

Beyond C4d: the ultrastructural appearances of endothelium in ABO-incompatible renal allografts

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ABSTRACT

Background. ABO incompatibility is no longer a barrier in kidney transplantation. C4d is frequently positive in ABO-incompatible (iABO) biopsies without further signs of micro-circulation injury. This phenomenon is assumed to represent graft accommodation. However, ultrastructural examination of glomerular and peritubular capillary endothelium might reveal subtle endothelial damage.

Methods. We studied the ultrastructural appearance of the endothelium in 67 biopsies from 21 patients with iABO allografts and compared it with 20 patients (29 biopsies) with ABO-compatible (cABO) grafts with C4d positivity and 25 ABO-compatible control patients (25 biopsies) without serological or histological evidence of humoral rejection (C4d negative). Ten ultrastructural parameters indicative of chronic and

acute glomerular and peritubular capillary damage in transmission electron microscopy (TEM) were semi-quantitatively graded and expressed in a sum score. Clinico-pathological data were compared as well as graft function at the time of biopsy and follow-up.

Results. Ultrastructural parameters did not significantly differ between iABO and controls. In contrast, C4d-positive cABO had the highest TEM sum score ($P = 0.001$ versus iABO, $P = 0.002$ versus controls). The sum score did not differ between C4d-positive and C4d-negative iABO but did differ between patients with and without anti-HLA donor-specific antibodies (DSA). Graft function in iABO at the time of biopsy and at follow-up was similar to controls.

Conclusions. Our ultrastructural observations support the concept of endothelial accommodation in iABO renal transplants. C4d positivity in the ABO-incompatible situation does not indicate injurious activation of the complement cascade

and does not seem to impact on the graft function, in contrast to C4d deposition in cABO with antibody-mediated rejection.

INTRODUCTION

ABO-incompatible (iABO) renal transplantation is a well-established procedure that has helped to increase the donor pool [1]. Long-term graft outcome has been shown to be similar to conventional ABO-compatible (cABO) kidney transplantation [2]. This is all the more noteworthy since the grafts are functioning despite the presence of donor-specific anti-blood group antibodies. This specific condition has been referred to as accommodation [3–5]. It is also well known that biopsies from iABO renal allografts frequently show strong C4d positivity in peritubular capillaries, indicating complement activation, without further signs of microcirculation injury or impairment of graft function [6, 7].

C4d is a split product from classical complement activation. Identification of its deposition on the graft endothelium led to a more profound understanding of antibody-mediated rejection (ABMR) in renal allografts [8, 9]. Although recently, the sensitivity of C4d immunohistochemistry has been questioned [10, 11], C4d staining is still considered highly specific for ABMR in a cABO setting [12].

Acute and chronic endothelial damage can be assessed using transmission electron microscopy (TEM). Acute endothelial damage causes endothelial swelling, expansion of the glomerular lamina rara interna and loss of endothelial fenestration (LEF) [13]. Chronic microcirculation injury is reflected in multilayering of glomerular and peritubular capillary basement membranes [13–16]. Systematic ultrastructural assessment of endothelial changes in iABO allografts has not been reported in the scientific literature.

In contrast to cABO, the specificity of positive C4d staining is completely lost in iABO renal transplants, where it is frequently strongly positive in the absence of additional light microscopic signs of microcirculation injury. This raises the question whether there is any evidence of subtle complement-mediated endothelial injury which is not identified by light microscopy.

To answer this question, we examined ultrastructural signs of microcirculation injury in iABO patients and compared it with cABO with positive C4d staining and ABO-compatible C4d negative controls. We related the findings to histopathology, presence of donor-specific HLA antibodies and graft function, aiming to further clarify the relevance of C4d in iABO transplants.

SUBJECTS AND METHODS

Patients and study design

Sixty-six patients with 121 transplant biopsies taken between 2001 and 2012 were included in the study. Twenty-one patients received an iABO transplant and had 67 biopsies in total. All available biopsies until February 2010 from all iABO transplants in our centre were included. Twenty patients had a cABO transplant and 29 biopsies in total. All of them showed C4d positivity

(Banff score >1) in peritubular capillaries, with or without additional morphological features of microcirculation injury. To select these patients, we browsed our transplant database for C4d-positive cABO biopsies. From the patients that were found in the database, all C4d-positive biopsies were included, provided that a sample for TEM and clinical follow-up was available. Twenty-five patients with 25 biopsies from cABO transplants without C4d positivity and without signs of microcirculation injury served as controls. These patients were selected from our transplant database based on the following criteria: no microcirculation injury (Banff ptc and g score = 0) in any of the patient's biopsies, C4d always negative, time between transplantation and biopsy comparable with other groups; TEM and clinical follow-up available. Demographical and clinical data are shown in Table 1. Experiences with iABO kidney transplantation in our centre, including the induction protocol and maintenance immunosuppression (IS), have been described previously [17]. According to our centre's policy, approved by the Hannover Medical School Ethics Committee, protocol biopsies were performed after obtaining a written consent at 6 weeks, 3 and 6 months after transplantation.

