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Risk stratification by the virtual crossmatch: a prospective study in 233 renal transplantations

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Summary

The virtual crossmatch (virtual-XM) has been proposed for accurate identification of donor-specific HLA-antibodies, but large prospective studies assessing its value for pretransplant risk stratification are lacking. A total of 233 consecutive renal allograft recipients were prospectively stratified according to the virtual-XM. In patients with a negative virtual-XM (n = 190, 82%), prospective cytotoxicity crossmatches (CDC-XM) were omitted, and they received standard immunosuppression. Virtual-XM positive patients were only transplanted if CDC-XM were negative. They received additional induction with anti-T-lymphocyte-globulin and intravenous immunoglobulins (n = 43, 18%). The cumulative incidence of clinical/subclinical antibody-mediated rejection (AMR) at 1 year was lower in the negative virtual-XM than in the positive virtual-XM group [15/190 (8%) vs. 18/43 (42%); $P < 0.0001$]. After a median follow-up of 2.6 years, allograft loss because of AMR occurred less often in the negative virtual-XM group (1% vs. 7%; $P = 0.04$) and death-censored allograft survival at 2 years was higher (98% vs. 91%; $P = 0.02$). Serum creatinine was not different at the last follow-up (129 μM vs. 130 μM; $P = 0.58$). We conclude that a negative virtual-XM defines patients at low risk for AMR and early allograft loss, while a positive virtual-XM represents a significant risk for AMR despite enhanced induction therapy. This supports the utility of the virtual-XM for risk stratification and treatment allocation.

Introduction

Donor-specific HLA-antibodies (HLA-DSA) are associated with a high risk for early rejection and allograft loss [1,2]. For decades cell-based crossmatch assays [i.e. by complement-dependent cytotoxicity (CDC-XM) or flow-cytometry] have been used to determine the presence of HLA-DSA immediately before transplantation. However, it became evident that cell-based crossmatches have important limitations with respect to sensitivity and specificity to detect HLA-DSA, and their performance requires substantial resources in HLA-laboratories [2,3].

In the last few years, development of microparticles coated with single HLA-molecules (= single HLA-antigen flow-beads; SAFB) allow for precise determination of the specificity of the recipient's HLA-antibodies with high sensitivity [4]. By comparing the recipient's HLA-antibodies with the HLA-antigens of the donor, the presence of HLA-DSA can be determined virtually (= virtual crossmatch; virtual-XM). The virtual-XM has the potential to improve allocation of organs to compatible recipients, accelerate the allocation process, decrease the workload for HLA-laboratories, and improve risk stratification [5–8].

The predictive value of the virtual-XM has been investigated in several retrospective studies with conflicting results [9–14]. Given the inherent limitations of retrospective studies, a prospective evaluation of the virtual-XM for prediction of clinically relevant outcomes is

required. So far, Zangwill *et al.* [15] and Bielmann *et al.* [8] have reported promising results in small prospective studies (i.e. 10 pediatric heart and 65 adult renal transplantations, respectively) with a short follow-up time. The current study describes our extended experience using the virtual-XM prospectively as a single test for risk stratification and adaptation of immunosuppression in 233 consecutive renal transplantations with a minimal follow-up of 1 year.

Materials and methods

Procedure of the virtual crossmatch

A positive virtual-XM was defined as the presence of at least one detected HLA-antibody of the recipient, which was directed against an HLA-molecule of the donor. HLA-antibodies of the recipients were specified by SAFB in all cases. The reagents used during the study period changed, but always included SAFB covering HLA-A/B/DR/DQ and since available also HLA-Cw/DP. From 11/2004 to 12/2006 FlowPRA SAFB (OneLambda, Canoga Park, CA, USA) have been used, and since 01/2007 detection of HLA-antibodies was performed by LabScreen SAFB (OneLambda) on a Luminex platform.

FlowPRA SAFB were considered positive when their fluorescence signal were higher than the signals of the negative control bead and the corresponding bead in the negative control serum. LabScreen SAFB were considered positive when the baseline normalized mean fluorescence intensity (MFI) was >500. HLA-DSA detected by FlowPRA SAFB were retrospectively confirmed by LabScreen SAFB. Since March 2006, screening for HLA-antibodies was performed by SAFB, before by FlowPRA screening beads. Patients with sensitizing events were evaluated for the presence of HLA-antibodies in the current and historic sera. HLA-antibodies detectable only in historic sera were included for virtual-XM analysis. HLA-antibody analysis by SAFB was repeated yearly or after sensitizing events.

