectively) (Figure 3C-D). Depending on the threshold ($aMFI\geq 500$ or $aMFI\geq 2000$), patients with both DSA and anti-ARHGDIB antibodies had a 14.4-fold (95%CI, 5.18-40.2; $p<0.0001$) or 24-fold (95%CI, 6.60-87.0; $p<0.0001$) increased risk for transplant failure, compared to DSA-negative and anti-ARHGDIB negative patients in adjusted multivariate Cox analysis, (Table 2, model-2). Patients with only DSA had a 4.5-fold (95%CI, 2.42-8.25; $p<0.0001$) increased risk for transplant failure. The independent effect of anti-ARHGDIB antibodies on graft outcome in the multivariate analysis was confirmed only for the patients with $aMFI\geq 2000$ (HR=4.07; 95%CI, 1.42-11.70; $p=0.009$). Finally, the multivariate model-3 showed that the effect of anti-ARHGDIB antibodies on graft failure was independent of the MFI value of the HLA-DSA.

ARHGDIB gene expression and graft rejection

To understand better how anti-ARHGDIB antibodies associate with graft failure without associating with the risk of allograft rejection, we further studied posttransplant expression of ARHGDIB gene in kidney allograft biopsies. We performed biopsy microarray analysis on 33 patients of this cohort (selected from our previous study²⁷): 15 patients had biopsies with AMR_h, of which 7 had DSA, and 18 patients had biopsies without AMR_h. The transcript probe sets (201288_at and 1555812_a_at) for ARHGDIB were significantly overexpressed in AMR_h cases compared to NoAMR_h cases (4.2 and 5.0-fold change), independent of the presence of DSA (Figure 4).

Finally, as ARHGDIB is overexpressed in biopsies with AMR_h, we evaluated the impact of anti-ARHGDIB antibodies on graft outcome separately in patients with AMR_h and NoAMR_h. In all multivariate models with only AMR_h cases (n=203), anti-ARHGDIB antibodies associated with impaired graft survival (Table S5, SDC, <http://links.lww.com/TP/B826>). However, in patients without AMR_h, anti-ARHGDIB antibodies did not associate with graft failure (Table S6, SDC, <http://links.lww.com/TP/B826>).

Discussion

In this study, we described that antibodies against ARHGDIB are associated with impaired graft outcome and contribute in risk stratification of patients with DSA. Although the presence of anti-ARHGDIB antibodies was not associated with increased rejection rates of any histological phenotype (AMR_h, TCMR or both), there was a 4-fold increased expression of ARHGDIB gene in biopsies with AMR_h, which could explain the prognostic importance of these antibodies in cases with DSA and AMR_h. Pretransplant antibodies against other targets, agrin, APMAP, ARHGEF6, AT1R, ETAR, LMNB1, LPLUNC1, PECR, PLA2R, PRKCZ, TUBB4b and vimentin also could not explain the histology of AMR in the absence of DSA, and were not associated with an increased risk of graft failure.

The association between antibodies against ARHGDIB and reduced graft survival was recently described in a large nationwide cohort study.²⁵ In our current study, we confirm this association and shed more light on this association. We showed that the concomitant presence of DSA and anti-ARHGDIB antibodies in kidney transplant recipients leads to an importantly increased risk of graft failure. Patients positive for both DSA and anti-ARHGDIB antibodies (aMFI $\geq$ 1000) had a 20-fold increased risk for transplant failure compared to DSA-negative and anti-ARHGDIB negative patients, while patients with only DSA had a 4-fold increased risk for graft failure. This effect was independent of the MFI of the DSA. The mechanism behind the additive effect in patients with DSA on graft outcome by anti-ARHGDIB antibodies remains unclear, and increased expression of ARHGDIB in AMR_h cases could be a hint towards the underlying pathophysiology.

