

**Risk factors and prognostic significance
of microvascular inflammation in the kidney allograft**

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Received: 25 October 2016

Introduction: *the prognostic significance of microvascular inflammation (MVI) in the absence of donor-specific antibodies (DSA) in kidney allotransplantation (KTx) is not well defined, and the predictors of such immune conflict type remain unclear.*

The study *was aimed to define the long-term prognosis of MVI and to identify clinical factors associated with MVI.*

Material and methods. *Two hundred eighty four kidney allograft (KA) recipients were enrolled into the study according to inclusion criteria (AB0-compatibility, negative cytotoxic crossmatch, and at least one KA biopsy in post-transplant period). One hundred fifty patients had MVI. The control group included 134 KTx recipients without MVI. The following clinical parameters were registered: the recipient's gender and age, donor age, cadaveric/alive donor, cold/warm ischemia time, renal replacement therapy duration, last donor creatinine level, a delayed graft function*

(DGF), an immunological risk (IR) (number of HLAm, panel-reactive antibodies, previous KTx), the level of creatinine and proteinuria at the time of biopsy. The long-term KA survival was estimated by Kaplan-Meier analysis. The combined end-point was determined as the return to dialysis or the estimated glomerular filtration rate 15 mL/min/1.73m² or less. Multivariate Cox regression analysis was used for the evaluation of independent risk factors associated with the presence of MVI and risk of KA loss.

Results. According to MVI phenotypes, the patients were distributed into the following groups: MVI+DSA+ cases (n=31) that met criteria for antibody-mediated rejection (Banff 2013); MVI+DSA- (n=62); and MVI+DSA? cases (n=57) with undetermined DSA. In MVI+DSA- group, 28 recipients had an isolated MVI, while MVI was accompanied with T-cell mediated rejection (TCMR) in 34 cases. The median follow-up was 52 (23; 85) months from KTx and 39 (13; 77) months from the biopsy. KA survival in all MVI groups was significantly lower compared with controls. In the adjusted multivariable Cox regression model, MVI was associated with the relative risk of KA loss ($Exp(\beta)=4.2$ (CI 95% 2.3–7.7), along with DGF ($Exp(\beta)=1.9$ (CI 95% 1.1–3.4), and donor age ($Exp(\beta)=1.03$ (CI 95% 1.01–1.05). A higher estimated IR and the DGF were independent predictors for MVI+DSA+ and MVI+DSA-. DGF was the only predictor for TCMR and MVI+DSA?. No clinical factors associated with the isolated MVI were identified.

Conclusion. MVI is associated with a higher IR and a DGF and strongly predicts an unfavourable KTx outcome. Further investigations of MVI mechanisms and treatment approaches could be important steps toward an improvement of KTx efficacy.

Keywords: microvascular inflammation, glomerulitis, peritubular capillaritis, immunological risk, antibody-mediated rejection, kidney allograft survival.

Introduction

The efficacy of KTx is limited by the development of variously severe immune reactions due to gene polymorphism in HLA system [1, 2]. There are two main types of rejection: a T-cell mediated rejection (TCMR) and an antibody-mediated rejection (AMR) mediated by the activation of B-cells. The occurrence of the latter is known to lead to a significant reduction in graft survival [3, 4]. The AMR diagnostic criteria includes the presence of MVI in the form of monocyte-macrophageal reaction in the peritubular capillaries (peritubular capillaritis, PTC) and/or glomeruli (glomerulitis, G) in combination with DSA [5]. However, MVI signs in the KA may often be observed in the absence of DSAs and may be regarded as a specific variant of conflict in the immune system in KTx. A prognostic value of MVI course without DSA (MVI+DSA-) remains a matter of debate [6-11], and predictors of such immune conflict development have not been studied. The aim of the study was to assess the MVI prognostic value and to search for MVI-associated predictors.

Material and Methods

Study group, inclusion/exclusion criteria

The retrospective observational study was conducted in two transplant centers and included 150 cases with histological signs of MVI (G and/or PTC) who were selected from the 1270 KA recipients who had received a

transplant in 2000-2013. The control group comprised 134 cases selected from the database of all KA recipients (n = 1270) using the "case-control" methodology and taking into consideration the donor and recipient age, gender, transplant type (live related/cadaveric), and the year of KTx performed.

