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Flow cytometry crossmatching to investigate kidney-biopsy-proven, antibody-mediated rejection in patients who develop *de novo* donor-specific antibodies.

Running Title: Flow cytometry crossmatching in kidney transplant.

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ABSTRACT:

Introduction. The appearance of *de novo* donor-specific anti-human leukocyte antigen antibodies (*dn*DSAs) after kidney transplantation is independently associated with poor long-term allograft outcomes. The objective of the present study was to evaluate the predictive value of a flow cytometry crossmatching (FC-XM) assay after the appearance of *dn*DSAs related to antibody-mediated allograft rejection (ABMR) after kidney transplantation.

Materials and methods. A total of 89 recipients with *dn*DSAs after transplantation were included. The crossmatching results were compared with the *dn*DSA profile (the mean fluorescence intensity (MFI), the complement-binding activity, and the IgG subclass profile) and the biopsy's morphological features.

Results. Of the 89 patients, 59 (66%) were positive in an FC-XM assay, 17 (19%) had complement-binding DSAs, 55 (62%) were positive for IgG1 and/or IgG3 in a solid phase assay, and 45 (51%) had morphological biopsy features linked to ABMR.

Conclusion. An FC-XM assay was unable to discriminate between cases with or without ABMR on biopsy findings; it had a low positive predictive value (<70%) and a low negative predictive value (<42.9%), taking into account the sensitivity of our assay (limit of detection: DSAs with an MFI >3000). In this context, the height of the MFI of the *dn*DSAs might be enough for a high positive predictive value for ABMR and additional testing for complement binding activity can remain optional.

Keywords: Antibody mediated rejection; cross-matching; kidney transplant; crossmatch, crossmatching, HLA.

Abbreviations:

ABMR: antibody-mediated rejection

DSA: donor specific antibody

FC-XM: flow cytometry crossmatching

MFI: mean fluorescence intensity

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INTRODUCTION

Kidney transplantation is the optimal treatment for patients with end-stage renal disease on maintenance dialysis. Kidney allograft biopsies and assays of donor-specific anti-human leukocyte antigen (HLA) antibodies (DSAs) are complementary techniques for predicting early graft dysfunction.

Biopsies are useful for detecting signs of active antibody-mediated rejection (ABMR) of the kidney graft [1, 2]. Although biopsy analyses help to prevent disease progression and prolong graft function, this invasive procedure is burdensome for the patient.

The appearance of *de novo* DSAs (*dn*DSAs) after kidney transplantation may have a variety of effects on the allograft, ranging from the absence of obvious damage to active or chronic active ABMR [3]. It has been shown that the level of circulating DSAs (evaluated approximately by the mean fluorescence intensity (MFI) in a solid-phase assay [4]) and the DSAs' complement-binding activity are associated with poor allograft outcomes [2-5]. Moreover, information on the DSAs' IgG subclasses (which differ in their complement-binding activity) has prognostic value. It has been demonstrated that IgG3 *dn*DSAs (with high complement-binding activity) are associated with allograft failure [6].

The flow cytometry crossmatching (FC-XM) assay is currently the most sensitive cell-based method for detecting DSAs. Although this prospective technique is unable to discriminate between complement- and non-complement-binding antibodies, its prognostic value in a pre-transplant screening is well established [7].

1. OBJECTIVE

In the present study, we hypothesized that a positive FC-XM IgG assay performed upon the appearance of *dn*DSAs in the post-transplant period would be associated to the transplant outcome with distinct histological biopsy features and clinical findings. In control experiments, we compared the *dn*DSAs' characteristics (MFI, complement-binding activity, and IgG subclass profile) with the morphological biopsy features associated with ABMR.