The increased expression of ARHGDIB in kidney allografts with AMR_h is intriguing. ARHGDIB, previously suggested as a minor histocompatibility antigen, is an intracellular GTP-binding protein involved in many cell activities.^{28,29} Initially it was reported to be expressed only in hematopoietic tissues, predominantly in lymphocytes,³⁰ but it has become clear that this molecule is widely expressed in other organs and tissues.^{31,32} Recent work using an anti-ARHGDIB antibody staining has shown that ARHGDIB protein is expressed in endothelial cells of arteries, podocytes and lymphocytes of transplanted kidneys.²⁵ However, it is unlikely that anti-ARHGDIB antibodies can directly induce cell destruction or graft damage since the antigen target is intracellular. Consequently, it is expected that anti-ARHGDIB antibodies cannot be detected on endothelial cell cross matches. We hypothesize that anti-ARHGDIB antibodies can only have an additive effect on the cellular damage caused by DSA or other concomitant undetected antibodies. This hypothesis is supported by our findings that anti-ARHGDIB antibodies have a synergistic effect with DSA and, in absence of DSA, are associated with impaired graft survival only in kidneys with AMR_h.

Next to the role of ARHGDIB antibodies, we found an association between anti-PECR antibodies and AMR_h. If we chose the lowest threshold for positivity of aMFI $\geq$ 500, in about half of DSA negative patients (44.5%) the histological lesions suggestive for AMR could be explained by anti-PECR antibodies. However, this association should be viewed with caution and need further investigation as anti-PECR antibodies with the same threshold were found at the time of transplantation in 26.9% of the patients that did not develop AMR_h.

We did not find associations of any other tested non-HLA antibodies including anti-AT1R antibodies, with AMR_h or graft failure. This finding contrasts with several previously published studies where non-HLA antibodies, particularly those directed at AT1R, were linked to increased rejection rates and allograft failure,^{18-21,33-34} although this was not confirmed in all studies.^{25,35,36} The main explanation could be that we evaluated only the impact of pretransplant anti-AT1R antibodies and did not investigate the relevance of de novo antibodies occurring posttransplant. The use of different technologies to assess the AT1R antibodies could additionally explain these discrepancies, as we used sensitive Luminex-based assays for the detection of non-HLA antibodies, while all other published studies used ELISA-based assays. A recent study suggested that an ELISA-based method for anti-AT1R testing lacks specificity, false positive cases, and thus over-estimation of the prevalence of these antibodies.³⁷ The lack of a standardized cutoff for determining positive results for anti-AT1R antibodies could also play an important role in the discrepancies between studies on non-HLA antibodies, and challenges the comparison between studies. To address this relevant issue for our assay, we adjusted target MFI values by subtraction with MFI of the negative control and investigated the clinical relevance of the studied antibodies with different thresholds for positivity. However, the lack of association of non-HLA antibodies other than ARHGDIB with AMR_h or graft failure, with any of the calculation approaches or thresholds, illustrates that we could not confirm the clinical utility of measuring these antibodies prior to transplantation and that we should be

careful in implementation of such nonvalidated antibody profiling in clinical decisions or reporting on causality in research projects.

Next to the discussion on these previously described non-HLA antibodies, a recent study has illustrated the immense broadness of non-HLA immunity in kidney transplantation.³⁸ This study showed that the abundance of mismatches in nonsynonymous single nucleotide polymorphisms (SNPs) of different transmembrane and secreted proteins is associated with an increased risk of graft failure. In cases with chronic AMR, donor-specific antibody responses could be identified to these genetically predicted mismatched epitopes. This implies that besides antibodies against currently known non-HLA epitopes, there are potentially a vast number of other mismatched non-HLA molecules that can raise immune responses. Focusing on only a limited number of molecules and not looking to all mismatched epitopes thus grossly ignores the true complexity of alloimmunity. For the *ARHGDIB* gene, it was reported that SNPs located in the coding exon could be mismatched between the transplant pairs and that the relevance of anti-*ARHGDIB* antibodies is primarily allo-reactive in nature.^{29,39} Further exploring the genetic mismatch in the *ARHGDIB* gene is necessary for more in-depth understanding the underlying pathophysiology of these antibodies.