The estimation of glomerular filtration rate (eGFR) was carried out using the simple Modification of Diet in Renal Disease equation. The renal function was evaluated at baseline (best clearance during the first 6 weeks after transplantation), at the time of biopsy and at the time of last follow-up. For patients who lost their graft, the date of last follow-up marks the date of return to dialysis. The impairment of graft function per month was expressed as $eGFR\text{-slope} = (eGFR \text{ at follow-up} - \text{baseline } eGFR) / \text{time to follow-up in months}$.

Histopathology and transmission electron microscopy

Paraffin sections from formalin-fixed tissue were prepared for H&E, PAS, Jones Silver and C4d stains. Biopsies were graded according to the Banff criteria [12].

'Suspicious for ABMR' was defined as the presence of glomerulitis or peritubular capillaritis and C4d > Banff score 1 (cABO) or as glomerulitis and peritubular capillaritis with or without C4d (iABO). 'Possible ABMR' was defined as the presence of glomerulitis and peritubular capillaritis >0 and C4d ≤ Banff score 1. Mixed cellular/humoral rejection was defined as borderline or acute cellular rejection according to the Banff criteria plus glomerulitis or peritubular capillaritis and C4d > Banff Grade 1 (cABO); plus glomerulitis and peritubular capillaritis with or without C4d (iABO).

A minute sample of each biopsy was fixed in glutaraldehyde to be processed for TEM. TEM was available for 63 of 67 biopsies in the iABO group, 29 of 29 for cABO and 25 of 25 in the control group.

The following ultrastructural parameters were semi-quantitatively graded as 0, 1, 2 and 3 as follows: foot process effacement (FPE): <10, 10–25, 26–50, >50% of the glomerular tuft involved; expansion of the lamina rara interna (LRIE): none, <25, 26–50, >50%; lamellation of glomerular basement membranes (LGBM): none, <25, 26–50, >50%; double contours of glomerular basement membranes (DKGBM): none, <25, 26–50, >50%; swelling of glomerular endothelial cells (SGE): none, <25, 26–50, >50%; loss of endothelial fenestration

Table 1. Demographical and clinical data

	iABO	cABO	controls	P-value
No. of patients/no. of biopsies	21/67	20/29	25/25	N/A
No. of biopsies per patient	3.19 ± 1.89	1.45 ± 0.67	1 ± 0	<0.001
No. of protocol/indication bx	36/31	12/17	19/6	0.03
Male/female	14/7	7/13	15/10	0.73
Age at tx (years)	40.33 ± 15.15	47.31 ± 14.53	43.00 ± 12.86	0.41
Donor age (years)	48.38 ± 12.39	52.2 ± 12.85	50.56 ± 7.71	0.553
Time tx to bx (days) ^a	138.1 ± 163.46	169.0 ± 378.142	116.36 ± 110.44	0.196
Time to follow-up (months)	34.71 ± 18.84	57.30 ± 39.59	72.52 ± 24.04	<0.001
No. of tx (1st/2nd/3rd)	19/2	11/6/3	22/2/1	0.008 (1 versus >1)
IS (1 ^b /2 ^c /3 ^d)	21/0/0	7/5/8	2/9/14	N/A
No. of HLA-mismatches per patient	3.57 ± 1.63	2.95 ± 1.70	2.08 ± 1.61	0.016
Donor-type (living/deceased)	21/0	4/16	13/12	<0.001

^aEach biopsy considered separately.

^bTriple immunosuppression (IS) including tacrolimus, mycophenolate mofetil (MMF), prednisone.

^cTriple IS including cyclosporin, MMF, prednisone.

^dDouble IS including tacrolimus in combination with MMF or cyclosporin or MMF in combination with prednisone.

(LEF): none, <25, 26–50, >50%; adhesion of inflammatory cells to glomerular endothelium (AGE): none, <25%, 26–50%, >50%; peritubular capillary basement membrane multilayering (PTCML): none, <3, 4–5, >5 lamellae. Adhesion of inflammatory cells to peritubular capillary endothelium (APE) and swelling of peritubular capillary endothelial cells (SPE) was noted if present but not further graded. A summary of ultrastructural parameter abbreviations is provided in Table 2.

To include all available biopsy information for each patient, but to avoid bias due to variable numbers of biopsies per patient, we combined all biopsies from each individual patient into one ‘virtual biopsy’. The ‘virtual biopsy’ consists of the mean value of every histopathological and ultrastructural parameter. For further comparisons between groups, the virtual biopsies were used.

