

## C4d staining in post-reperfusion renal biopsy is not useful for the early detection of antibody-mediated rejection when CDC crossmatching is negative

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### Abstract

**Background.** Sensitized patients (pts) may develop acute antibody-mediated rejection (AMR) due to preformed donor-specific antibodies, undetected by pre-transplant complement-dependent cytotoxicity (CDC) crossmatch (XM). We hypothesized that C4d staining in 1-h post-reperfusion biopsies (1-h Bx) could detect early complement activation in the renal allograft due to preformed donor-specific antibodies.

**Methods.** To test this hypothesis, renal transplants ( $n = 229$ ) performed between June 2005 and December 2007 were entered into a prospective study of 1-h Bx and stained for C4d by immunofluorescence. Transplants were performed against a negative T-cell CDC-XM with the exception of three cases with a positive B-cell XM.

**Results.** All 229 1-h Bx stained negative for C4d. Fourteen pts (6%) developed AMR. None of the 14 protocol 1-h Bx stained positive for C4d in peritubular capillaries (PTC). However, all indication biopsies—that diagnosed AMR—performed at a median of 8 days after transplantation stained for C4d in PTC.

**Conclusions.** These data show that C4d staining in 1-h Bx is, in general, not useful for the early detection of AMR when CDC-XM is negative.

**Keywords:** antibody-mediated rejection; C4d staining; complement activation; renal transplantation

### Introduction

Over the last decade, the acute rejection (AR) rate in renal transplantation (Tx) has decreased to <10% due to modern immunosuppression [1] and therapeutic drug monitoring.

However, after this decline, the incidence has levelled off possibly due to a more recently recognized form of AR: the antibody-mediated rejection (AMR) that repre-

sents 6–9% of the acute rejection episodes [2]. The clinical use of C4d staining in renal biopsies led to a more feasible recognition of this entity [3,4]. AMR constitutes a new challenge in renal transplantation.

At our transplant region, pre-transplant complement-dependent cytotoxicity crossmatch with anti-human globulin (CDC-AHG XM) is the current method to detect preformed donor-specific antibodies (DSA) in recipients of deceased-donor kidneys. However, these patients may have low anti-HLA antibody levels, undetected by the CDC-AHG XM at the time of transplantation.

C4d staining in renal biopsy can demonstrate complement activation at the tissue level. C4d-positive staining is a common feature of acute AMR [2] as well as chronic antibody-mediated rejection [5]. Nevertheless, the sensitivity of C4d staining in 1-h post-reperfusion biopsies in this setting has not been largely analysed.

We have, since June 2005, developed a policy of 1-h post-reperfusion zero-biopsies (1-h Bx) in renal transplanted patients in order to verify the value of C4d staining in detecting complement activation due to preformed DSA.

In this study, we report our analysis of C4d staining in a large series of 1-h Bx.

### Materials and methods

#### Patients

At our center, all transplanted kidneys from deceased donors received a protocol zero-biopsy. Between June 2005 and December 2007, our protocol was changed in order to perform zero-biopsies at least 60 min after revascularization. This observational study was conducted according to the Helsinki Declaration of 1975 and was approved by the local Ethical Committee for Clinical Research. All enrolled patients signed an informed consent.

Out of the 354 kidney transplants performed during this time period, 229 entered this protocol, and only their data were analysed. Also, from this population, the data of those who developed AMR ( $n = 14$ ; 6%) were more intensively analysed.

AMR was defined as the presence of acute tissue injury plus C4d-positive staining in peritubular capillaries (PTC) and also the finding of circulating DSA detected by any method, according to the Banff classification [2].

#### *One-hour post-reperfusion biopsies*

The biopsies were performed at least 1 h after removing the arterial and venous clamps and before the closure of the surgical wound. The time for all these procedures is usually <60 min and requires the surgeon to wait for the planned biopsy time. After a minimum of 60 min, two core biopsies were performed; one of them was saved for immunofluorescence (IF) studies.

#### *C4d staining*

C4d staining, in all protocol 1-h Bx, carried out done by the IF method. For the indication biopsies, IF was always analysed when enough biopsy material was available. Otherwise, immunohistochemistry (IHC) method was performed in paraffin-embedded samples.

C4d staining was classified according to the percentage of PTC stained as: negative (<10% of staining in PTC) or positive (a strong staining in a range of PTC: 10–24%, 25–50% and >50% PTC). Positive cases were evaluated in four different microscopy fields, counting a minimum of 20 PTC, starting in an area where there was a positive staining. The relative positive percentage staining was then calculated.