The control group included the KA recipients in whom TCMR was registered (n = 44) and the cases without any variant of immune conflict (no rejection, NR; n = 90). All the KA recipients included in the study (n = 284) met the inclusion criteria: donor-recipient blood group compatibility, negative cytotoxic cross-match test, available results of one or more KA biopsies containing 7 or more glomeruli, according to Banff biopsy reliability criteria [12]. The following cases were excluded: recipients with morphological signs of recurrent primary renal pathology in the KA, poliovirus infection (confirmed by immunohistochemistry), recurrent KA infection, and the cases of the graft loss in the early postoperative period due to surgical complications.

Pre- and peri-transplant demographics and clinical data were studied for all the patients. They are presented in Table. 1.

Table 1. Demographic and clinical characteristics of the groups at the time of allotransplantation

	All patients (N = 284)	MVI (N = 150)	Control group (N = 134)	P
Male, %	58.8	56	62	0.18
A living donor KA,%	16.5	18.7	14.2	0.2
Patient's age, years, M ± SD	48 ± 13	47 ± 13	49 ± 13	0.14

RRT duration, months, m (25-75%)	73 (34; 102)	77 (32; 115)	69 (36; 91)	0.27
Cold ischemia time, min, M ± SD	713 ± 362	727 ± 391	699 ± 328	0.69
Warm ischemia time, min, M ± SD	40 ± 16	41 ± 18	40 ± 15	0.49
Last measured creatinine level in donor's blood, mmol/L, M ± SD	0,09 ± 0,05	0,09 ± 0,05	0.09 ± 0.06	0.9
Donor's age, years, M ± SD	52 ± 15	50 ± 15	53 ± 16	0.08

Note: RRT, renal replacement therapy; M ± SD, mean and standard deviation; m (25-75%), the median and interquartile range (25; 75 percentile).

In addition, the following clinical parameters were recorded: the DGF, IR (pre-existing antibodies [PEA], the number of HLAm, the number of previous KTx), serum creatinine values and proteinuria at the time of biopsy. DGF was defined as the condition requiring at least one session of hemodialysis to be performed after KTx.

Morphological analysis

The DGF, serum creatinine increase by 25% from baseline or higher, the daily urine study demonstrating the occurrence of proteinuria or its 50% increase were the standard indications for KA biopsy. The histologic examinations were performed in 26% of patients according to the standard protocol within the post-transplant monitoring program adopted in centers.

The methodology of histologic examination and immunological assay were previously described in detail [13]. Immunohistochemistry (IHC) study for C4d was performed on paraffin-embedded sections after dewaxing and dehydration according to standard procedures. A diffuse linear peritubular capillary (PTC) C4d deposition was considered a C4d positive test result [14]. Routine IHC for IgA, IgG, IgM, C1q, C3 and SV40 was performed to exclude a recurrent primary glomerular disease and poliovirus infection of KA. All graft alterations, including MVI manifestations (G and PTC) were evaluated by pathomorphologists using Banff 1993-2013 Classification [5, 13, 14].

HLA-typing and antibody identification

HLA system genotyping of donor and recipient blood samples were performed for HLA-A, -B, -C, DR, DQ, DP loci and MICA-antigens using the Sequence Specific Primers and Sequence-based Typing techniques, and the presence of DSAs was determined by means of the following techniques: by enzyme immunoassay (ELISA) using multiplex flow Luminex analyzer and by Single-antigen bead method (described in detail previously [15]). Tests for DSA were considered positive when the mean fluorescence intensity was equal to or exceeding 1000. Tests for DSA in the control groups were negative in all cases.

Stratification of patients by immunological risk

The patients were stratified by pretransplant IR according to the approach previously developed [13] on the basis of three IR factors: PEA more than 0%, HLAm equal to or exceeding 2, and the previous history of KTx. KA recipients having 1 IR factor or lower were referred to the

subgroup of low immunological risk (LR; n = 195), the KA recipients with 2 or more IR factors were referred to an increased immunological risk (IncrR) subgroup (IncrR; n = 89).

The follow-up period and outcomes

The median follow-up period was 39 (13; 77) months from the time of biopsy, 52 (23; 85) months after KTx. The registered parameter was the achievement of the combined endpoint uniting two variants of KTx outcome: a complete KA loss with the return to dialysis or the glomerular filtration rate less than 15 ml/min/1.73 m² calculated using the MDRD equation [16]. The mortality with the date of death in the patients with a functioning KA were also recorded.