Analysis of HLA antibodies

Screening for anti-HLA class I and II antibodies was performed using the Luminex™ technology (LABScreen™ Mixed assay, One Lambda Inc., Canoga Park, CA) according to the manufacturer's instructions. This assay enables the detection of antigen-bound antibodies on fluorescently tagged polystyrene microbeads. Samples were subsequently analysed on a Luminex™ machine (Luminex™ Corporation, Austin, TX), which is able to discriminate up to 100 unique bead populations in one reaction vial. The discrimination of HLA specificities in LABScreen™ mixed-positive samples was performed using LABScreen™ single antigen beads (One Lambda Inc.) for anti-HLA class I and II antibodies. In addition, all samples were tested for the presence of lymphocytotoxic antibodies using a complement-dependent

Table 2. Abbreviations of ultrastructural parameters

Ultrastructural parameter	Abbreviation
Foot process effacement	FPE
Expansion of lamina rara interna	LRIE
Lamellation of glomerular basement membranes	LGBM
double contours of glomerular basement membranes	DKGBM
Swelling of glomerular endothelial cells	SGE
Loss of endothelial fenestration	LEF
Adhesion of inflammatory cells to glomerular endothelium	AGE
Peritubular capillary basement membrane multilayering	PTCML
Adhesion of inflammatory cells to peritubular capillary endothelium	APE
Swelling of peritubular capillary endothelial cells	SPE

cytotoxicity assay with the addition of dithiothreitol to exclude lymphocytotoxic autoantibodies of the IgM type. In two cases, the detection of class I and II anti-HLA-antibodies was determined using the Lifecodes Single Antigens Class I and Class II assay (Gen-Probe, San Diego, CA) on a Luminex platform.

Statistics

Statistics were performed with SPSS 18.0/19.0. For comparison of continuous variables between groups, ANOVA, Kruskal–Wallis (for overall comparison of the three groups) and Mann–Whitney *U* test (for group-by-group comparison of significant variables) were used as appropriate. A chi-square exact test was used for count data. Correlations were performed using Spearman's rho test. Due to the small size of the groups, a multivariate test model was not appropriate. A *P*-value of <0.05 was considered statistically significant in two-sided tests. To control the error rate of multiple comparisons, the Sidák correction was used (significance threshold for each test = $1 - (1 - \alpha)^{1/\text{number of tests}}$). A *P*-value of <0.0169 was therefore considered statistically significant for three possible pairwise comparisons between our three cohorts.

RESULTS

Histopathology in iABO, cABO and controls

Twenty of the 21 iABO patients were C4d positive in at least one of their biopsies: 14 of 21 had maximum Grade 3, 2 of 21 maximum Grade 2 and 4 had maximum Grade 1. The

C4d score was significantly correlated with Banff ptc and g score (Spearman's: $r = 0.260$, $P = 0.033$; $r = 0.318$, $P = 0.009$, respectively) when looking at each biopsy as an independent event but was not when using the virtual biopsies.

C4d was positive (score >1) in all cABO patients and negative in all control patients according to the inclusion criteria for the respective group.

In iABO patients, 4 of 21 (19%) had an episode of acute rejection: 2 borderline rejections, 1 Banff II acute cellular rejection and 1 mixed cellular/humoral rejection (for definition, see Subjects and Methods). None of the patients presented with features of chronic (active) ABMR in light microscopy. In cABO patients, acute rejection was significantly more frequent (12/20, $P = 0.011$ versus iABO): one patient showed borderline changes, three patients had features 'suspicious for ABMR' and eight patients had mixed cellular/humoral rejection.

In the control group, 6 of the 25 patients ($P > 0.05$ versus iABO and $P = 0.03$ versus cABO) had acute cellular rejections (5 borderline rejections, 1 Banff Grade I). By definition, none had ABMR.

Interstitial fibrosis/tubular atrophy did not differ between the three groups. Three of 21 iABO patients had BK-virus