HLA-A/B/DRβ/DQβ antigens were determined by serology (Biotest, Dreieich, Germany) and confirmed by SSP DNA-typing (Protrans, Hockenheim, Germany). In 32/132 deceased donors (24%), HLA-DQβ antigens were not determined but inferred from the HLA-DRβ antigens, which are in strong linkage disequilibrium [16]. In living donors (n = 101), HLA-Cw and HLA-DP typing was performed prospectively, if the recipient had HLA-Cw or HLA-DP antibodies. In deceased donor transplantations (n = 132), HLA-Cw and HLA-DP typing was not performed prospectively. Only two patients had potential isolated donor-specific HLA-Cw (n = 1; treated as negative virtual-XM) or HLA-DP (n = 1; treated as positive virtual-XM) antibodies, which were both confirmed by retrospective DNA-typing.

Risk stratification by the virtual crossmatch

Since November 2004, our policy was to prospectively use the virtual-XM for pretransplant risk stratification and adaptation of immunosuppression. These prospectively obtained data were analyzed with approval from the ethics committee of the University of Basel.

Risk stratification and adaptation of immunosuppression according to the virtual-XM is outlined in Fig. 1a. Flow-cytometric crossmatches were not performed pretransplant. Patients with a negative virtual-XM were considered as normal risk for rejection and a prospective CDC-XM was omitted for deceased donor transplantations and, although available, not used for risk stratification in living donor transplantations. These patients received an induction therapy consisting of 20 mg basiliximab (Simulect; Novartis, Basel, Switzerland) on day 0 and 4 and a triple therapy either with tacrolimus (Tac, Prograf; Astellas, Wallisellen, Switzerland), mycophenolate-mofetil (MMF, CellCept; Roche, Basel, Switzerland) and steroids, or a steroid-free regimen consisting of Tac, mycophenolate-sodium (MPS, Myfortic; Novartis, Basel, Switzerland) and sirolimus or everolimus [Rapamune (Wyeth, Zug, Switzerland) or Certican (Novartis, Basel, Switzerland)]. Immunosuppression was reduced within the first 6 months with the aim to establish a dual therapy in the long-term with Tac–MMF/MPS or sirolimus-MPS or everolimus-MPS.

In patients with a positive virtual-XM, a prospective standard T-cell and B-cell CDC-XM was performed to determine the level of HLA-DSA [17]. Patients with a positive virtual-XM and current positive CDC-XM were not transplanted because the risk for early antibody-mediated rejection (AMR) and allograft loss was considered as too high [1,18]. Patients with negative current T- and B-cell CDC-XM were considered to have low-level HLA-DSA and classified as high risk for rejection. They received an induction therapy consisting of a polyclonal anti-T-lymphocyte-globulin (ATG; ATG-Fresenius, Fresenius, Stans, Switzerland) 9 mg/kg prior to reperfusion and 3 mg/kg on day 1–4, as well as intravenous immunoglobulin (IvIg) 0.4 g/kg on day 0–4. Maintenance immunosuppression consisted of Tac-MMF-steroids as reported previously [17]. Therapeutic protocols were approved by the local ethics committee.

Diagnosis and treatment of rejection

Clinically indicated allograft biopsies were preformed when serum creatinine increased by more than 20%. Surveillance biopsies were performed at month 3 and 6 post-transplant. Biopsy specimens were evaluated using light microscopy and immunofluorescence for C4d-deposition in peritubular capillaries. Findings were graded according