Immunosuppressive therapy

All patients received intravenous glucocorticosteroids (GCS) as an induction therapy with further switch to an oral dose of 1 mg/kg, and a gradual tapering to a maintenance dosage. In IncrR, the induction was supplemented with the administration of basiliximab or anti-thymocyte immunoglobulin (ATG). The measures of ABMR prevention in highly sensitized patients included the plasma exchange, and/or the intravenous immunoglobulin (IVIG) infusions and anti-CD20-antibodies (rituximab) in a dose of 375 mg/m². Basic immunosuppression, in addition to corticosteroids, included calcineurin inhibitors (cyclosporine or tacrolimus) and mycophenolate mofetil. The therapy for ABMR included the PE sessions, IVIG infusions, and rituximab and/or of bortezomib in case of persistence. The MVI without DSA was treated using pulsed GCS therapy with further switch to an oral dose of 0.5-1 mg/kg/day and a reduction to a maintenance

dose within a month. The patients who received cyclosporine A at the time of diagnosing MVI were switched to tacrolimus; and in cases of persisting morphological changes, ATG and/or rituximab were administered.

Statistical analysis

Statistical analysis of the data was performed using the licensed SPSS Statistical Software Package for Windows 14.0 (Chicago, IL, USA). The groups were compared using the following tests: Student's t-test for paired and unpaired comparisons, Mann-Whitney test, a single-factor analysis of variance (ANOVA), Fisher exact test and the χ^2 test. KA survival analysis was assessed by the Kaplan-Meier estimate. The selected clinical and demographic parameters were studied for being potential predictors of outcome using a univariate Cox regression analysis. A multivariate regression analysis included only those clinical parameters studied that were associated with the risk of KA loss in a univariate Cox regression analysis at $p < 0.1$. When analyzing the survival, the date of death that occurred in a recipient with a functioning KA was taken as the end of observation/follow-up, and such a case was considered censored. Between-group differences and regression coefficients were considered statistically significant at $p < 0.05$. Data are presented as mean values and standard deviations (M \pm SD), as the mean and the confidence interval (M; 95% CI) and as a median and an interquartile range (m; 25-75%).

Results

In 55% of KA recipients, both MVI components were present (G+ PTC+; n = 83); in 29% of cases only G was recorded (G+; n = 44), and in 15% of cases only PTC were present (PTC+; n = 23). DGF was detected

more frequently in the group with MVI than in the control group (61.3% vs. 44.8%; $p = 0.003$); biopsy was performed if indicated (82% vs. 65%; $p = 0.001$) and the creatinine higher values at the moment of biopsy were recorded (m(25-75%) 0.4 (0.18; 0.6) vs. 0.26 (0.13; 0.3) mmol/L, $p < 0.001$) as well as were the values of daily proteinuria (m(25-75%) 0.36 (0.18; 0.75) vs. 0.19 (0.11; 0.34) g/day; $p < 0.001$). The median time from KTx to the diagnostic biopsy in MVI group was 30 (10; 224) days and was naturally shorter than that in the control group (74 (19; 249) days; $p = 0.035$). Pretransplant IR factors were more frequently reported in the group with MVI (Table. 2).

Table 2. Pre-transplant immunological risk factors in the study groups

Pre-transplant immunological risk factors	All patients (N = 284)	MVI (N = 150)	Control group (N = 134)	P
PEA, M (95% CI)	18 (14; 22)	25 (19; 31)	10 (5; 15)	0.005
PEA > 0%, %	31.7	40	22.4	0.001
Repeated KTx%	19.7	26.7	11.9	0.001
HLAmm, m (25-75%)	3 (1.5; 3)	3 (2; 3)	2 (1; 3)	0.034

Note: HLAmm, the number of HLA mismatches; M (95% CI), the mean and the confidence interval.

With regard to the DSA presence (Luminex assay), the MVI Group was represented by: 1) MVI+ DSA+ (n = 31) cases that corresponded to AMR by Banff 2013 criteria [5]; 2) MVI+ DSA- (n = 62); 3) the patients were referred to undetermined DSA cases: DSA+ MVI? (n = 57) if they

were negative for DSA as confirmed by ELISA, in whom a multiplex assay (Luminex) had not been performed. In the MVI+ DSA- group, the course was characterized by concurrent TCMR in 34 cases, and by isolated MVI in the remaining 28 cases. There were no differences between the patients of these subgroups (MVI+ DSA-) in the main clinical and demographic characteristics or pre-transplantation IR factors (data not presented). A peritubular capillary C4d deposition was detected in 38% of MVI+DSA+ cases, in 12% of MVI+DSA- cases, and in 14% of MVI+ DSA? cases.