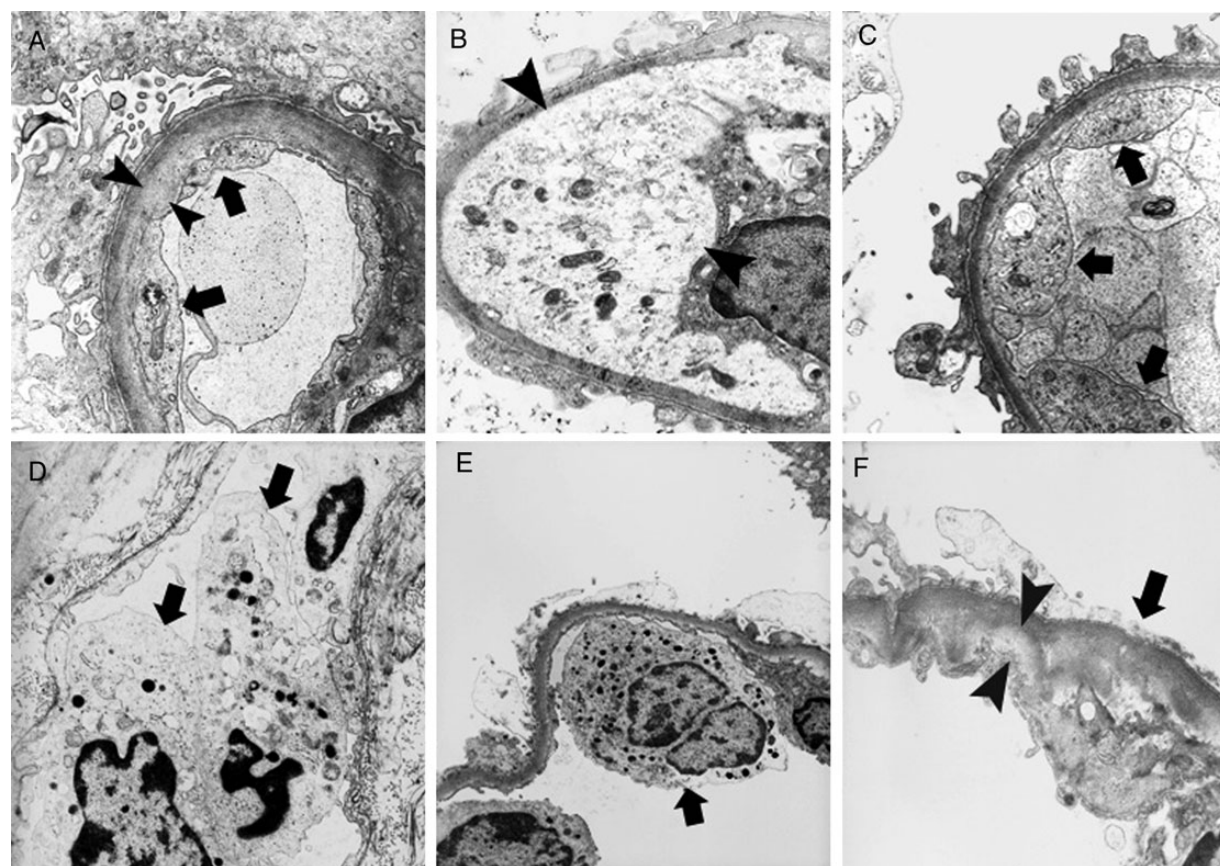


FIGURE 1: Examples of ultrastructural findings. (A) Glomerular capillary loop showing mild expansion of the lamina rara interna (LRI) (▶) and segmental endothelial swelling with LEF (➡) ($\times 10\,000$). (B) Glomerular capillary loop showing marked LRI (▶) ($\times 10\,000$). (C) Glomerular capillary loop showing endothelial swelling and segmental LEF (➡) ($\times 10\,000$). (D) Peritubular capillary with inflammatory cells within the lumen attached to the endothelium (➡). The basement membrane appears to be unremarkable ($\times 12\,000$). (E) Glomerular capillary loop with an inflammatory cell attached to the endothelium (➡). The basement membrane and the endothelium appear to be unremarkable ($\times 9500$). (F) Glomerular capillary loop showing subtle LRI (▶) and FPE (➡). The endothelial fenestration is intact ($\times 11\,000$).

nephropathy (BKVN) in the analysed biopsies. One patient developed BKVN later during follow-up. None of the patients in the other groups had BKVN in the investigated biopsies. However, two patients in the cABO group developed BKVN during follow-up.

Ultrastructure in iABO, cABO and controls

Representative pictures of ultrastructural findings are shown in Figure 1 and the results of TEM are presented in Table 3.

None of the ultrastructural parameters significantly differed between iABO and controls. In contrast, all parameters indicated a higher degree of endothelial injury in cABO with 4 of 10 parameters being significantly elevated (after Sidak correction for multiple testing) in comparison to iABO and controls, respectively. No association between the time after transplantation and TEM results was found for any of the parameters analysed (assessed for all 'non-virtual' biopsies together, data not shown).

Transmission electron microscopy sum score

As a single ultrastructural parameter might be less meaningful than the entire picture of microcirculation changes, we summed the results of the individual parameters to give the total TEM sum score of each patient. The mean score in iABO patients was not found to be different from the controls, but was significantly higher in cABO compared with both iABO and controls (Figure 2A). Interestingly, the score was significantly higher in patients with DSA (Figure 2B) and those with graft failure (Figure 2C), while it did not differ between iABO with and without C4d positivity (not shown). The TEM

sum score significantly correlated with graft function at different points of time, examined for all patients together (Figure 2D–F).