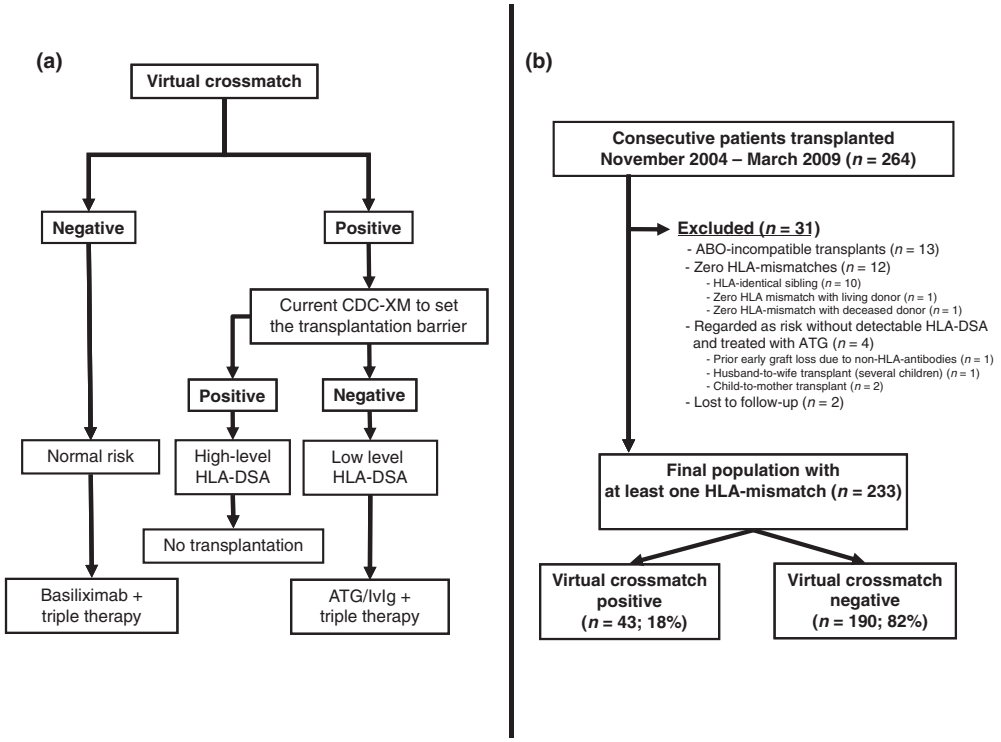

Figure 1 Risk assessment algorithm and patient population for the study. (a) Risk stratification algorithm by the virtual crossmatch. Patients with a positive virtual crossmatch were mandatory evaluated by a prospective standard T- and B-cell CDC-crossmatch. In deceased donor transplantations with a negative virtual crossmatch, the pretransplant CDC-crossmatch was omitted and performed retrospectively. In living donor transplantations with a negative virtual crossmatch, the CDC-crossmatch was been performed at the time of the immunological workup (i.e. HLA-typing, HLA-antibody analysis), but was not used for risk stratification and adaptation of the immunosuppressive regimen. (b) Patient population for the study and risk assignment according to the virtual crossmatch result. HLA-DSA, donor-specific HLA-antibodies; ATG, polyclonal anti-T-lymphocyte globulin; IvIg, intravenous immunoglobulins; CDC-XM, complement-dependent cytotoxicity crossmatch.

to the Banff 2007 classification [19]. Phenotypes of AMR were subdivided as previously reported [17]: (i) C4d-positive acute AMR, (ii) C4d-negative acute AMR, (iii) C4d-positivity only.

Clinical AMR was treated with steroid pulses i.v. and IvIg. Plasmapheresis and rituximab (Mabthera; Roche) were added depending on the severity and clinical response to the previous treatment. Subclinical AMR was treated in most cases with steroid pulses i.v. and IvIg ± rituximab depending on the severity. Subclinical C4d positivity only was not treated in most cases. Clinical T-cell-mediated rejection (TCR) (rejection grade: borderline to IIA) were treated with steroid pulses iv and in three cases with additionally ATG. Subclinical TCR (rejection grade: borderline to IIA) were treated with steroid pulses i.v. or p.o. according to the severity.

Investigated outcomes

Primary outcome was incidence of biopsy-proven clinical/subclinical AMR within the first year post-transplant. Secondary outcomes were allograft survival and function.

Statistical analysis

We used JMP software version 8.0 (SAS Institute, Cary, NC, USA) for statistical analysis. For categorical data, Fisher's exact test or Pearson's chi-square test were used. Parametric continuous data were analyzed by Student's *t*-tests. For nonparametric continuous data, the Wilcoxon rank sum test was used. Survival analysis was performed using the Kaplan–Meier method and groups compared using the log-rank test. A P-value <0.05 was considered to indicate statistical significance.

Results

Patient population and characteristics

Between November 2004 and March 2009, 264 kidney transplantations were performed at the University Hospital Basel. Thirty-one patients were excluded from the analysis, mainly because of blood group incompatible or zero HLA-mismatch transplantations. In addition, four patients, who formally belonged to the negative virtual-XM group, were excluded because they received an

enhanced induction therapy despite the absence of HLA-DSA in all available sera. One patient had lost her first living donor allograft from the mother because of early AMR very likely related to non-HLA-antibodies [20]. Thus, she received ATG/IvIg induction for her second transplant despite the absence of any HLA-DSA. Furthermore, three women received allograft from living donors, against whose mismatched HLA-antigens they were exposed during pregnancies [i.e. husband-to-wife transplant with several children ($n = 1$), child-to-mother transplant ($n = 2$)]. As sensitization against the mismatched HLA-antigens could not be excluded because of lack of historic sera dating back to the time of the pregnancies around 30 years ago, we regarded these transplants as high risk for rejection despite absence of HLA-DSA in current sera and treated them with ATG induction (Fig. 1b). The final population consists of 233 patients with at least one HLA-mismatch. Forty-three of 233 patients (18%) had a positive virtual-XM, 190/233 patients (82%) had a negative virtual-XM. Three of 43 patients (7%) with a positive virtual-XM had HLA-DSA only in historic sera, but not at the time of transplantation.