2. METHODS

2.1. Study participants

The study was approved by the local independent ethics committee (*CPP Nord Ouest II*, Amiens, France; reference: In accordance with French legislation and the Declaration of

Helsinki, all participants gave their prior, written, informed consent to genetic testing. All patients having undergone kidney transplantation at Amiens University Hospital (Amiens, France) between 2002 and 2016 were screened for eligibility. All donor-recipient pairs were ABO-compatible. At the time of transplantation, all recipients were negative for DSAs in a T- and B-cell complement-dependent cytotoxicity crossmatch assay. Recipients were assayed for DSAs on day 0, month 4, and month 12 post-transplantation, and annually thereafter. Our final analysis included recipients (n=89) having undergone a kidney allograft biopsy after a positive *dn*DSA assay during follow-up and for whom sufficient samples were available in our laboratory. In this cohort of patients, 44 patients had received an induction regimen with thymoglobulin and steroids, and 45 had received basiliximab and steroids. This was followed by combination maintenance immunosuppressive therapy using tacrolimus (44 patients) or cyclosporine (45 patients) plus, in some cases, mycophenolate and/or prednisone. Most of the 89 patients had received a corticosteroid-sparing regimen: indeed, 7 patients had not received corticosteroids at all, 55 had received a low-dose regimen (prednisolone equivalent <7.5 mg/d), 25 had received a prednisolone equivalent of 10 mg/d, and only two had received higher doses.

Cases of active ABMR were mainly treated with intravenous immunoglobulins and pulses of corticosteroids, together with modulation of the maintenance immunosuppression regimen. These patients variously received rituximab (n=1), therapeutic plasma exchange (n=1) and bortezomib (n=1). In cases with borderline changes in the biopsy and *dn*DSAs, the effectiveness of the maintenance immunosuppressive therapy was checked by assaying the plasma drug concentration and then increasing the dose and/or switching the drug.

2.2.Kidney biopsies

The indication for a kidney biopsy was the appearance of *dn*DSAs, regardless of the presence or absence of elevated serum creatinine or new-onset proteinuria. All biopsies were performed in the 3 months following the appearance of *dn*DSA, and were evaluated according to the Banff classification (category 2: Antibody-mediated changes; active ABMR and chronic active ABMR) for C4d deposits (detected by immunofluorescence staining or immunohistochemistry on paraffin-fixed tissue sections), glomerular or peritubular capillary inflammation, and transplant glomerulopathy [8]. A biopsy was considered to be positive for C4d deposits when more than 10% of the biopsy surface was stained.

2.3.Characterization of *dn*DSAs

*2.3.1. Screening for *dn*DSAs*

The Luminex™ single-antigen bead assay (One Lambda, Canoga Park, CA, USA) was used to screen serum samples for anti-HLA class I and II *dn*DSAs. The MFI cut-off for a positive assay was 1000. If two or more *dn*DSAs were present, we considered the immunodominant DSA (iDSA, i.e. the *dn*DSA with the highest MFI) and the sum of total MFI of all DSAs (sumDSAs) in our statistical analysis. To avoid prozone effects, the samples were pretreated with EDTA, as described previously [9]. Each recipient was tested before transplantation, at least, once a year, even if screening assay is negative.

2.3.2. Flow cytometry crossmatching

On the day of the transplantation, donor mononuclear cells (collected from spleen or lymph nodes on a Ficoll gradient) in 20% DMSO were frozen in nitrogen liquid. On the day of the study, the cells were thawed in a water bath at 37°C and incubated with 50 µl of recipient serum obtained before the appearance of *dn*DSA (the negative control) and recipient serum containing *dn*DSAs. We performed a pan-IgG FC-XM that did not specifically detect the IgG subclasses IgG1 to IgG4. For samples with low MFI DSA levels, we established previously that our pan-IgG FC-XM assay has an MFI limit of detection of around 3000 [10]. Fluorescein-isothiocyanate (FITC)-conjugated pan-anti-human IgG was used (Life Technologies, Villebon sur Yvette, France). Anti-KO-CD45, A750-CD3 and ECD-CD19 antibodies were used to screen for T- and B-lymphoid cells, as described previously. Two negative controls were used: a serum obtained from to a male donor (blood group: AB) that lacked MHC antibodies in a class I and II single-bead assay, and a sample of the recipient's serum obtained before the appearance of *dn*DSAs. The positive control serum sample came from to a pool of highly polyimmunized recipients. A positive FC-XM result corresponded to a sample MFI/negative pool MFI ratio greater than 1.5 [10].