Kidney function

While the eGFR in iABO was similar to controls, cABO had the lowest baseline eGFR (Figure 3A), lowest eGFR at the time of biopsies (Figure 3B) and lowest eGFR at follow-up (Figure 3C). The estimated GFR-slope (the impairment of renal function per month from baseline to follow-up) was not different between the groups (Figure 3D).

In the iABO group, 2 of the 21 patients lost their transplant during follow-up (eGFR ≤ 10 mL/min/1.73 m²), one from BKVN and one from humoral rejection with thrombotic microangiopathy, both in the second year after transplantation. The latter was the only patient with detectable DSA in the iABO group. There were 6 of 20 transplant losses in the cABO group: 1 from BKVN (<1 year after transplantation), 1 from BKVN and concurrent humoral rejection (<1 year), 3 from rejection (<1 year, second and fifth year) and 1 from unknown reasons (fifth year). There were no transplant losses in the control group.

Correlation between graft function and ultrastructural parameters

Weak-to-moderate significant correlations between ultrastructural parameters and graft function at different points of time were seen for LRIE, LEF, FPE, SGE, AGE and SPE, especially with the eGFR at follow-up (Table 4). None of the ultrastructural parameters correlated with the eGFR slope.

Table 3. Results of ultrastructural examination

Parameter ^a	iABO (<i>n</i> = 21)	cABO (20)	Control (25)	P (Mann–Whitney U)	P (Kruskal–Wallis)
FPE (mean \pm SD) (number of patients affected)	0.47 \pm 0.37 (16)	0.91 \pm 0.77 (14)	0.6 \pm 0.91 (10)	ns	0.128
LRIE	0.80 \pm 0.48 (18)	1.34 \pm 0.67 (18)	0.72 \pm 0.74 (15)	0.006^b (0.004)^c	0.005
LGBM	0.08 \pm 0.17 (4)	0.18 \pm 0.49 (3)	0	ns	0.093
DKGBM	0.02 \pm 0.11 (1)	0.38 \pm 0.63 (6)	0.04 \pm 0.2 (1)	ns	0.011
SGE	0.91 \pm 0.56 (20)	1.27 \pm 1.56 (14)	0.84 \pm 0.99 (13)	ns	0.379
LEF	0.24 \pm 0.34 (10)	1.14 \pm 0.99 (15)	0.48 \pm 0.77 (9)	0.002^b (0.01)^c	0.005
AGE	0.40 \pm 0.31 (16)	0.75 \pm 0.7 (14)	0.44 \pm 0.65 (9)	ns	0.097
PTCML	0.11 \pm 0.19 (6)	0.64 \pm 0.85 (11)	0.12 \pm 0.33 (3)	0.015^b (0.003)^c	0.004
APE	0.19 \pm 0.24 (9)	0.35 \pm 0.49 (7)	0.12 \pm 0.33 (3)	ns	0.113
SPE	0.05 \pm 0.13 (3)	0.45 \pm 0.48 (10)	0.12 \pm 0.33 (3)	0.005^b (0.008)^c	0.003

Significant results in bold letters.

^aFor abbreviations of ultrastructural parameters, see Table 2.

^biABO versus cABO.

^ccABO versus control.

