The Significance of Vascular Alterations in Acute and Chronic Rejection for Vascularized Composite Allotransplantation.

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Abstract
Vascularized composite allotransplantation (VCA) has emerged as a useful reconstructive option for patients suffering from major tissue defects and functional deficits. While the technical feasibility has been optimized and more than 130 VCAs have been performed during the last two decades, hurdles such as acute and chronic allograft rejection, graft deterioration, and eventual functional impairment need to be addressed. Recently, chronic graft rejection and progressive failure have been linked to vascular alterations observed in the allografts. Graft vasculopathy (GV) may play a pivotal role in long-term graft deterioration. The understanding of the underlying pathophysiological processes and their initial triggers is of utmost importance in the prevention, attenuation, and therapy of GV. While there are reports on the etiology and development of GV in solid organ transplantation, there are limited data with respect to chronic rejection and GV in the realm of VCA. Nevertheless, recent reports from long-term VCA recipients suggest that GV could truly jeopardize allografts in the follow-up evaluation. Chronic rejection and GV include different entities and might have different pathways in distinct organs. Herein, we reviewed the current literature on vascular changes during both acute and chronic allograft rejection, with a focus on their clinical and translational significance for VCA.

Introduction

Over the last two decades, vascularized composite allotransplantation (VCA) has evolved as a viable reconstructive option for carefully selected patients with devastating facial defects or of the upper extremities, and thus far, over 130 procedures have been performed worldwide [1]. Improvement in both surgical technique and immunosuppressive regimen, largely adopted from solid organ transplantation (SOT), allowed to control acute rejection and guarantee short- and mid-term graft survival. The functional and immunological results in these patients are encouraging. Recently, chronic rejection and loss of vascularized composite allografts (VCAGs) drew parallels to SOT with jeopardized long-term graft survival [2]. The half-life of transplanted solid organs ranges between 4 and

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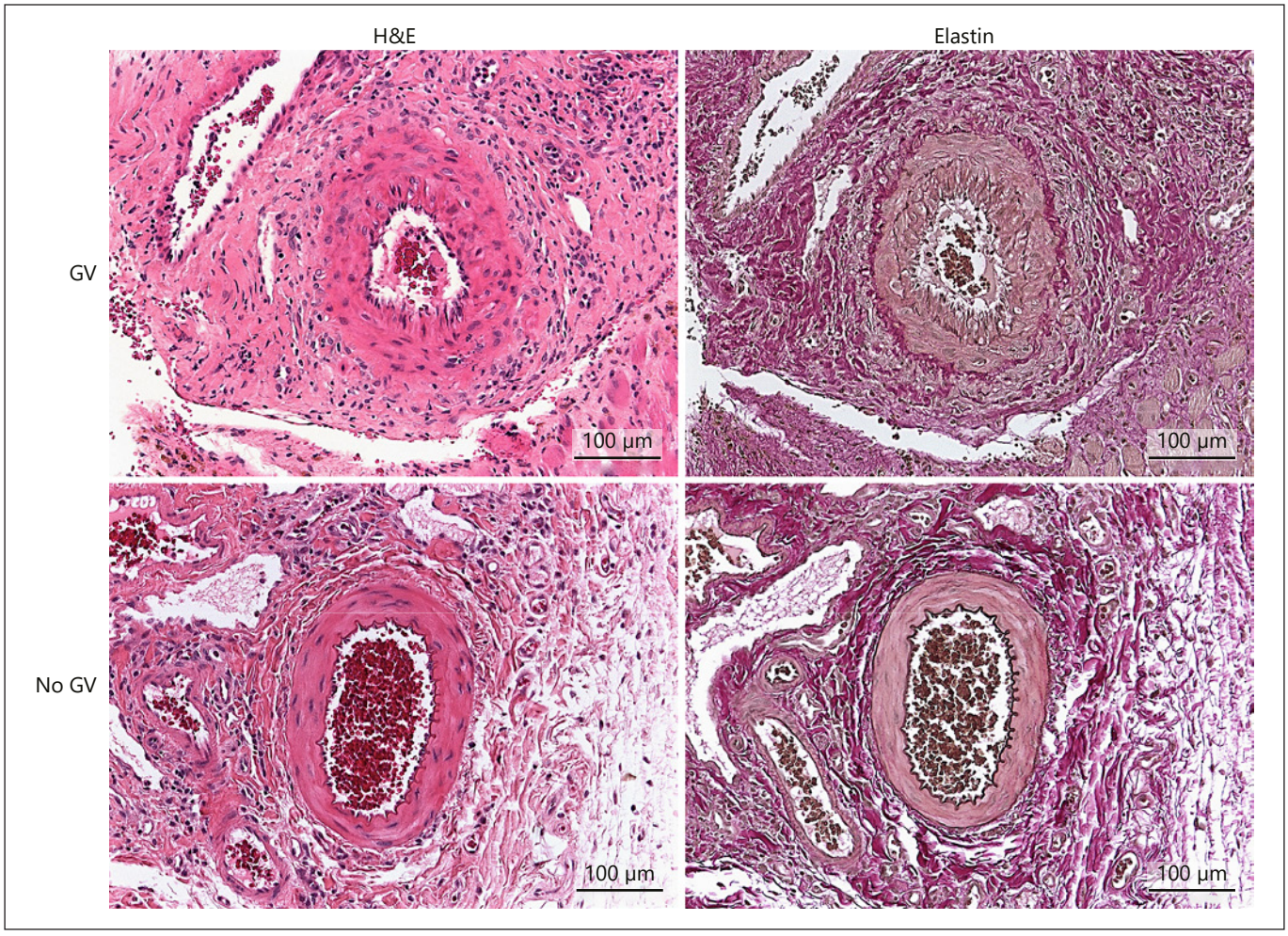