2.3.3. IgG subclasses:

The IgG subclass profile was determined by replacing the phycoerythrin-conjugated anti-pan human IgG reporter antibody with monoclonal antibodies that were specific for each of the four IgG subclasses (IgG1: clone HP6001; IgG2: clone 31–7-4; IgG3: clone HP6050; IgG4: clone HP6025; Southern Biotech, Birmingham, AL, USA), as described previously [6]. We

used the same negative and positive control sera as in the FC-XM assay. The MFI cut-off for a positive assay was 500.

2.3.4. Complement-binding assay:

The presence of C1q-binding donor-specific anti-HLA antibodies was measured using single-antigen flow bead assays (C1qScreen™, One Lambda), according to the manufacturer's instructions. We again used the same negative and positive control sera as in the FC-XM assay. In our laboratory, the positive serum reliably gave positive crossmatches in a complement-dependant cytotoxicity assay. The MFI cut-off for a positive assay was 500. In cases with two or more *dn*DSAs, we first considered the complement-binding assay of the *i*DSA in our statistical analysis. If this assay was negative, we considered complement binding assay of others *dn*DSA, if positive.

2.4. Statistical analysis

The data that support the findings of this study are available from the corresponding author upon reasonable request. The highest single-*dn*DSA MFIs were used in the statistical analysis. Fisher's test was used to assess the putative link between the assay results (FC-XM, C1qScreen™, and the presence of IgG1 and/or IgG3 subclasses) and morphological features of the biopsy (ABMR and C4d deposits). The threshold for statistical significance was set to $p < 0.05$. The DSA MFIs for each group were compared using Student's t-test.

3. RESULTS

3.1. Demographic characteristics and biopsy findings

The baseline characteristics of the study cohort ($n=89$) are summarized in **Table 1**. The mean \pm SD (range) duration of follow-up after the first positive *dn*DSA assay was 46.5 ± 27.7 months (12-130). The biopsy findings at the time of the first positive *dn*DSA assay are also summarized in **Table 1**. None of the maintenance suppression regimens protected the patients against the development of *dn*DSA and subsequent ABMR, observed in 21 of the 44 patients on tacrolimus and 24 of the 45 patients on cyclosporine ($p=0.85$).

Ten patients presented normal features or non-specific changes in the biopsy. We observed that 45 of the 89 patients (50.5%) had a diagnosis of active or chronic, active ABMR and transplant glomerulopathy at the time of the first positive *dn*DSA assay. Moreover, 27 of the 89 biopsies (30%) featured C4d deposits.

There was no difference ($p=0.854$ in Student's t-test) in the time interval between transplantation and first positive *dn*DSA assay between patients with ABMR and those without ABMR.

3.2. Characteristics of the *dn*DSA assays

3.2.1. *dn*DSA MFIs

70 patients had only one DSA, and 19 patients had 2 to 4 DSAs. The mean \pm SD (range) MFI for the iDSA and sum DSAs were respectively 6196 ± 4786 (1000-20000) and 7635 ± 6530 (1000-37000). The *dn*DSAs were directed primarily against (i) HLA class II antigens in 58 of the 89 patients (65%), (ii) HLA class I antigens in 21 patients (24%), and (iii) both HLA class I and II antigens in 10 patients (11%). In 15 cases (17%), the *dn*DSAs disappeared during the first year of follow-up; this almost always concerned a *dn*DSA with an MFI between 1000 and 2000 (data not shown).

3.2.2. FC-XM distribution

Fifty-nine of the 89 patients (52.5%) were positive in the pan-IgG FC-XM assay. The MFI of the iDSA or the total sum of *dn*DSAs (when more than one *dn*DSA were identified in the recipient) was above 3000 (our limit of detection) in all of these cases. In six cases, the FC-XM was negative – even though an iDSA with an MFI of between 3000 and 5000 had been detected in a serum assay. These DSAs had these following specificities: Anti-A*02:01 (MFI=4500); C*04:01 (MFI=3500); DQB1*05 :01 (2 cases, MFI=3000 and 5000); DQA1*05 :01 (MFI=6000 in association with a second DSA anti-B*44:03 MFI=1000), DPB1*04 :02 MFI=5000). The main FC-XM results are summarized in **Table 2**.