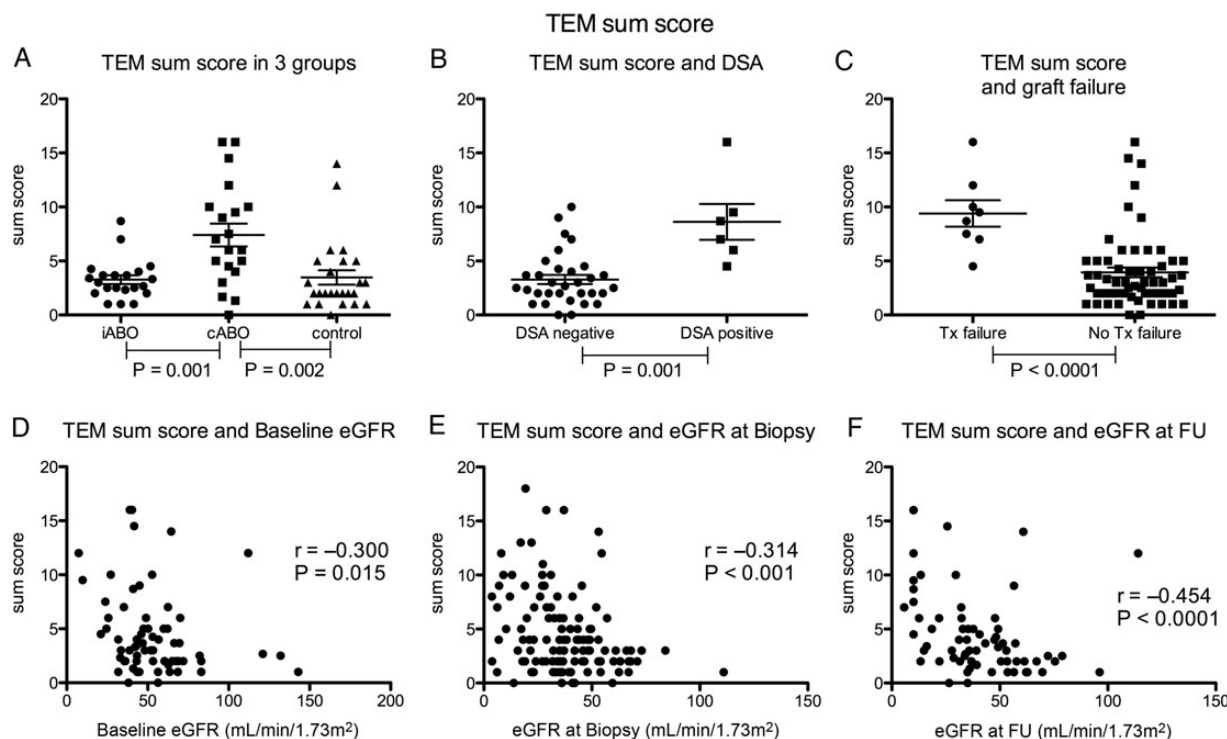


FIGURE 2: Transmission electron microscopy sum score. Each point represents the mean TEM sum score of all biopsies of an individual patient, except for (E), where each biopsy was regarded as an independent event. (A) The TEM sum score differs significantly between iABO, cABO and controls. (B) In 38 patients, sera were available for retrospective DSA testing. (C) The TEM sum score differed significantly between patients with and without transplant failure during follow-up. (D) 'Baseline eGFR' is defined as the best value recorded for an individual patient during the first 6 weeks after transplantation. (E) Each point represents the eGFR at the time of the individual biopsy. (F) Note that two patients in the iABO group and six patients in the cABO group had lost their transplant during the follow-up (eGFR ≤ 10 mL/min/1.73 m²).

Comparison between C4d-negative and C4d-positive ABO incompatible grafts

We analysed whether iABO patients with and without C4d staining were different with regard to histology, TEM and clinical parameters. 'C4d negative' was defined as complete absence or only minimal C4d positivity (Banff ≤ 1) in all of the patient's biopsies. Neither histopathological nor ultrastructural parameters were different between C4d negative ($n = 5$) and C4d positive ($n = 16$) patients. The number of HLA mismatches and number of kidney transplants were not different between the two groups. Graft function was not significantly different between the two groups at any point of time.

Presence of DSAs

For retrospective analysis of donor-specific HLA-antibodies (DSAs) post transplant, 56 sera from 38 patients were available (18/21 iABO, 12/20 cABO and 8/25 controls). DSAs were detected in 1/18 iABO (HLA class II) and 5/12 cABO (1 \times HLA class I, 3 \times HLA class II and 1 \times HLA class I and II). Thus, DSAs were significantly more frequent in cABO compared with iABO ($P = 0.026$). In those patients in whom serial sera were tested (one to three different points of time were available), all were positive for DSAs.

In the ultrastructural examination, patients with DSAs had significantly worse FPE ($P = 0.003$), LRIE ($P = 0.011$), LEF ($P < 0.001$) and SPE ($P = 0.016$) compared with patients

without DSAs. There was no association between the number of HLA mismatches and the presence of DSAs but patients with DSAs more often had had more than one kidney transplant ($P = 0.012$).

Patients with DSAs compared with patients without DSAs had significantly worse eGFR at baseline (36.0 mL/min/1.73 m² ± 20.5 versus 59.81 ± 27.74 ; $P = 0.035$), at the time of biopsies (24.1 ± 10.41 versus 41.84 ± 20.72 ; $P = 0.014$) and at follow-up (17.97 ± 11.1 versus 44.88 ± 21.26 ; $P = 0.002$). Three of six patients with DSA lost their transplant during follow-up compared with 3 of 32 in the DSA-negative group ($P = 0.039$). All DSA-negative patients with transplant failure had BKVN.