IF method. Briefly, frozen sections of 2–4 µm were placed in a dry surface for 30 min, flushed with PBS and received the avidin/biotin complex (Vector Laboratories, Burlingame, CA, USA) for 20 min. They were then incubated with anti-human C4d monoclonal antibodies (ABs) (Biogenesis, Sandown, NH, USA) flushed 2–3 times with PBS, then received a horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) for 30 min and then received FITC-streptavidin (Vector Laboratories, Burlingame, CA, USA) for 30 min.

IHC method. Briefly, sections of 3 µm were deparaffinized, and endogenous peroxidase activity was blocked with hydrogen peroxide. Sections were stained with rabbit polyclonal anti-human C4d antibody (Biomedica, Wien, Austria) in a dilution of 1/50 with overnight incubation at 4°C, after antigen retrieval using pressure cooking in 0.01 M citrate buffer at pH 6.0 for 2 min. Detection was made with secondary antibody polymer peroxidase complex (Novolink Max Polymer, Novocastra, Newcastle, UK). Diaminobenzidine was used as the chromogen. Counterstaining was carried out with Harris haematoxylin [3].

Positive and negative controls were used for both methods. For IF, a positive glomerular staining with negative PTC staining can be used as control.

#### *Crossmatch (XM)*

Panel-reactive antibodies (PRA) were performed pre-transplant by the ELISA method. All transplants were performed against a negative T-lymphocytes CDC-AHG XM with the exception of three cases transplanted with a positive B-lymphocyte CDC-XM.

Post-transplant flow cytometry XM (FCXM) was performed, after transplantation, in patients with a suspicious AMR, using both the pre-transplant sera (when available,  $n = 8$ ) and the current post-transplant sera.

DSA were detected by single-antigen (class I and II) beads (One Lambda Inc, Canoga Park, CA, USA).

For CDC-XM, T and B cells were isolated using Ficoll–Hypaque gradient. The presence of anti-HLA IgM antibodies was excluded by testing serum in the presence of dithiothreitol (DTT). For T-cell XM, AHG was added to the assay.

Briefly, for FCXM,  $0.5 \times 10^6$  donor lymphocytes were incubated with 25 µL of serum for 30 min at room temperature. After three washes with phosphate-buffered saline solution containing 2% fetal bovine serum and 0.1% sodium azide (PBS-FCXM), 25 µL of a pre-titred fluorescein isothiocyanate-conjugated goat anti-human IgG (Sigma, MO, USA) were added, and 25 µL of phycoerythrin-conjugated monoclonal mouse anti-human T-cell (CD3) and 5 µL Cy-Chrome-conjugated monoclonal mouse anti-human B-cell (CD19) (Sigma, MO, USA) were added to each tube. After 30 min of incubation at 4°C in the dark, the cells were washed three times with cold buffered solution and re-suspended in 200 µL of PBS-FCXM until analysis. For all FCXM, donor lymphocytes were incubated with patient's test serum, a positive and a negative control serum. FCXM analysis was performed using the FACSCalibur system [6].

## Results

### *Patients*

Out of the 354 transplants performed at our center during the study period, 229 (65%) performed 1-h Bx for C4d staining, and 125 did not, either due to no adequate record of the time for the biopsy procedure in the patients' files ( $n = 30$ ) or because the biopsy was not performed at all ( $n = 95$ ). These patients were not analysed. Patients who did not perform the 1-h Bx were mainly live donors with pre-transplant zero PRA ( $n = 95$ ).

Out of the 229 studied patients with a mean age of  $42 \pm 15$  years, 115 (50%) were male, 158 (69%) were white recipients, 208 (91%) were first transplants and 113 (49%) received kidney from a deceased donor. Induction therapy with anti-thymocyte globulin was used in 53 (23%) patients (pts), and IL-2R antibodies in 154 (67%) pts. Delayed graft function occurred in 71 (63%) grafts from deceased donors. Immunosuppression consisted of TCL in 214 pts (93%) and mycophenolic acid in 199 (87%). Forty-four pts (19%) were considered sensitized (PRA-ELISA >10%) against class I, and 34 (15%) against class II HLA antigens.

### *AMR*

Out of the 229 studied patients, 14 patients (6%) developed acute AMR with the initial diagnosis suggestion (either by a positive C4d in the indication biopsy or by a positive post-transplant XM), at a median of 8 (6–12) days after transplantation.