3.2.3. IgG *dn*DSA subclasses

It is noteworthy that 26 (29%) of the patients with a positive Luminex™ single antigen IgG assay were negative for all four IgG subclasses (mean \pm SD (range) pan-IgG MFI: 1646 ± 644 (1000-3000)). This was related to a lower detection threshold in the subclass assay - even when an MFI cut-off of 500 was used.

Of the 63 patients with IgG *dn*DSAs, IgG1 *dn*DSAs were predominant in 55 (87%), and IgG3 *dn*DSAs were predominant in 30 (48%). To establish a link between cytotoxicity complement dependent of *dn*DSA and IgG subclasses, we pooled the patients with IgG1 and/or IgG3

*dn*DSAs, and pooled the patients with IgG2 and/or IgG4 but not IgG1 or IgG3 in the solid-phase assay. Fifty-five (87%) patients fell into the first pooled class, and 6 of the 63 patients (10%) fell into the second (**Table 2**).

3.2.4. Complement-binding antibodies:

C1q-binding *dn*DSAs were detected in only 17 of the 89 patients (19%) with a high MFI in the pan-IgG standard bead assay (MFI >8000, in our experiments), which was related to low detection threshold in the complement-binding antibody assay. All of these patients were positive for IgG1 and/or IgG3 subclasses. In only one patient, we had two DSAs positive for complement-binding antibodies. In only one other patient, we did not consider for complement binding antibodies the iDSA (anti-A1 with 9000 of MFI) but the second DSA (anti-DQ9 with 8000 of MFI).

3.3. Comparison of the characteristics of *dn*DSA assays in patients with ABMR

Among the 45 patients with ABMR and transplant glomerulopathy:

- *dn*DSAs were directed against (i) HLA class I in 4 cases (9%), (ii) HLA class II in 34 cases (75%), and (iii) HLA classes I and II in 7 cases (16%). The mean MFI was 8522 ± 5115 .
- 41 (91%) patients were positive in the FC-XM.
- 40 (89%) patients were positive for IgG1 and/or IgG3.
- 16 (36%) patients were positive for C1q binding.

The characteristics of the *dn*DSA assays as a function of the presence or absence of ABMR are shown in **Figure 1**.

Other important characteristics of the *dn*DSAs are summarized in **Table 2**. An FC-XM result for pan-IgG, complement-binding activity, and the presence of IgG1 and/or IgG3 subclasses were significantly associated (all $p < 0.05$) with the presence of biopsy features (i.e. ABMR and/or C4d deposits). However, in view of our FC-XM assay's limit of detection and when considering only DSAs cases with MFI > 3000, we noted that the FC-XM assay results were not closely associated with the presence or absence of ABMR even if we considered sum DSAs. Indeed, the FC-XM assay was poorly specific (13.7%). FC-XM assay should be used with great care in this indication. This data clearly shows that its good ABMR-negative

predictive value in case of low MFI (under 3000) is only due to its detection threshold and not to a good sensitivity.

The MFI of *i*DSA 1000 to 2999 vs up to 3000 is respectively related to absence and presence of ABMR. The study of *sum*DSAs showed a stronger link with ABMR with MFI up to 6000 ($p = 0.01$). See results in Table 3.

Each assay's sensitivity, specificity, and positive and negative predictive values for ABMR and for C4d deposits are listed in **Table 4**.

3.4. Investigation of patients with ABMR and loss of graft function

While following up of our cohort, we observed the loss of kidney function (i.e. resumption of dialysis) in 15 patients with *dn*DSAs and active ABMR in graft biopsies. Cases of active ABMR were treated mainly with intravenous immunoglobulins and corticosteroid pulses. Seven of the 15 patients lost graft function less than six months after the onset of ABMR. All 15 patients had *dn*DSAs with MFI >3000 and IgG1 and/or IgG3 *dn*DSAs, and 12 patients were positive in an FC-XM assay for pan-IgG subclasses. The 3 patients with a negative FC-XM assay had the following *dn*DSAs: anti-DQ5 (DQB1*05:01) (MFI=3000), anti-DQ5 (DQB1*05:01) (MFI=5000), and anti-DP4 (DPB1*04:02) (MFI=5000). Only 2 patients had a positive C1qScreen® assay, and only 9 patients had C4d deposits on the biopsy.