DISCUSSION

To the best of our knowledge, this is the first study to systematically examine ultrastructural changes in C4d-positive biopsies in iABO and cABO renal allografts. C4d positivity of at least some degree was present in 95% of patients in the iABO cohort. However, the light microscopic picture of ABMR as defined by the Banff classification was rarely seen in iABO. The ultrastructural changes as assessed by the TEM sum score did not significantly differ between iABO and controls, while patients with C4d-positive cABO allografts and/or detection of DSA had the highest TEM sum score. Consistently, graft function was similar in iABO and control patients, but significantly

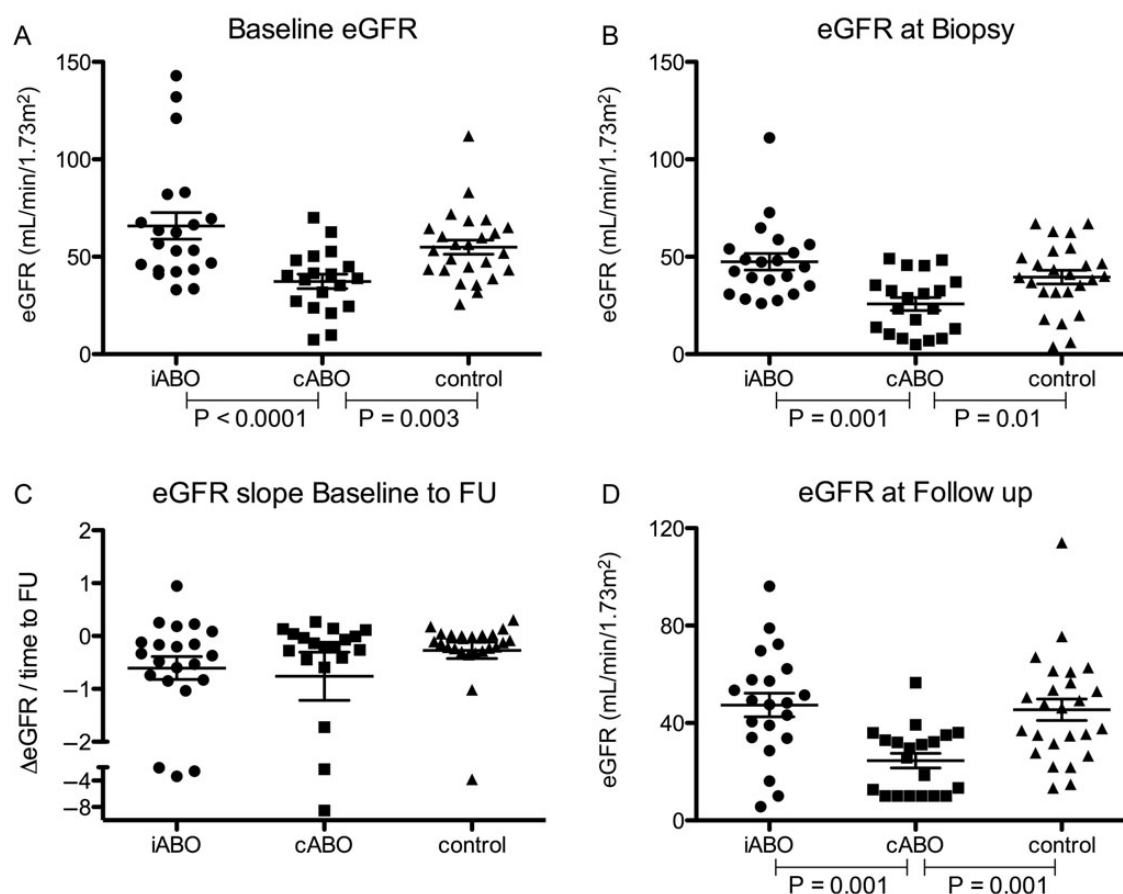


FIGURE 3: Kidney function in iABO, cABO and controls at different points of time. Kidney function was not significantly different between iABO and controls at any point of time. (A) ‘Baseline eGFR’ is defined as the best value recorded for an individual patient during the first 6 weeks after transplantation. (B) Each point represents the mean eGFR of all biopsies of an individual patient. (C) Note that two patients in the iABO group and six patients in the cABO group lost their transplant during follow-up (eGFR ≤ 10 mL/min/1.73 m²). (D) No significant difference is seen in the impairment of renal function/months to follow-up between the three groups. This might be explained by the fact that the groups start with significantly different baseline eGFR already.

impaired in C4d-positive cABO patients. C4d-positive and -negative iABO patients did not differ in terms of graft function, outcome of the graft, ultrastructural changes or immunological risk factors such as HLA mismatches, presence of HLA-DSA or the number of renal transplants. Although C4d is a very specific marker of ABMR in cABO, it does not seem to add information in the iABO setting.