Our study has several other limitations, including the retrospective case-control design and possible selection bias. The relevance of anti-*ARHGDIB* antibodies was however demonstrated recently in a large unselected cohort study, which suggests that our conclusions are not affected by our case mix.²⁵ Another limitation is that we only investigated the impact of selected pretransplant non-HLA antibodies on allograft outcome, while other non-HLA antibodies and evolution or de novo occurrence of these antibodies after transplantation could also be relevant but are missed. Finally, we did not have insight into the donor-recipient genetic mismatch for non-HLA genes, including

ARHGDIB, and thus in the alloreactivity of these non-HLA antibodies. Further work is needed to validate our findings in a larger prospective cohort and to investigate the kinetics, de novo occurrence and the donor-specificity of the anti-ARHGDIB antibodies.

In conclusion, anti-ARHGDIB antibodies are associated with impaired kidney allograft survival and have an additive effect in patients with DSA. Overexpression of the ARHGDIB gene in kidney allografts with signs suggestive for AMR could play a role in this association. Therefore, detection of anti-ARHGDIB antibodies before transplantation may improve individual risk assessment of kidney recipients with DSA. Other non-HLA antibodies did not contribute to risk stratification and could not explain the histology of AMR in the absence of DSA, including anti-AT1R antibodies. Further work is necessary to confirm our findings and elucidate the pathophysiology of anti-ARHGDIB antibodies in kidney transplant recipients.

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Figure legends:

Figure 1. Kaplan-Meier analysis of death-censored graft survival after transplantation, according to pretransplant anti-ARHGDIB antibody status (N=422). Survival analysis in patients with different thresholds of positivity for anti-ARHGDIB antibody A) aMFI ≥ 500 , B) aMFI ≥ 1000 , C) aMFI ≥ 1500 and D) aMFI ≥ 2000 . Survival in the anti-ARHGDIB antibody positive group was significantly decreased only when the aMFI value was above 1000 (log-rank, $p < 0.05$).

Figure 2 Occurrence of AMR_h within the first 5 years after transplantation according to non-HLA antibody status with aMFI ≥ 500 as a threshold for positivity (N=422). The p-value is calculated with Gray's test. None of the p-values were significant after applying the Benjamini-Hochberg procedure for multiple testing with a false discovery rate of 0.10. Anti-PLA2R antibody was not evaluated in this analysis due to the very low number of positive patients ($n=2$). The cumulative incidence should be interpreted cautiously as this is the representation of a selected cohort, with enrichment of the dataset with AMR_h cases.

Figure 3. Kaplan-Meier analysis of death-censored graft survival after transplantation, according to pretransplant DSA and anti-ARHGDIB antibody status (N=422). Four different thresholds for positivity of anti-ARHGDIB antibodies are shown, with A) aMFI ≥ 500 , B) aMFI ≥ 1000 , C) aMFI ≥ 1500 and D) aMFI ≥ 2000 . Compared to DSA-negative and anti-ARHGDIB negative patients, survival of grafts with both DSA and anti-ARHGDIB antibodies was significantly decreased for all 4 different thresholds for positivity (log-rank, $p < 0.0001$). In contrast, survival in patients with only anti-ARHGDIB antibodies, compared to DSA-negative and anti-ARHGDIB-negative patients, was decreased only when the aMFI was above 1500 (log-rank, $p = 0.03$) and 2000 (log-rank, $p = 0.002$).

Figure 4. Microarray expression data of ARHGDIB transcripts measured in kidney allograft biopsies (N=33). A), B), C) ARHGDIB expression was increased in biopsies with AMR_h when compared to NoAMR_h biopsies independent of DSA ($p < 0.05$). D) When we compared the ARHGDIB expression between DSA_{neg}AMR_h and DSA_{pos}AMR_h biopsies, the difference was not significant ($p = 0.69$). The groups were compared using Mann-Whitney U analysis.