4. DISCUSSION

The histological features of ABMR vary markedly from one individual to another - especially on biopsies taken soon after transplantation. Hence, the accurate diagnosis of ABMR can be challenging as a result of the slow disease progression, potential fluctuations in DSA levels, variable C4d positivity, and microvascular inflammatory activity [11]. Indeed, a combination of positive C4d staining, tissue injury, and presence of *dn*DSAs is required for a highly specific diagnosis of ABMR, according to the Banff classification.

In the present study, we used an FC-XM assay with pan-IgG determination to predict the risk of developing biopsy-proven ABMR in a cohort of kidney transplant recipients. We also used several non-invasive assays to detect and characterize anti-HLA *dn*DSA profiles (MFI values, IgG subclasses and complement-binding ability in a solid-phase assay).

Our present results confirm the specific association between this triad (C4d staining, tissue injury, and *dn*DSAs) for ABMR and graft loss – even though we found a non-negligible proportion of recipients (5 out of 89) displaying T-cell-mediated rejection episodes before or at the *dn*DSA onset. The histological features at the time of the first positive *dn*DSA assay were not broadly consistent with ABMR; in line with previous reports, only 60% of the biopsies with microvascular inflammation were C4d-positive [12, 13]. We found that 50.5% of our *dn*DSA-positive patients displayed biopsy-proven ABMR at the time of diagnosis, which is consistent with Yamamoto et al.'s [14] reported value of 40%.

In this context, we tried to reproduce *in vitro* the mechanisms of tissue injury evidenced on the biopsy by performing an FC-XM assay with cryopreserved donor cells and recipient *dn*DSA-containing serum. However, this technique is unable to discriminate between complement- and non-complement-binding antibodies [7]. Unfortunately, this FC-XM assay of the *dn*DSAs' ability to bind to donor cells was unable to discriminate between cases with or without ABMR on biopsy findings. The assay had a low positive predictive value (<70%) and a low negative positive predictive value (42.9%), after taking account of its sensitivity (detection of DSAs with MFI >3000).

After having explored the *dn*DSAs' characteristics, our main finding was that the combination of complement-binding ability and an elevated MFI is predictive of the development of ABMR in *dn*DSA-positive patients; this is consistent with previous reports [15, 16]. However, the high specificity and positive predictive value of the complement-binding assay is also due to its low detection threshold and its positivity for *dn*DSAs with a high MFI (8000, in our experiment) only. By way of a comparison, Bouatou et al. found that 48% of *dn*DSA-positive patients showed complement-binding activities, which were linked to an elevated incidence of ABMR [15]. Conversely, the observation of *dn*DSAs in a patient lacking complement-binding activity and despite the presence of C4d deposits on biopsy is consistent with reports of complement-binding antibodies that are not detected by a standard C1q-binding assay [17].

In conclusion, our results showed that a pan-IgG FC-XM assay is sensitive to the appearance of *dn*DSAs with an MFI >3000. Unfortunately, the assay is also positive in some cases with *dn*DSAs but no biopsy-proven ABMR. Even if the *dn*DSAs' complement binding activity (detected using a C1qScreen® assay for *dn*DSAs with a high MFI) had a higher positive

predictive value for ABMR than a pan-IgG FC-XM assay or a solid-phase IgG subclass assay, the height of the MFI of iDSA (up to 3000) might be enough in this context.

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Legends

Figure 1: *dn*DSA profiles, as determined by each assay.

*dn*DSA determined from complement-binding ability (C1q assay, C1qScreen®), IgG1/3 subclasses (a bead assay), and pan-IgG FC-XM, as a function of the biopsy findings : ABMR (active or chronic active) versus no ABMR.