Table 1 shows the characteristics of these cases. There were more female patients ( $n = 11$ , 78%) and five re-transplants. Median HLA-Class I ELISA-PRA was 44% (31–79%), and median HLA-Class II PRA was 54% (23–89%). One case was zero PRA for both HLA class I and II. Patients received kidneys with a median of 3 (2–5) HLA-A, HLA-B and HLA-DR mismatches.

Out of the 14 patients who developed AMR, four cases were transplanted against a B-positive XM. One of these cases was detected by FCXM only, and three cases with both CDC-XM and FCXM. FCXM was performed after transplantation using pre-transplant sera.

After transplantation, DSA were identified in all cases either by FCXM ( $n = 11$ ) or by CDC ( $n = 3$ ) and individualized by class I and II single-antigen bead assays. Crossmatch became positive against both T and B lymphocytes in 11 cases, and against B-lymphocytes in 3 cases.

FCXM, using available pre-transplant sera, was performed post-transplant in eight patients. Out of these cases, four were shown to be FCXM-positive for B lymphocytes but negative for T lymphocytes, and the remaining four were T- and B-lymphocyte FCXM negative.

### *C4d staining in protocol and indication renal biopsies*

None of the 229 protocol 1-h Bx was positive for C4d in PTC. There was no neutrophil margination in PTC. Biopsies that were considered abnormal revealed acute tubular necrosis (ATN) in various degrees. No evidence of throm-

**Table 1.** Characteristics of antibody-mediated rejection in 14 patients

Patient number	Tx#	Sex	Age	Race	Donor	PRA I	PRA II	Pre-Tx CDC-XM	Pre-Tx FCXM	PO-AMR	Post-Tx XM	%PTC	C4d+
1	1	F	51	C	LR	82	50	T&B neg	T&B neg	90	T&B pos	10–24	IHC
2	1	F	43	C	DC	36	92	T&B neg	NA	7	T&B pos	>50	IF
3	3	M	33	C	LUR	79	0	T&B neg	T&B neg	8	T&B pos	>50	IF
4	2	F	43	C	DC	50	92	T&B neg	T&B neg	4	T&B pos	10–24	IHC
5	1	F	49	O	DC	0	58	Tneg/Bpos	Tneg/Bpos	17	T&B pos	25–50	IF
6	2	M	42	C	LU	38	0	T&B neg	T&B neg	9	T&B pos	>50	IF
7	1	F	44	O	DC	57	25	Tneg/Bpos	Tneg/Bpos	4	T&B pos	>50	IHC
8	1	F	16	C	DC	100	100	T&B neg	NA	13	T&B pos	10–24	IHC
9	1	F	52	C	DC	93	89	T&B neg	NA	11	T&B pos	>50	IF
10	1	F	62	C	DC	38	37	T&B neg	NA	2	Tneg/Bpos	>50	IF
11	2	M	28	C	DC	31	23	Tneg/Bpos	Tneg/Bpos	7	Tneg/Bpos	>50	IHC
12	1	F	30	C	LR	11	89	T&B neg	NA	2	T&B pos	>50	IHC
13	1	F	41	AF	DC	67	86	T&B neg	Tneg/Bpos	12	Tneg/Bpos	>50	IF
14	1	F	29	C	LR	0	0	T&B neg	NA	8	T&B pos	>50	IF

Tx #, number of transplant; F, female; M, male; C, Caucasian; O, Oriental; AF, African-Brazilian; LR, living related donor; LUR, living unrelated donor; DC, deceased donor; PRA, panel-reactive antibodies; Pre-Tx CDC-XM, results of pre-transplant complement-dependent cytotoxicity cross-matching with anti-human globulin; Pre-Tx FCXM, results of pre-transplant flow cytometry crossmatching; PO-AMR, post-operative day of the initial diagnosis of antibody-mediated rejection; %PTC C4d+, percentage of complement fraction C4d staining in peritubular capillaries; IF, immunofluorescence; IH, immunohistochemistry; NA, not available; Tneg, T-cell negative; Bpos, B-cell positive; T&B neg, T- and B-cell negative; T&B pos, T- and B-cell positive; Tneg/Bpos, T-cell negative and B-cell positive.

botic microangiopathy was seen in any biopsy. Arteriosclerosis was seen in some deceased-donor kidney biopsies and attributed to systemic arterial hypertension. Two cases had an increased number of mononuclear cells in the glomerular capillaries. Besides these findings, the remaining biopsies were considered normal. There was no correlation between the presence of ATN and immediate renal function, although severe ATN at zero-biopsy was usually followed by delayed graft function.