The kidney allograft survival in various microvascular inflammation phenotypes

The lowest KA survival was identified in the humoral rejection group (MVI+ DSA+). KA Survival in MVI+ DSA- was higher than that in MVI+ DSA+ ($p_{\log\text{-rank}} = 0.04$), but significantly lower than in the control group (MVI-) ($P_{\log\text{-rank}} < 0.001$). There was no difference in KA survival between the MVI+ DSA- and MVI+ DSA? groups ($p_{\log\text{-rank}} = 0.43$) (Fig.1). In MVI+ DSA-, the KA survival did not differ between the subgroups with the isolated MVI (MVI+ DSA- TCMR-) and the MVI combined with TCMR ($p_{\log\text{-rank}} = 0.54$), and between the subgroups of the control groups (MVI- TCMR- vs. MVI- TCMR+; $p_{\log\text{-rank}} = 0.13$). The AK survival rate in the group with TCMR free from MVI was higher than in MVI+ DSA- TCMR+ ($p_{\log\text{-rank}} = 0.05$) (Fig.2).

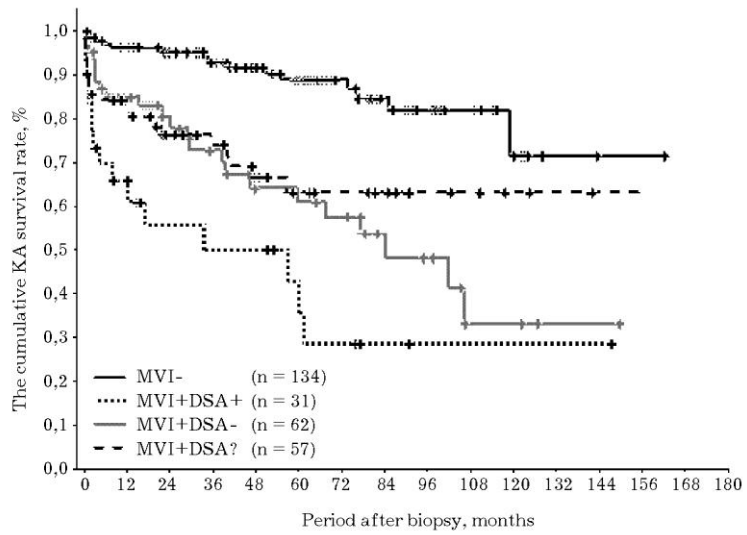


Fig. 1. The kidney allograft survival in various microvascular inflammation phenotypes

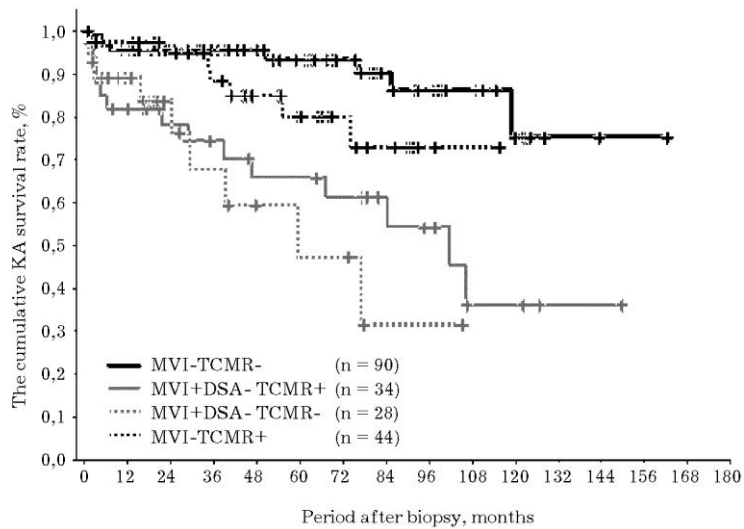


Fig. 2. The kidney allograft survival in MVI+ DSA- and TCMR subgroups

In a multiple regression analysis with the model being adjusted by DSA, IncrR, RRT duration, and the cold ischemia time, the MVI appeared

an independent predictor of KA loss (Exp (β) = 4.2 (95%, CI: 2.3-7.7), as did the DGF (Exp (β) = 1.9 (95%, CI: 1.1-3.4), and the donor age (Exp (β) = 1.03 (95%, CI: 1.01-1.05).