*i*DSA: immunodominant donor-specific antibody; *sum*DSAs: sum of total MFI of all donor-specific antibodies; ABMR: antibody-mediated rejection (active, chronic active); FC-XM: flow cytometry crossmatching assay

Figure 1

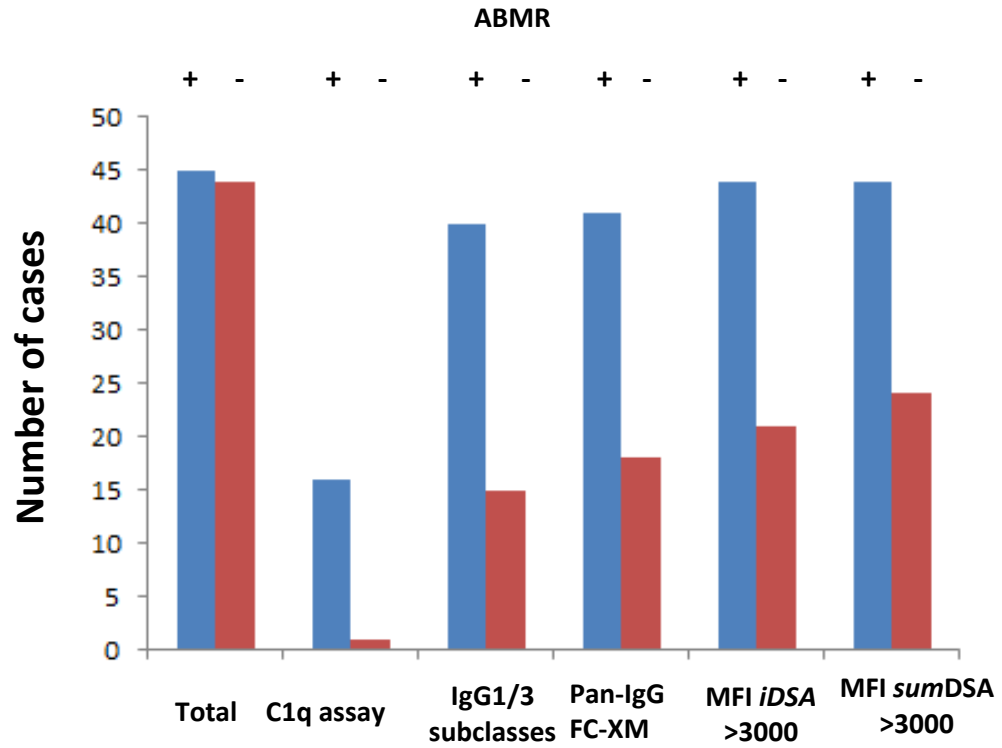


Table 1: Demographic variables, biopsy findings and DSA characteristics in the study population

	N = 89	ABMR (N=45)	No ABMR (N=44)
Demographic variables			
Sex, M/F	53/36	26/19	27/17
First transplant n,%	83 (93%)	42 (93%)	41 (93%)
Age at first positive <i>dn</i>DSA assay (years)	49.5 ± 12.8 (24-73)	48.3 ± 12.4 (26-73)	50.6± 13.2 (24-71)
Induction Therapy			
Basiliximab	45 (50.5%)	23 (51%)	22 (50%)
Thymoglobulin	44 (49.5%)	22 (49%)	22 (50%)
Maintenance Immunosuppression			
Cyclosporine	45 (50.5%)	24 (53%)	21 (48%)
Tacrolimus	44 (49.5%)	21 (47%)	23 (52%)
Corticosteroid (mg/day) (prednisolone equivalent)			
No corticosteroid	7 (8%)	3 (7%)	4 (9%)
Under 7.5 mg/day	55 (62%)	30 (66.5%)	25 (57%)
10 mg/day	25 (28%)	12 (26.5%)	13 (30%)
More than 10 mg/day	2 (2%)	0	2 (4%)
Biological data			
eGFR (ml/min/1.73 m²)	46.3 ± 21.3 (15-99)	45.4 ± 21.5 (15-99)	47.4 ± 21.1 (21-95)
Increase in creatinine of more than 20% above baseline, and/or new-onset proteinuria	25/89 (28%)	14 (31%)	11 (25%)
Time interval between transplantation and first positive <i>dn</i>DSA assay (months)	83 ± 51 (4-192)	81 ± 48 (4-179)	85 ± 50 (12-192)
Duration of follow-up after the first positive <i>dn</i>DSA assay (months)	46.5 ± 27.7 (12-130)	44.2 ± 25.3 (12-128)	47.2 ± 26.9 (14-130)
Biopsy findings n,% according to the Banff classification			
Range time to perform biopsy according to <i>dn</i>DSA identification (0-3 months)			
ABMR or transplant glomerulopathy	45 (50.5%)	45 (100%)	NA
<i>Glomerulitis (g) and/or peritubularcapillaritis (ptc) lesions</i>	33 (37%)	33 (73%)	
<i>Interstitial inflammation (i) and/or tubulitis (t)</i>	16 (18%)	16 (36%)	