Even in the 14 patients who developed AMR, C4d staining in protocol 1-h Bx was negative in PTC by IF. In four cases with a pre-transplant B-lymphocyte-positive FCXM, the 1-h Bx was also negative for C4d in PTC. In three of these cases, both pre-Tx CDC-XM and FCXM were positive for B lymphocytes.

Nevertheless, all 14 indication biopsies—that diagnosed AMR—performed at a median of 8 days after transplant revealed a positive C4d staining in PTC by IF and/or IH.

## Discussion

Acute AMR has been easily identified due to the large clinical use of C4d staining in renal biopsies and to the introduction of new solid-phase assays that facilitate the detection of DSA. Management of AMR became a challenge to transplant centers, and it seems that the earlier the diagnosis, the higher the possible chances of treating AMR by the use of one or all methods to decrease AB levels [7–13].

Many transplant the centers, due to logistic reasons, perform only CDC-AHG XM pre-transplantation for deceased-donor kidneys. Currently, the analysis of pre-Tx PRA results, by solid-phase assays, may identify pre-transplant antibodies ('virtual crossmatch') eventually not detected by CDC-XM.

Theoretically, another way to detect the presence of pre-Tx DSA that activates classical complement pathway would be a positive C4d staining in PTC of renal biopsies obtained after organ reperfusion [14]. This hypothesis is very attractive and was tested in this study.

We chose the IF method for C4d staining in the 1-h zero protocol biopsies in order to use the most sensitive assay. Comparison of IHC and IF methods showed IF as more sensitive [15,16].

C4d is a split-fraction of the complement, covalently bound and stable during histological preparation. In renal transplantation, C4d staining usually indicates the activation of the complement cascade after the recognition of HLA antigens by the recipient's anti-HLA antibodies, for example in the endothelium of PTC. The presence of positive C4d staining in PTC is usually used as a surrogate marker of DSA participating in the process of graft inflammation, as these cases are frequently associated with DSA [17].

Haas *et al.* studied C4d staining in 1-h post-reperfusion biopsies in 47 patients—including 13 who developed AMR. They found a C4d-positive staining in two patients who developed AMR at 5 and 34 days after transplantation, respectively [14]. To our knowledge, this is the only study addressing this issue in post-reperfusion biopsies.

Our study performed in a larger number of transplants—including 14 patients who developed AMR—shows that C4d staining in 1-h post-reperfusion zero-biopsies is not useful for the early detection of ongoing AMR rejection. All 229 1-h post-reperfusion zero-biopsies stained negative for C4d by IF, including the 14 patients who, at a later stage, developed AMR. Nevertheless, it is noteworthy that our patients were all pre-Tx CDC-AHG XM negative, although patients in the study of Haas *et al.* were in a desensitization programme due to a positive



crossmatch (CDC and flow cytometry) and continued to have a weak positive flow crossmatch at the time of transplantation [14].

Our findings are in accordance with the findings of Nicleleit *et al.* who, in 125 zero-biopsies, also failed to demonstrate C4d staining by IF. However, different to our study, where all the biopsies were performed after 1-h after post-reperfusion, in the study of Nicleleit *et al.*, only 10% of the biopsies were collected after graft reperfusion [4].

In our series, all patients had negative, pre-Tx, T-cell CDC-AHG XM. Four cases had pre-Tx B-cell positive XM, possibly indicating pre-Tx anti-class II DSA as documented later on. Even in these cases, C4d staining in the post-reperfusion zero-Bx was also negative.

Additionally, considering (i) the short time period for the initial detection of AMR (8 days) in the 14 cases, (ii) that the patients were highly sensitized (with the exception of one case) and (iii) that there were four cases with a pre-Tx B-lymphocyte-positive FCXM (three pre-transplant B-lymphocyte-positive CDC-XM), it is possible to believe that most of the 14 patients had low titres of preformed ABs at the time of transplantation rather than developing *de novo* ABs, although we cannot prove this for the entire group.

There is a need for a certain amount of ABs to start activating complement. The addition of AHG to the CDC-XM assay increases the detection of low AB levels *in vitro*. However, this may not be the case *in vivo*. Haas *et al.* analysed six patients with a positive FCXM but a negative CDC-XM showed that none of the cases stained positive for C4d in the post-reperfusion zero-biopsies [14].