The incidence of microvascular inflammation, and pretransplant immunologic risk

G and PTC were more frequently detected in patients with IncrR than LR (60.7% vs. 37.4%; $p = 0.005$), and (51.7% vs. 30.8%; $p < 0.001$), respectively. A complete phenotype of humoral rejection was also more often recorded in patients with IncrR (21.3% vs. 2.7%; $p < 0.001$). The incidence of MVI+ DSA? was not significantly different between the groups with IncrR and LR (7.9% vs. 13.8%; $p = 0.15$), the same trend was observed with the incidence of an isolated MVI (11.2% vs. 9.2%; $p = 0.6$). TCMR was recorded in IncrR and in LR in nearly equal proportions (17.9% vs 25.1%; $p = 0.26$). The cases of a combined T-cell rejection with MVI+ DSA- were more often detected in IncrR than in LR (19.1% vs. 8.7%; $p = 0.012$).

Factors associated with the development of microvascular inflammation

A univariate regression analysis was used to assess the relationship between the development of various MVI phenotypes and the pretransplant risk factors: the donor age, recipient's gender and age, the type of donor (living donor vs. cadaveric KTx), the last measured creatinine level in donor's blood, cold and warm ischemia times, the RRT duration, DGF, and IR. IncrR and DGF were the predictors of all variants of the immune conflict except the isolated MVI (Table. 3). In a multivariate regression analysis, the IncrR and DGF were the independent predictors associated with the

development of MVI, as a whole, with its components (G and PTC), and phenotypes MVI+ DSA- and MVI+ DSA+ (see Table 3). The recipient's age was also a risk factor of MVI development as a part of humoral rejection. The DGF was the only predictor of TCMR, the same as of MVI+ DSA?. None of the studied factors was associated with the development of an isolated MVI (see Table. 3).

Table 3. The regression analysis of the relationship between the pre-transplant factors and the development of various types of KA immune conflict (only statistically significant values are presented, $\text{Exp}(\beta)$ at $p < 0.05$)

Predictors	Increased IR (vs. Low IR)	Recipient's age	Delayed KA function (vs. immediate function)	RRT duration (1 month)
Univariate regression analysis				
Variants of immune conflict				
MVI	1.9 (1.4-2.7)	-	2.2 (1.6-3.1)	1.003 (1.001-1.005)
MVI+ DSA+	4.8 (2.6-10.0)	0.97 (0.95-0.99)	2.6 (1.2-5.6)	1.006 (1.002-1.010)
MVI+ DSA-	2.6 (1.4-3.7)	-	2.1 (1.2-3.5)	1.003 (1.000-1.006)
MVI+ DSA?	-	-	2.2 (1.3-3.9)	-
An isolated MVI	-	-	-	-
MVI+ DSA-TCMR+	2.6 (1.3-5.1)	-	3.4 (1.6-7.3)	-

TCMR without MVI	-	-	1.7 (1.1-4.2)	-
TCMR, all cases	-	-	2.2 (1.4-3.6)	-
G+	2.1 (1.4-2.9)	-	2.6 (1.8-3.7)	1.003 (1.001-1.005)
PTC+	2.1 (1.4-3.1)	-	2,2 (1.5-3.3)	1.004 (1.001-1.006)
Multivariate regression analysis				
MVI	1.9 (1.4-2.7)	-	2.2 (1.6-3.1)	-
MVI+ DSA+	5.4 (2.5-11.6)	0,96 (0.93-0.99)	2.7 (1.2-5.8)	-
MVI+ DSA-	2.2 (1.3-3.6)	-	2.1 (1.2-3.5)	-
MVI+ DSA?	-	-	-	-
An isolated MVI	-	-	-	-
MVI+ DSA-TCMR+	2.5 (1.3-4.9)	-	3. (1.5-7.4)	-
TCMR without MVI	-	-	1.7 (1.1-4.2)	-
TCMR, all cases	-	-	2.2 (1.4-3.6)	-
G+	2.1 (1.4-2.9)	-	2.6 (1.8-3.7)	-
PTC+	2.1 (1.4-3.1)	-	2.2 (1.5-3.3)	-

Note: "-" indicates no statistically significant relationship.