<i>Intimal arteritis (v)</i>	4 (4.5%)	4 (9%)	
<i>Mesangial matrix expansion (mm) and/or transplant glomerulopathy (cg)</i>	11 (12%)	11 (24%)	
<i>Interstitial fibrosis (ci) and/or tubular atrophy (ct)</i>	23 (26%)	23 (51%)	
<i>Vascular fibrous intimal thickening (cv) and/or arteriolar hyalinosis (ah)</i>	5 (5.5%)	5 (11%)	
<i>C4d+</i>	27 (30%)	27 (60%)	
T-cell mediated rejection	7 (8%)	NA	7 (8%)
Bordeline changes - Others (not considered to be due to rejection)	27 (30%)		27 (30%)
Normal kidney or non specific changes	10 (11%)		10 (11%)
Pre transplant HLA antibody (no DSAs)			
HLA class I only	14 (16%)	7 (15%)	7 (16%)
HLA class II only	27 (30%)	15 (33%)	12 (27%)
HLA classes I and II	5 (6%)	3 (7%)	2 (5%)
Post transplant DSAs			
Number of DSAs	129	71	58
MFI between 1000 and 2999	32 (25%)	9 (13%)	23 (39%)
MFI between 3000 and 5999	52 (40%)	18 (25%)	34 (59%)
MFI >6000	45 (35%)	44 (62%)	1 (2%)
Mean MFI for the <i>i</i>DSAs	6196 ± 4786	8522 ± 5115*	3388 ± 2172*
Mean MFI for the <i>sum</i>DSAs	7635 ± 6530	11144 ± 7003*	4045 ± 3319*

Abbreviations: M: male, F: female, *dn*DSA: *de novo* donor-specific anti-HLA antibodies, eGFR: estimated glomerular filtration rate, ABMR: antibody-mediated rejection (active, chronic active or chronic), MFI: mean fluorescence intensity, HLA: human leukocyte antigen, *i*DSA: immunodominant donor-specific anti-HLA antibodies, *sum*DSAs: sum of total MFI of all donor-specific antibodies.

*p value between these two subgroups (Student's test): p<0.0001. Others variables in this table are not statistically significant.

Table 2: characteristics of *dn*DSAs associated with morphological biopsy features. DSA: donor-specific antibody; FC-XM: Flow cytometry crossmatch; ABMR: antibody-mediated rejection (active, chronic active)