Patient characteristics are summarized in Table 1. In the positive virtual-XM group, female gender ($P = 0.02$), sensitizing events ($P < 0.0001$), and allograft from deceased donors ($P = 0.03$) were more common than in the negative virtual-XM group. Notably, 90/190 patients (47%) in the negative virtual-XM group had any sensitizing events, and 18/41 re-transplantations (44%) fell into the negative virtual-XM group. There were no statistical differences regarding recipient age, renal disease, donor age, and HLA-mismatches. Every patient had a minimal follow-up of 1 year.

Table 1. Baseline characteristics.

	Virtual-XM positive ($n = 43$)	Virtual-XM negative ($n = 190$)	*P*-level
Recipient			
Females, *n* (%)	21 (49)	55 (29)	0.02
Age, median (range)	55 (19–73)	54 (18–74)	0.99
Renal disease			
Glomerulopathies	14 (33)	62 (33)	0.33
Vascular	4 (9)	18 (9)	
Diabetic	2 (5)	26 (14)	
ADPKD	7 (16)	37 (19)	
Other	16 (37)	47 (25)	
Donor			
Age, median (range)	53 (2–73)	54 (1–85)	0.28
Deceased donor, *n* (%)	31 (72)	101 (53)	0.03
Cold ischemia time deceased donors [h], median (range)	11 (7–25)	10 (2–37)	0.41
HLA-A/B/DR mismatches			
1 mismatch	2 (4)	9 (5)	0.86
2 mismatch	7 (16)	22 (11)	
3 mismatch	11 (26)	45 (24)	
4 mismatch	12 (28)	49 (26)	
5 mismatch	9 (21)	47 (25)	
6 mismatch	2 (4)	18 (9)	
Known sensitizing events*			
Any sensitizing event, *n* (%)	41 (95)	90 (47)	<0.0001
Prior transplants, *n* (%)	23 (53)	18 (9)	<0.0001
Blood transfusions, *n* (%)	30 (70)	66 (35)	0.0001
Pregnancies, *n* (%)	18 (42)	36 (19)	0.002
PRA [%], median (range)	44 (0–97)	0 (0–94)	<0.0001
Number of HLA-DSA, *n* with 1/2/3/4/5	22/13/6/1/1	NA	
Class of HLA-DSA			
Class I, *n* (%)	19 (44)	NA	
Class II, *n* (%)	15 (35)		
Class I + II, *n* (%)	9 (21)		
Cumulative strength of HLA-DSA [MFI]**			
Median (range)	2287 (543–26537)	NA	
Induction therapy			
ATG/Ivlg, *n* (%)	43 (100)	–	<0.0001
Basiliximab, *n* (%)	–	189 (99)	
None, *n* (%)	–	1 (1)	
Initial immunosuppression			
Tac–MMF–P, *n* (%)	43 (100)	64 (34)	<0.0001
Tac–MPS–mTOR, *n* (%)	–	115 (60)	
Other, *n* (%)	–	11 (6)	

Tac, tacrolimus; MMF, mycophenolate-mofetil; MPS, mycophenolate-sodium; P, prednisone; mTOR, sirolimus or everolimus; MFI, mean fluorescence intensity.

*A patient can contribute to more than one group.

**The cumulative strength of HLA-DSA was calculated by adding the individual mean fluorescence intensity (MFI) of all present HLA-DSA.

Rejection episodes

The phenotypes and severities of biopsy-proven rejection episodes are shown in Table 2. Clinical rejection within the first year post-transplant occurred in 7/43 patients (16%) in the positive virtual-XM group and in 24/190 patients (13%) in the negative virtual-XM group ($P = 0.62$). Clinical AMR was observed more often in the positive virtual-XM than in the negative virtual-XM group (14% vs. 4%; $P = 0.03$).

The overall incidence of clinical/subclinical rejection within the first year post-transplant was significantly higher in patients with a positive virtual-XM (18/43; 42%) than in patients with a negative virtual-XM (50/190; 26%) ($P = 0.02$; Fig. 2a). Most strikingly, the incidence of clinical/subclinical AMR within the first year post-transplant was significantly higher in the positive virtual-XM (18/43; 42%) than in the negative virtual-XM group (15/190; 8%) ($P < 0.0001$; Fig. 2b). Accordingly, the prevalence of subclinical AMR at 3 and 6 months post-transplant was higher in the positive virtual-XM group (27% and 35%, respectively) than in the negative

Table 2. Phenotype and severity of clinical and subclinical rejection episodes within the first year post-transplant.