Color version available online

Fig. 1. Representative pictures of GV in a rodent model of vascularized composite allotransplantation. The top row depicts arteries with GV in both H&E (left) and Elastica van Gieson's stain (right). These present myointimal proliferation with luminal narrowing in contrast to normal, naïve arteries in the bottom row. GV, graft vasculopathy.

16 years or more [3]. Owing to the relatively small number of VCA patients as well as limited long-term patient follow-up, it is difficult to predict the longevity of a VCAGs. Currently no precise definition of chronic rejection in VCA exists. Features of chronic rejection in VCA such as loss of adnexa, skin and muscle atrophy, fibrosis of deep tissue, formation of tertiary lymphoid follicles, nail changes, or capillary thrombosis might be associated with vascular alterations [4, 5]. Additionally, a handful of reports suggest that vascular smooth muscle cell (SMC) proliferation and endothelial deterioration resemble graft vasculopathy (GV) and may play a crucial role in late graft loss, being a hallmark of chronic rejection in VCA [2, 6, 7]. GV is characterized by progressive concentric de novo myointimal proliferation and luminal narrowing of predominantly arterial vessels (Fig. 1), resulting in subsequent graft ischemia, dysfunction, and eventual failure. Indeed, in heart transplantation, cardiac allograft vasculopathy (CAV) is one of the leading causes of graft loss and mortality beyond the first year after transplantation [8].

Despite the high clinical relevance of GV, this topic remains insufficiently clarified and only a discrete body of knowledge is available, making it impossible to establish proper treatment options or prevention strategies to significantly ameliorate the long-term outcomes. It is imperative to understand the pathophysiology of GV so as to enable better prognosis and treatment of VCAG. The aim of this article is to review the current evidence and to summarize relevant aspects regarding vascular alterations in VCA.

Reported Evidence for GV in Human Upper Extremity and Face Transplantation

We searched the PubMed database for all available reports of histologically proven cases of GV in human upper extremity and face transplantation. The database was searched from September 23, 1998 (date of the first human hand transplantation) to March 15, 2019 using the following keywords: "hand transplant," "face transplant," "vascularized composite allotransplantation," and "graft vasculopathy." To match and remove duplicates, the search was refined using the Boolean operators AND and OR. Reports in languages other than English were excluded. In order to provide a comprehensive review of the literature, the relevant full-text reports together with associated references were profoundly scanned for information regarding putative mechanisms, experimental models, and clinical management of GV in upper extremity and face transplantation.