	Biopsy features						
	Total (89 cases) N cases (%)	ABMR (45 cases) N cases (%)	No ABMR (44 cases) N cases (%)	p	C4d + (27 cases) N cases (%)	C4d- (18 cases) N cases (%)	p
anti-HLA class I DSAs	21 (24%)	4 (9%)	17 (37%)	p=0.002	3 (11%)	1 (5.5%)	ns
anti-HLA class II DSAs	58 (65%)	34 (75%)	24 (56%)		18 (67%)	16 (89%)	ns
anti-HLA class I and II DSAs	10 (11%)	7 (16%)	3 (7%)		6 (22%)	1 (5.5%)	ns
Positive for complement-binding activity (C1q)	17 (19%)	16 (36%)	1 (2%)	p<0.001	25 (93%)	11 (61%)	p<0.002
Negative for complement-binding activity (C1q)	72 (81%)	29 (64%)	43 (91%)		2 (7%)	7 (39%)	
Presence of IgG1 and/or IgG3 subclasses	55 (62%)	40 (89%)	15 (34%)	p=0.012	27 (100%)	16 (89%)	ns
Presence of IgG2 and/or IgG4 subclasses only	6 (7%)	1 (2%)	5 (11%)		0 (0%)	2 (11%)	
FC-XM, positive for pan-IgGs	59 (66%)	41 (91%)	18 (41%)	p<0.001	27 (100%)	14 (78%)	p=0.02
FC-XM, negative for pan-IgGs	30 (34%)	4 (9%)	26 (59%)		0 (0%)	4 (12%)	
	iDSA>3000 (65 cases) N cases (%)	ABMR (44 cases) N cases (%)	No ABMR (21 cases) N cases (%)	p	C4d + (27 cases) N cases (%)	C4d- (17 cases) N cases (%)	p
FC-XM, positive for pan-IgGs	59 (91%)	41 (93%)	18 (86%)	ns	27 (100%)	14 (82%)	p=0.05
FC-XM, negative for pan-IgGs	6 (9%)	3 (7%)	3 (14%)		0 (0%)	3 (18%)	
	sumDSA>3000 (68 cases) N cases (%)	ABMR (44 cases) N cases (%)	No ABMR (24 cases) N cases (%)	p	C4d + (27 cases) N cases (%)	C4d- (17 cases) N cases (%)	p
FC-XM, positive for pan-IgGs	59 (87%)	41 (93%)	18 (75%)	p=0.06	27 (100%)	14 (82%)	p=0.05
FC-XM, negative for pan-IgGs	9 (13%)	3 (7%)	6 (25%)		0 (0%)	3 (18%)	

Table 3: Link between *dn*DSAs MFI and morphological biopsy features. *i*DSA: immunodominant donor-specific antibody; *sum*DSAs: sum of total MFI of all donor-specific antibodies; ABMR: antibody-mediated rejection (active, chronic active); ref: reference.

		Biopsy features						
	Total (89 cases) N cases (%)	ABMR (45 cases) N cases (%)	No ABMR (44 cases) N cases (%)	p		C4d + (27 cases) N cases (%)	C4d- (18 cases) N cases (%)	p
<i>i</i>DSA						<i>i</i>DSA		
1000 - 2999	24	1	23	p < 0.001		0	1	p = 0.4
3000 - 5999	19	11	8	p = 0.38		5	6	p = 0.29
6000 - 9999	29	19	10	p = 0.31		11	8	p = 0.28
Up to 10000	17	14	3	ref		11	3	ref
<i>sum</i>DSAs						<i>sum</i>DSAs		
1000 - 2999	21	1	20	p < 0.001		0	1	p = 0.4
3000 - 5999	18	7	11	p = 0.01		1	6	p = 0.08
6000 - 9999	28	18	10	p = 0.11		11	7	p = 0.30
Up to 10000	22	19	3	ref		15	4	ref

Table 4: Diagnostic sensitivity, specificity, and positive and negative predictive values for each *dn*DSA assay.

FC-XM: Flow cytometry crossmatching; ABMR: antibody-mediated rejection (active or chronic active)

	Sensitivity	Specificity	Positive predictive value	Negative predictive value
C1qScreen™ vs. ABMR	35,6%	97,7%	94,1%	59,7%
C1qScreen™ vs. C4d deposits	92,5%	38,9%	69,5%	78,8%
IgG1 and/or IgG3 subclasses vs. ABMR	88,9%	65,9%	72,7%	85,9%
IgG1 and/or IgG3 subclasses vs C4d deposits	100%	11,1%	62,8%	100%
FC-XM vs. ABMR	91,1%	59,1%	69,5%	86,7%
FC-XM vs. ABMR (DSA with MFI>3000)	93,8%	14,3%	69,5%	50%
FC-XM vs. C4d deposits	100%	22,2%	65,8%	100%
FC-XM vs. C4d deposits (DSA with MFI>3000)	100%	17,6%	65,8%	100%
<i>i</i>DSA with MFI>3000 vs. ABMR	97,8%	52,3%	67,7%	97,8%
<i>sum</i>DSA with MFI>3000 vs. ABMR	97,8%	45,4%	64,7%	95,2%
<i>i</i>DSA or <i>sum</i>DSAs with MFI>3000 vs. C4d deposits	100%	5,6%	61,4%	100%