	Virtual-XM positive (n = 43)	Virtual-XM negative (n = 190)	P-level
Patients with any biopsy, n (%)	42 (98)	186 (98)	1.0
Patients with clinical rejection episodes*			
Overall, n (%)	7 (16)	24 (13)	0.62
Antibody-mediated rejection, n (%)	6 (14)	8 (4)	0.03
C4d positive acute AMR, n	5	3	
C4d negative acute AMR, n	–	4	
C4d positivity only, n	1	1	
T-cell-mediated rejection, n (%)	1 (2)	16 (8)	0.21
Borderline, n	–	7	
IA and IB, n	–	3	
IIA, n	1	6	
Surveillance biopsy at 3 months			
Biopsies performed/patients at risk, n/n (%)	37/42 (88)	160/185 (86)	
Antibody-mediated rejection, n (%)	10 (27)	5 (3)	<0.0001
C4d positive acute AMR, n	5	–	
C4d negative acute AMR, n	2	3	
C4d positivity only, n	3	2	
T-cell-mediated rejection, n (%)	5 (14)	26 (16)	0.81
Borderline, n	5	21	
IA and IB, n	–	1	
IIA, n	–	4	
Polyomavirus BK nephropathy, n (%)	1 (3)	6 (4)	1.0
Surveillance biopsy at 6 months			
Biopsies performed/patients at risk, n/n (%)	37/42 (88)	169/185 (91)	
Antibody-mediated rejection, n (%)	13 (35)	4 (2)	<0.0001
C4d positive acute AMR, n	5	1	
C4d negative acute AMR, n	2	3	
C4d positivity only, n	6	–	
T-cell-mediated rejection, n (%)	6 (16)	47 (28)	0.21
Borderline, n	6	29	
IA and IB, n	–	10	
IIA, n	–	8	
Polyomavirus BK nephropathy, n (%)	2 (5)	8 (5)	1.0

AMR, antibody-mediated rejection.

*Two patients in the virtual-XM negative group demonstrated combined antibody- and T-cell-mediated rejection. They were included in the antibody-mediated rejection phenotype group.

virtual-XM group (3% and 2%, respectively) ($P < 0.0001$; Table 2). Prediction of AMR by the virtual-XM was not different in the era using FlowPRA SAFB (11/2004 to 12/2006) or LabScreen SAFB (01/2007 to 03/2009); furthermore, there was no difference between deceased and living donor transplantations (data not shown).

Antibody-mediated rejection in patients with a negative virtual crossmatch

Fifteen of 190 patients (8%) experienced AMR despite a negative virtual-XM. According to the time period post-transplant until AMR occurred, these patients can be divided into three groups.

Six of 190 patients (3%) experienced clinical AMR within the first 9 days post-transplant consistent with missed preformed donor-specific antibodies. In 4/6 patients, we could not detect any HLA-DSA pretransplant, at the time of rejection, and at follow-up by SAFB analysis and retrospectively performed flow-cytometric crossmatches. This suggests that non-HLA antibodies were responsible for these early AMR episodes, leading to allograft loss in 2/4 patients despite intensive treatment [20]. The other two patients experienced AMR because of a missed HLA-Cw DSA (HLA-Cw-typing of the deceased donor was lacking) and because of a memory response in a broadly sensitized multiparous woman (no detectable HLA-DSA in recent pretransplant sera, but at day of rejection). These two AMR episodes related to HLA-DSA responded to treatment with steroids ± IvIg, and surveillance biopsies at 3 and 6 months were free of AMR.

Five of 190 patients (3%) experienced subclinical AMR in surveillance biopsies 3 months post-transplant. None had circulating HLA-DSA at the time of the biopsy. In