The underlying reasons why antibody binding and complement activation can occur without causing endothelial injury are incompletely understood. Various mechanisms have been investigated (reviewed in [18, 19]). Most studies addressing accommodation have been done *in vitro* or in xenograft models showing increased expression of cell protective molecules such as haemoxygenase-1 or nitric oxide, upregulation of anti-apoptotic genes such as A20, Bcl-2 and Bcl-x or increased expression of complement regulatory proteins such as DAF (CD55) or CD59 [5, 18–22]. The latter would explain why the complement cascade can be initiated but does not result in cell lysis. Only a few studies have been performed in human tissue, showing immunomodulation via reduced mRNA expression of SMAD5,

TNF β and TGF β in accommodated grafts [4]. Ding *et al.* [5] speculated that the kinetics of anti-graft antibody titres rather than the titre itself seems to be crucial to allow the establishment of accommodation. One may speculate that once accommodation is established, this protects the graft not only against anti-blood group, but also anti-HLA antibody-mediated injury. However, the only DSA-positive patient in the iABO group lost his graft due to ABMR.

General concerns have been raised over the presence of any anti-graft antibody, either anti-HLA or anti-ABO, which may cause accumulative subtle allograft injury resulting in long-term graft impairment [23].

In our cohort, however, there is no evidence of endothelial damage in iABO beyond that observed in uncomplicated renal allografts. Sekijima *et al.* [24] briefly mentioned ultrastructural ‘signs of humoral injury’ in iABO. However, electron microscopy was not further described, findings were not compared with cABO transplant biopsies and no follow-up was available. Our results indicate that mild endothelial changes can be observed, even in ‘normal’ ABO-compatible transplant biopsies.

Table 4. Correlation between graft function and ultrastructural parameters

eGFR (mL/min/1.73 m ²)	Ultrastructural parameter					
	LRIE ^a	LEF ^b	FPE ^c	SGE ^d	AGE ^e	SPE ^f
At baseline						
r	−0.393	−0.312	ns	ns	ns	ns
P	0.001	0.011				
At the time of biopsies						
r	−0.357	−0.290	−0.260	ns	ns	ns
P	0.003	0.018	0.035			
At follow-up						
r	−0.498	−0.401	−0.281	−0.251	−0.272	−0.284
P	<0.001	0.001	0.022	0.042	0.027	0.021
Only significant correlations are shown in this table. ^a Lamina rara interna expansion. ^b LEF. ^c FPE. ^d Swelling of glomerular endothelial cells. ^e Adhesion of inflammatory cells in glomerula. ^f Swelling of peritubular capillary endothelial cells.						

However, this does not seem to affect the long-term outcome. No significant differences regarding electron microscopy were seen between iABO and controls for any of the parameters analysed. Over-interpretation of transient abnormalities at a single point of time should therefore be avoided.

Two parameters in particular seem to indicate relevant acute endothelial injury: lamina rara interna expansion and loss of endothelial fenestration. These parameters inversely correlated with the transplant function at different points of time, differed between cABO and both iABO and controls and differed between patients with and without HLA-DSA. As electron microscopy was mostly performed within the first year after transplantation, chronic changes such as glomerular double contours or advanced multilayering of peritubular capillary basement membranes were infrequently seen (present in 11 and 5 of 117 biopsies, respectively). Transplant glomerulopathy (TG) detected by light microscopy is normally seen >2 years after transplantation [13], although ultrastructural endothelial abnormalities in patients with later TG can be observed in the very early post-transplant period [13]. Ultrastructural studies on transplant renal biopsies have shown that a mild degree of PTCML can be present in renal diseases other than chronic rejection, especially those that cause endothelial injury, and even in normal transplants, similar to our results [14, 25–27]. Recently, Roufosse *et al.* showed that PTCML predicts TG and that the risk of TG increases with every additional layer of basement membrane [16]. Our results do not necessarily support the value of PTCML to predict TG, especially if it is of mild degree: Only two of six patients in the cABO group with ultrastructural evidence of glomerular double contours had concurrent PTCML. Five patients developed overt TG by light microscopy subsequently and only two

had PTCML at the time of our investigation. One was of higher degree. Conversely, some degree of PTCML was present in 17 patients in the iABO and cABO groups combined, only three of whom developed light-microscopic TG during follow-up.

There are several shortcomings in our study, which are the limited number of patients in each group and incomplete DSA testing. This is inevitable due to the retrospective nature of our study. The fact that several biopsies per patient were available in both cABO and iABO reduces the risk of over-interpretation of potentially transient findings.

To the best of our knowledge, this is the first study to examine the ultrastructural appearances of the endothelium in the early course after ABO-incompatible transplantation in correlation with histopathological findings and clinical course. The results indicate that a mild degree of endothelial injury in ABO-incompatible patients may be identified. However, this is not different from normal controls and does not seem to impact on the graft outcome. This supports the concept of endothelial accommodation in ABO-incompatible renal allografts.

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CONFLICT OF INTEREST STATEMENT

All authors of this manuscript declare to have no financial conflicts that are specific to the paper. We also declare that the results presented in this paper have not been published previously in whole or part, except in abstract form.

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