Table 1. Main demographic and clinical characteristics of the study cohort (N=422), according to DSA status and histology of AMR (AMR_h).

Characteristic	All (N=422)	NoAMR _h (N=219)	AMR _h (N=203)	NoAMR _h vs. AMR _h	DSA _{neg} AMR _h (n=119)	NoAMR _h vs. DSA _{neg} AMR _h
<i>Recipients</i>				p-value ^a		p-value ^a
Age (years), mean ±SD	53.1 ±13.6	53.9 ±13.0	52.3 ±14.2	0.21	53.4 ±13.3	0.71
Gender (female), n (%)	173 (41.0)	83 (37.9)	90 (44.3)	0.18	45 (37.8)	0.99
Caucasian ethnicity, n (%)	415 (98.3)	217 (99.1)	198 (97.5)	0.21	115 (96.6)	0.12
Repeat transplantation, n (%)	77 (18.2)	22 (10.1)	55 (27.1)	<0.0001	12 (10.1)	0.99
<i>Donors</i>						
Age (years), mean ±SD	47.8 ±15.2	47.0 ±14.9	48.6 ±15.5	0.29	50.7 ±14.5	0.03
Gender (female), n (%)	194 (46.0)	104 (47.5)	90 (44.3)	0.52	55 (46.2)	0.82
Deceased donor, n (%)	397 (94.1)	208 (95.0)	189 (93.1)	0.42	112 (94.1)	0.74
Donation after brain death, n (%)	331 (83.4)	171 (82.2)	160 (84.7)	0.51	94 (83.9)	0.70
Cold ischemia time (h), mean ±SD	14.3 ±5.5	14.1 ±5.5	14.5 ±5.6	0.53	14.7 ±5.8	0.41
<i>Treatment at the time of transplantation and follow-up</i>						
Delayed graft function, n (%)	76 (18.0)	29 (13.2)	47 (24.2)	0.008	21 (17.6)	0.28
Immunosuppression regimen: TAC-MPA-CS, n (%)	367 (87.0)	184 (84.0)	183 (90.2)	0.06	102 (85.7)	0.68
Induction therapy, n (%)	189 (44.8)	83 (37.9)	106 (52.2)	0.003	46 (38.7)	0.89
Median time of follow-up (IQR)	7.7 (4.8)	7.81 (4.3)	7.2 (5.2)	0.004	8.3 (4.9)	0.56
<i>Immunological profile at the time of transplantation</i>						
HLA-A/B/DR mismatches, mean ±SD	2.9 ±1.3	2.7 ±1.3	3.0 ±1.2	0.007	3.2 ± 1.2	0.0009
A mismatch, mean ±SD	1.0 ±0.7	1.0 ±0.7	1.1 ±0.7	0.08	1.2 ±0.6	0.004
B mismatch, mean ±SD	1.1 ±0.6	1.1 ±0.7	1.1 ±0.6	0.18	1.2 ±0.6	0.13
DR mismatch, mean ±SD	0.7 ±0.6	0.7 ±0.6	0.8 ±0.5	0.03	0.8 ±0.6	0.03
HLA antibodies, n (%)	159 (37.8)	50 (22.8)	109 (53.7)	<0.0001	25 (21.0)	0.70
Pretransplant DSA, n (%)	83 (19.7)	9 (4.1)	74 (36.5)	<0.0001	0 (0.0)	0.03

Statistically significant p-values are indicated in bold font.

^aThe 2-sample t-test was used for continuous variables, the Pearson's chi-square test for categorical variables and the Wilcoxon test for comparison of medians.

Abbreviations: SD, standard deviation; HLA, human leukocyte antigens; DSA, donor-specific HLA antibodies; TAC, tacrolimus; MPA, mycophenolic acid; CS, corticosteroids.

Table 2. Univariate and multivariate Cox proportional hazards analysis of death-censored graft failure according to the presence of pretransplant non-HLA antibodies and DSA (N=422).