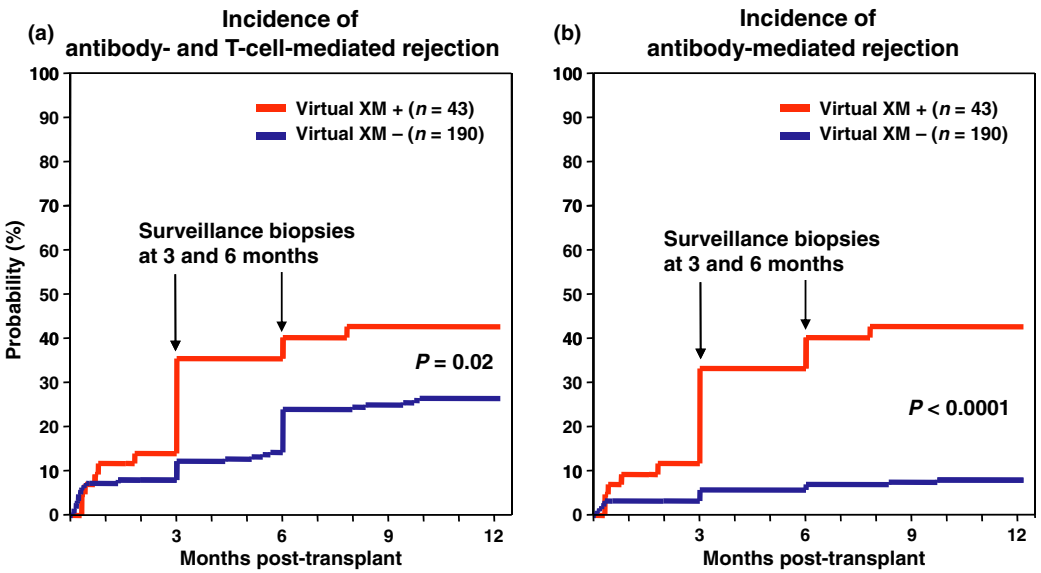

Figure 2 Incidence of rejection episodes. (a) Incidence of combined antibody- and T-cell-mediated rejection. (b) Incidence of antibody-mediated rejection (AMR). Surveillance biopsies were obtained at 3 and 6 months post-transplant. In this analysis, subclinical borderline tubulitis was not considered as rejection. If antibody- and T-cell-mediated rejection occurred together, it was classified as AMR.

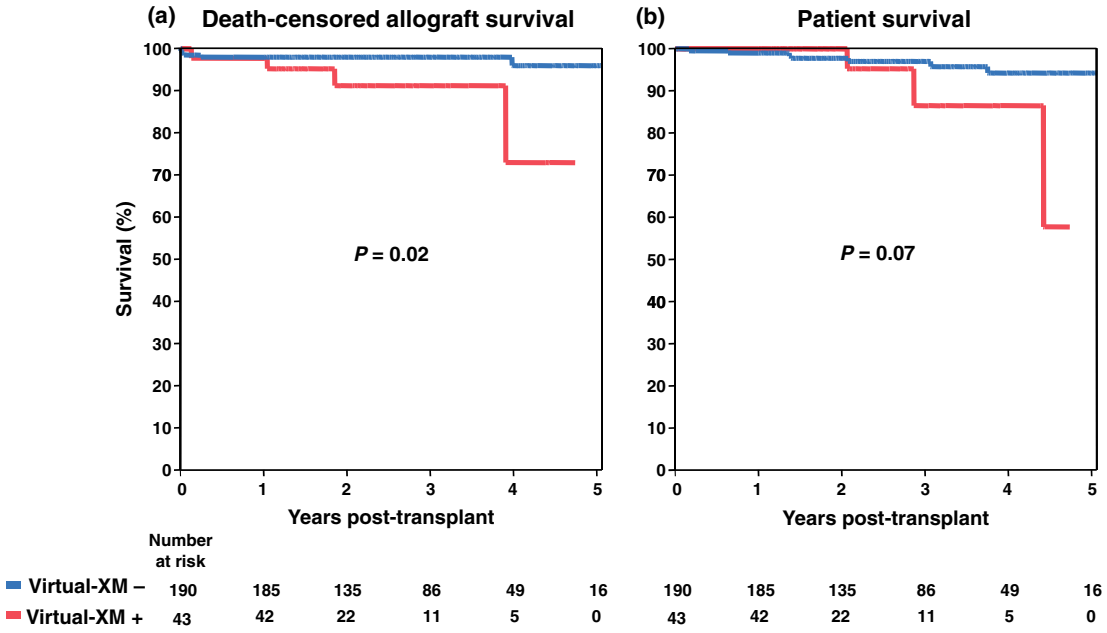

Figure 3 Survival analysis. (a) Death-censored allograft survival. (b) Patient survival.

3/5 patients subclinical AMR resolved by the next surveillance biopsy 6 months post-transplant; however, it persisted in 2/5 patients.

Four of 190 patients (2%) experienced AMR between 6 and 10 months post-transplant with unremarkable surveillance biopsies 3 months post-transplant, consistent with AMR because of *de novo* donor-specific antibody production.

Allograft and patient survival

Allograft and patient survival curves are shown in Fig. 3a,b. Because <50% of patients were still at risk beyond the first 2-year post-transplant, the curves at later time points have to be interpreted with caution. Death-censored allograft survival at one/two years was 98%/91% in the positive virtual-XM and 98%/98% in the negative

virtual-XM group. So far, four allograft were lost in the positive virtual-XM group: three because of ongoing AMR at 1, 2, and 4 years; one because of a donor-transmitted infection of the allograft with *Aspergillus fumigatus* requiring transplant nephrectomy 6 weeks post-transplant. Five allograft losses occurred in the negative virtual-XM group: early AMR presumably induced by non-HLA antibodies ($n = 2$); primary non-function without biopsy-proven rejection ($n = 2$); malcompliance 4 years post-transplant ($n = 1$). Allograft loss because of AMR occurred more often in the positive virtual-XM (3/43; 7%) than in the negative virtual-XM group (2/190; 1%) ($P = 0.04$).