Discussion

Traditionally MVI has been seen as a manifestation of antibody-mediated mechanism of the immune system activation [8, 9, 17] and has served a criterion of the ABMR [5]. In the study group of KA recipients with MVI, only 21% of cases met the criteria of a humoral rejection, while a

significant proportion of MVI cases (41%) had the course without DSA. In 38% of cases, MVI remained suspicious of ABMR which could not be excluded without a multiplex assay (Luminex), the most sensitive of the known methods for a DSA detection. The lowest KA survival rate was registered in MVI+ DSA+ group, which confirms the idea of an adverse prognostic significance of MVI in KTx within ABMR [7-10, 18-20]. Obviously, a dramatically increased risk of KA loss in ABMR is predetermined by the tendency of this immune conflict type to persist, despite the treatment conducted; that leads to the glomerulopathy development, as demonstrated by the series of morphological studies [21, 22]. The estimates of the relationship between the DSA-free MVI and the KA survival remained controversial [6-11]. We presented the data that clearly indicate a poor prognosis of KTx at any MVI variant, including DSA-free MVI. Previous studies indicate that among two MVI components - G and PTC - the former could have a more significant prognostic significance [8, 15]. The KA survival in the MVI group with undetermined DSAs did not differ from that in the MVI+ DSA- group, suggesting that the major part of these cases had no circulating antibodies and, consequently, no humoral rejection. The negative impact of MVI on KA prognosis, in our opinion, could be explained by the mechanisms by which fibroplastic changes develop and progress in various compartments of the organ, the mechanisms triggered both by the interaction of macrophages with resident cells in the micro-vessel walls, and also by focal ischemia and an impaired traffic of macromolecules.

The MVI+ DSA- group and the control group without MVI included the cases with the TCMR, and its role in the prognosis still remains controversial [3, 8, 23]. The obtained data suggest that the concomitant

TCMR has no significant effect on the KA survival either in the absence or in the presence of microvascular inflammation. The lack of a significant correlation between TCMR and the KA survival can be explained by a routine approach to an early morphological diagnosis and morphological monitoring of the treatment effect, the approach that has been established in both transplant centers. Moreover, the TCMR cases were mostly represented with more responsive to therapy borderline forms, or I A and B types [24, 25].

The mechanisms of DSA-mediated MVI comprise the induction of adhesive molecules such as MCP-1 (monocyte chemotactic protein-1), MIP1 β (macrophage inflammatory protein 1 β) and others in the endothelium with the further involvement of monocytes and macrophages [26-28]. Such mechanisms can be complement-dependent or complement-independent that has been indirectly confirmed by the identification of C4d deposits only in the third of the investigated MVI cases, including the cases of an evident humoral rejection. The etiopathogenesis of DSA-free MVI mediated by the development of a monocyte-macrophageal reaction in KA capillaries has not been sufficiently studied. A humoral mechanism can not be excluded for some of these cases, even without an evident presence of circulating anti-HLA/anti-MICA antibodies, since it is known that DSAs against other donor antigens can also be formed [29, 30]. In the described study, as in the routine practice, we did not screen for antibodies other than the anti-HLA/anti-MICA antibodies; and hence we can not completely exclude other antibody-mediated mechanisms in the development of MVI+ DSA- in some recipients. The formation of some small amounts of antibodies also seems possible when they cease to be detectable in serum after their interaction with target antigens on the KA endothelium [31, 32]. It is not an exception

that such cases may include a small portion of KA recipients with MVI and with the signs of the complement local activation (the C4d deposition in peritubular capillaries). On the other hand, the MVI can be independent of the immune system B-cell activation and of the DSA formation, since it is known that the inflammatory response in KA capillaries may be induced via the antigen presentation to T-cells directly by the endothelium [27, 33]. Moreover, the KA monocyte-macrophageal microvascular inflammation mediated by MSR-1 and MIP-1 β expression in the post-transplant period may be induced by a number of non-immune and other stimuli leading to an endothelial damage [25, 26].

The IncrR and DGF were the independent predictors associated with the risk of the most morphological and immune MVI variants under study. From a practical point, these predictors might be used to assess the risk of MVI when on alert, together with the planning of diagnostic measures: histological studies and monitoring for DSA. On the other hand, the identified predictors generally reflect the probable mechanisms of monocyte-macrophageal reaction. The relationship of MVI development with IncrR in the post-transplant period reflects a more pronounced tendency to the activation of B-cellular mechanisms in such recipients, and can be the reason for discussing the appropriate treatment and prevention measures. The DGF associated with the MVI development suggests other mechanisms being involved in the monocyte capillary reaction in the graft and induced by an ischemic/reperfusion injury [34-36], as was shown in relation to TCMR [1]. However, none of the analyzed factors was associated with an isolated MVI development that requires their further search, along with studying the causes and mechanisms of KA adverse immune reactions.

Conclusion

The MVI associated with an increased pre-transplant immunological risk and a delayed KA function, appears a clear-cut predictor of a poor prognosis. Further studies of mechanisms, approaches to MVI primary/secondary prevention may be essential steps to improve the KTx efficacy.

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