There is also the possibility that the 1-h time may not be enough to activate complement *in vivo*. We chose the 1-h time due to surgical feasibility. Anti-HLA antibodies bind to their specific cell membrane target antigens, and the complement is activated by the antigen-antibody complex on the cell, which leads to cell lysis. The time required for each of these two steps is not clearly defined and strongly depends on the antibody strength. For this reason, hyperacute rejection has been reported immediately post-reperfusion or several hours later. However, complement activation is another issue. In the CDC test, cells are incubated with serum for 60 (class I) or 120 min (class II), and then, rabbit complement is added followed by a 60- (class I) or 120-min (class II) incubation. Rabbit complement was chosen in the late 1960s due to the excellent reproducibility of results, while human complement failed for this purpose. In rats, the activation of the alternate complement pathway *in vivo* after ligating the left coronary artery shows that focal deposition of C3 was observed at 2 h and the deposition of the early (C1 and C3) and late pathway (C8 and C9) components occurred at 3 h [18]. It is evident, considering all points made above, that it is practically impossible to establish when to perform an immediate post-reperfusion biopsy which satisfies the above requirements.

Therefore, two most possible and complementary explanations for negative C4d staining in post-reperfusion Bx with low DSA levels would be (i) that the very low AB

levels may not (immediately) activate complement *in vivo*, or (ii) that the intensity of complement activation at 1 h post-reperfusion is very low and below the threshold of the current C4d immunofluorescent method.

Regardless of the reasons, this study demonstrates that C4d staining in 1-h post-reperfusion zero-biopsies is in general not useful when CDC crossmatch is negative. This conclusion cannot be extended to other series of sensitized patients, desensitization programmes and ABO-incompatible transplantation. However, this information is essential to avoid the risks associated with a second core zero-biopsy for C4d staining and to decrease the time and costs of performing post-reperfusion C4d in zero-biopsies.

*Conflict of interest statement.* None declared.

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## Unexplained sudden death in patients on the waiting list for renal transplantation

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### Abstract

**Background.** The incidence of unexplained sudden death (SD) and the factors involved in its occurrence in patients with chronic kidney disease are not well known.

**Methods.** We investigated the incidence and the role of comorbidities in unexplained SD in 1139 haemodialysis patients on the renal transplant waiting list.

**Results.** Forty-four patients died from SD of undetermined causes (20% of all deaths; 3.9 deaths/1000 patients per year), while 178 died from other causes and 917 survived. SD patients were older and likely to have diabetes, hypertension, past/present cardiovascular disease, higher left ventricular mass index, and lower ejection fraction. Multivariate analysis showed that cardiovascular disease of any type was the only independent predictor of SD ( $P = 0.0001$ , HR = 2.13, 95% CI 1.46–3.22). Alterations closely associated with ischaemic heart disease like angina, previous myocardial infarction and altered myocardial scan were not independent predictors of SD. The incidence of unexplained SD in these haemodialysis patients is high and probably a consequence of pre-existing cardiovascular disease.

**Conclusions.** Factors influencing SD in dialysis patients are not substantially different from factors in the general population. The role played by ischaemic heart disease in this context needs further evaluation.

**Keywords:** cardiovascular disease; chronic kidney disease; dialysis; sudden death; transplantation

### Introduction

Sudden death (SD) is one of the most significant causes of death in patients with chronic kidney disease (CKD). It is estimated that ~20–30% of all deaths in patients treated by dialysis are sudden [1,2]. Despite its undeniable importance, it is still unclear why sudden death is prevalent in this population and what factors are involved. Part of the problem is that, by its very nature, this kind of death often does not happen in the hospital setting, so its immediate causes can only be surmised. Necropsy could shed some light on the subject [3], but it is not routinely performed today in patients dying of natural causes in most countries. To make things even more obscure, no universally accepted definition of sudden death exists [4]. Periods of up to 24, 6 and 1 h between the onset of symptoms and the event have all been used [3,5–8]. Some authors require that death be unexplained and not occurring in a hospital; others do not. For all these reasons, the factors involved in SD in dialysis patients are seldom accurately identified.

In the general population, most SDs are believed to be due to ventricular arrhythmias, usually occurring in individuals with some underlying cardiac disease, usually coronary artery disease (CAD) [5]. However, data suggest that other factors may also be important as a cause of SD or cardiac death in patients with advanced uraemia [9–12]. Many confounding factors in dialysis patients, unrelated to cardiac disease, may lead to sudden death. For instance,