Patient survival at one/two years was 100%/100% in the positive virtual-XM and 99%/98% in the negative virtual-XM groups. So far, three patients died in the positive virtual-XM group, all with good allograft function. Causes of death were cardiac arrest ($n = 1$), sepsis ($n = 1$), and cancer ($n = 1$). Six patients died in the negative virtual-XM group with well functioning allograft. Causes of death were cardiac arrest ($n = 2$), sepsis ($n = 1$), pulmonary disease ($n = 1$), and unknown ($n = 2$).

Allograft function and proteinuria

After a median follow-up of 2.6 years (range 1–5.4), serum creatinine values were not different among the two groups [positive virtual-XM: 129 μM (IQR 99–163); negative virtual-XM: 130 μM (IQR 98–168); $P = 0.58$]. Urine protein/creatinine ratio was not different as well [positive virtual-XM: 13 mg/mmol (IQR 9–26); negative virtual-XM: 12 mg/mmol (IQR 7–27); $P = 0.60$]. Immunosuppressive dual therapy was achieved in 150/185 patients (81%) with negative virtual-XM and functioning allograft. Only 6/39 patients (15%) in the positive virtual-XM group were on a dual therapy at the last follow-up.

Discussion

This large prospective study including serial surveillance biopsies provides robust data on the predictive value of the virtual-XM for development of AMR and related allograft loss. The key observation was that a negative virtual-XM is associated with a very low risk for early AMR and allograft loss, while a positive virtual-XM indicates a 42% risk for clinical/subclinical AMR despite an enhanced induction therapy.

Only 6/190 patients (3%) with a negative virtual-XM experienced early clinical AMR within the first 3 months suggesting a high negative predictive value. These six patients highlight two important limitations of the virtual-XM. First, non-HLA-antibodies are missed, because the virtual-XM is restricted to HLA-antibodies. Several targets of non-HLA-antibodies have recently been identified (e.g. major-histocompatibility-complex class I-related chain A, angiotensin II type 1-receptor, and glutathione-S-transferase T1) and these antibodies are associated with AMR and allograft loss [21–23]. However, our prospective study and a retrospective analysis suggest that early clinical AMR because of non-HLA-antibodies is rare and might be difficult to predict [20]. Second, the accuracy of the virtual-XM depends on a thorough and complete HLA-typing of the donor, ideally including the HLA-Cw and HLA-DP locus. An extended HLA-typing policy for all donors is still debated, because early AMR because of isolated HLA-Cw or HLA-DP antibodies is likely a rare event [24,25].

The clinical outcome of patients with a positive virtual-XM and treated with an enhanced induction therapy was variable. Only fourteen percent experienced clinical AMR, but the prevalence of subclinical AMR at 3 and 6 months post-transplant was 27% and 35%, respectively. Although the long-term impact of subclinical AMR on allograft survival is still unknown, emerging data demonstrate that subclinical AMR can progress to transplant glomerulopathy as well as interstitial fibrosis and tubular atrophy, which are common causes for allograft loss [26–28]. Indeed, allograft losses related to ongoing AMR were observed more often in the positive virtual-XM group and occurred beyond the first year post-transplant. In patients with functioning allograft, serum creatinine and proteinuria were not different between patients with positive and negative virtual-XM 2.6 years post-transplant, although more than a third of patients with a positive virtual-XM demonstrated subclinical AMR. This suggests that this higher rate of subclinical AMR did not affect medium-term allograft function. Nevertheless, a better control of the humoral immune response than provided by the ATG/IvIg induction regimen with Tac–MMF–P maintenance immunosuppression would be desirable for more than a third of the patients. A variety of treatment modalities (i.e. plasmapheresis or immunoadsorption, rituximab, bortezomib, ATG and IvIg) have been successfully used to transplant patients with HLA-DSA or treat AMR post-transplant [18,29–32], but their therapeutic efficacy and safety is difficult to compare. Ideally, prospective randomized trials should be performed to determine the best treatment regimens in this regard.

Notably, in the positive virtual-XM group only 1/43 patients (2%) experienced clinical TCR within the first year post-transplant compared to 16/190 patients (8%) in the negative virtual-XM group. This lower rate of TCR in the positive virtual-XM group is surprising, but is most likely related to the T-cell depleting induction therapy in these patients.

Fifty-eight percent of the patients with a positive virtual-XM treated with an enhanced induction therapy had no clinical or subclinical AMR within the first year post-transplant. There are three main explanations for this observation: (i) false positive virtual-XM related to technical reasons, (ii) successful control of HLA-DSA by the used immunosuppressive regimen, and (iii) intrinsic low pathogenicity of HLA-DSA. Unfortunately, we cannot assign the relative contribution of these three factors.