Antibody	No. of patients	No. of events	HR	95% CI	p-value
Univariate analysis^a					
Non-HLA antibodies (per 1000 MFI increment)					
Anti-agrin abs	422	66	0.31	0.20 - 4.84	0.41
Anti-APMAP abs	422	66	1.52	0.66 - 3.48	0.32
Anti-ARHGDIB abs	422	66	1.28	1.08 - 1.52	0.004
Anti-AT1R abs	422	66	0.83	0.65 - 1.06	0.13
Anti-ARHGEF6 abs	422	66	1.23	0.81 - 1.86	0.34
Anti-ETAR abs	422	66	1.48	0.85 - 2.57	0.17
Anti-LMNB1 abs	422	66	1.09	0.43 - 2.74	0.86
Anti-LPLUNC1 abs	422	66	1.19	0.85 - 1.66	0.30
Anti-PECR abs	422	66	0.78	0.57 - 1.06	0.11
Anti-PLA2R abs	422	66	0.00	0.00 - x.x	0.27
Anti-PRKCZ abs	422	66	0.94	0.81 - 1.09	0.42
Anti-TUBB4b abs	422	66	1.35	1.09 - 1.68	0.007
Anti-vimentin abs	422	66	0.98	0.82 - 1.18	0.85
Multivariate analysis					
Multivariate model - 1					
Anti-ARHGDIB abs (per 1000 MFI increment)	422	66	1.29	1.09 - 1.54	0.004
Pretransplant HLA abs (yes vs. no)	159	43	3.86	2.21 - 6.81	<0.0001
Multivariate model - 2					
Anti-ARHGDIB abs $\geq$ 500 aMFI and DSA	422	66			
None	290	29	1	–	–
Anti-ARHGDIB abs	49	8	1.53	0.70 - 3.36	0.29
DSA	72	23	4.32	2.28 - 8.19	<0.0001
DSA and anti-ARHGDIB abs	11	6	14.43	5.18 - 40.2	<0.0001
Anti-ARHGDIB abs $\geq$ 1000 aMFI and DSA	422	66			
None	315	32	1	–	–
Anti-ARHGDIB abs	24	5	1.97	0.76 - 5.08	0.16
DSA	77	25	4.39	2.36 - 8.16	<0.0001
DSA and anti-ARHGDIB abs	6	4	19.52	6.00 - 63.9	<0.0001
Anti-ARHGDIB abs $\geq$ 1500 aMFI and DSA	422	66			
None	324	33	1	–	–
Anti-ARHGDIB abs	15	4	2.75	0.97 - 7.83	0.06
DSA	78	26	4.46	2.42 - 8.25	<0.0001
DSA and anti-ARHGDIB abs	5	3	15.92	4.30 - 58.9	<0.0001
Anti-ARHGDIB abs $\geq$ 2000 aMFI and DSA	422	66			
None	328	33	1	–	–
Anti-ARHGDIB abs	11	4	4.07	1.42 - 11.69	0.009
DSA	79	26	4.42	2.39 - 8.18	<0.0001
DSA and anti-ARHGDIB abs	4	3	23.97	6.60 - 87.03	<0.0001

Multivariate model - 3

Anti-ARHGDIB abs (per 1000 MFI increment)	422	66	1.27	1.07 – 1.50	0.007
Pretransplant DSA (per 1000 MFI increment)	422	66	4.64	2.55 – 8.43	<0.0001

Statistically significant p values are indicated in bold font.

^a For the univariate analysis the Benjamini-Hochberg procedure for multiple testing with a false discovery rate of 0.10 was applied.

All multivariate Cox models were adjusted for repeat transplantation, delayed graft function, induction therapy, deceased donation and HLA-A/B/DR antigen mismatches.

Abbreviations: abs, antibodies; aMFI, adjusted MFI; HLA, human leukocyte antigens; DSA, donor-specific HLA antibodies.

Figure 1

Figure 2

Figure 3

Figure 4