It is now well known that during the production process of SAFB, HLA-molecules might be denatured, leading to false positive SAFB results [33]. Fortunately, most of these technology-related false positive results occur with SAFB carrying rather infrequent HLA-molecules limiting the clinical relevance of this issue [34,35]. Furthermore, it is expected that this technical problem can be resolved in the near future.

We have recently shown in transplantations across a positive virtual-XM that an induction therapy with ATG/IvIg reduce not only the incidence, but also the severity of AMR to a subclinical stage [17]. Therefore, induction therapy is a potential confounder when the clinical relevance of HLA-DSA defined by SAFB is investigated. This might also partially explain why in some retrospective studies no impact on the occurrence of clinical rejection and short-term allograft survival has been found [13,14,36].

Finally, many intrinsic biological factors (e.g. antibody titer and characteristics, occurrence and magnitude of the humoral memory response, protective mechanisms of endothelial cells) contribute to the clinical impact of HLA-DSA [11,17,18,37–41]. It will be of major importance to define those characteristics, which allow reliable distinction between harmful and presumably irrelevant HLA-DSA.

We believe that in patients with a positive virtual-XM, a prospective CDC-XM is still required to determine the cumulative amount of HLA-DSA in the circulation, because there is an insufficient correlation between the strength of HLA-DSA measured by the MFI on SAFB and the result of the CDC-XM [42]. A key element for this missing correlation might be the fact that most HLA-antibodies are directed against public epitopes shared by several HLA-molecules [43,44]. Indeed, such a HLA-antibody will be distributed across several SAFB, and thus the generally applied method to count only the MFI of the donor-specific SAFB will underestimate the true amount of HLA-DSA. By contrast, in the CDC-XM assay such a HLA-antibody will accumulate on the targeted HLA-molecule of the cells and better represent the amount of HLA-DSA.

The concept of the virtual-XM was around for decades, but its broad implementation has been hindered by the lack of sensitive assays to define the precise specificities of HLA-antibodies [45]. With the introduction of solid-phase assays – such as SAFB – the universal application of the virtual-XM will likely become clinical reality. In fact, several organ procurement organizations and transplant centers have already or are in the process to implement the virtual-XM for efficient organ allocation to compatible recipients [5,7]. Our study further supports these efforts. The current strategy at our center is to reinforce transplantation with a negative virtual-XM, because it allows (i) to safely omit a prospective crossmatch reducing cold ischemia time, and (ii) to use a reduced immunosuppression in these low risk patients. In this regard, a recently published study reported low rejection rates and good 5 year allograft survival in highly sensitized African American and non-African American patients (PRA >80%), who were transplanted with a negative virtual-XM and basiliximab Tac–MPS–P immunosuppression [46]. We regard transplantations in the presence of a positive virtual-XM with negative CDC-XM not as a contraindication, but as a risk requiring an enhanced immunosuppression and careful follow-up including surveillance biopsies.

This study has two major advantages to determine the clinical utility of the virtual-XM for pretransplant risk stratification. First, the prospective design with inclusion of consecutive patients reflects 'real life'. Second, the virtual-XM as a single test was correlated with important clinical and histological outcomes as the 'gold standard'.

However, this study has also certain limitations. The virtual-XM in deceased donors (132/233 transplantations; 57%) was incomplete because prospective HLA-Cw and HLA-DP typing was not performed. In our population, only 2/132 patients (1.5%) without any donor-specific HLA-A/B/DR/DQ antibodies had HLA-Cw or HLA-DP antibodies. This suggests that the isolated presence of donor-specific HLA-Cw or HLA-DP antibodies might be rare, but this could become more prevalent in broadly sensitized patients receiving well HLA-A/B/DR/DQ-matched allograft. In addition, HLA-DQα antibodies were not assessed, which have recently been described in sensitized patients [47]. Furthermore, our experience in 233 almost exclusively Caucasian patients requires validation in populations with different HLA background. Finally, although patients in the positive virtual-XM group received more often allograft from deceased donors (72% vs. 53%, $P = 0.03$), there was no difference with respect to the occurrence of AMR between living and deceased donor transplants. Therefore, a clinically relevant bias caused by the disparity of the donor source is unlikely.

In conclusion, a negative virtual-XM defines patients at very low risk for AMR and early allograft loss. A positive virtual-XM represents a significant risk for AMR despite

an enhanced induction therapy. This supports the utility of the virtual-XM for risk stratification and treatment allocation.

Authorship

PA, JS and SS: designed the research/study. PA, PHM, HH and SS: performed the research/study. PA, PHM and SS: collected and analyzed the data; PA, PHM, GH, LG, MJM, JS, HH and SS: wrote the paper.

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