As of March 2019, a retrospective study by Ng et al. [9] reported 11 GV cases (Table 1): 9 patients having upper extremity allografts and 2 patients having face allografts. Strikingly, more than half of the patients (6/11) who were diagnosed with GV eventually ended up losing their allograft (Table 1). Challenging enough, GV appears not to always correlate with clinical skin changes, making early diagnosis difficult. One Louisville patient (No. 4, Table 1) lost his allograft as early as 9 months after transplantation due to an aggressive course of GV, but in absence of any chronic skin changes [7]. Other than that, 2 other VCA recipients (No. 3 and No. 10, Table 1) showed evidence of allograft fibrosis and functional impairment despite minimal GV [10, 11]. This is also in accordance with the Boston face transplantation experience. Indeed, chronic rejection was recognized in 3 out of 7 VCA recipients, but mostly limited to sclerotic skin changes and adnexal atrophy with no clear evidence of GV [12]. Of importance, the incidence of GV in human VCA in the present literature might be underestimated, for instance because of lack of reporting or challenges in the diagnosis of GV.

Potential Mechanisms of Vasculopathy in VCA

Numerous immunological and nonimmunological factors and pathways have been the focus of research for their contribution to GV, depicting it as a complex and multifactorial process. The pathogenesis has been extensively studied in SOT, especially in cardiac transplantation, although the mechanism is not yet completely understood [13, 14]. However, several distinguishable processes have been identified and are summarized in Figure 2.

Disruption of Vascular Homeostasis

Vascular homeostasis is maintained by synergistic regulation between blood flow and the vascular endothelium. The pre- and perioperative procedures during transplantation disrupt this synergy, thereby initiating vasculopathy within the graft. In the perioperative period, vascular injury is caused by an absence of graft perfusion as in deceased donors and relates to delayed graft function and poor graft survival when compared to perfused grafts obtained from brain-dead donors [15, 16]. Simultaneously, the endothelium becomes dysfunctional, triggering an imbalance in the production of vasoactive factors. The occurrence and effect of this imbalance has been studied in SOT, but not yet in VCA. In liver transplantation, increased angiotensin II and decreased bradykinin levels were detected during the anhepatic phase [17]. An increase in the expression of endothelin-1 in ex vivo perfused lungs was shown to correlate with poor transplantation outcome [18]. Endothelial dysfunction is also characterized by changes in the endothelium phenotype by overexpression of adhesion molecules switching to a proinflammatory state that leads to GV as shown in aortic transplantation [19].

An added feature of endothelial dysfunction during transplantation that directly reflects GV is accumulation of glycocalyx and lipids in the intima and media as shown in SOT [20]. Increased accumulation of the glycocalyx directly contributes to thickening of the intima in GV. Lipids are entrapped in this dense network of accumulated glycocalyx, resulting in vascular hyperlipidemia. Several other factors also contribute to hyperlipidemia that collectively progresses into atherosclerosis and GV [21, 22]. The evidence of atherosclerosis contributing to GV is based on (1) histological similarities between atherosclerosis and GV and (2) therapeutic outcomes by the use of statins during transplantation. Both involve macrophage and T cell infiltration of the vascular wall, triggering intimal proliferation, with the exception of lipid-laden foam cells, which are more typical for atherosclerotic plaques. More specifically, low-density lipoproteins are associated with macrophage-like foam cells in atherosclerosis [23] and HLA antigens expressed mainly on the endothelial surface in GV [24].

With regard to therapeutic outcomes, a pooled analysis confirmed that statins improve survival in heart transplant recipients and may reduce the incidence of CAV

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Table 1. Reported cases of histologically proven GV in clinical upper extremity and face transplantation

Patient No. [ref.]	Country, year	VCA type	HLA mismatch	Pre-VCA PRA	Induction	Maintenance immunosuppression	Donor BMC infusion	Prior CMR episodes	C4d/DSA	GV diagnosis	Imaging	Biopsy	Outcome
1 [102]	China, 2000	UE	n/a	n/a	ATG	TAC, MMF, steroids	no	yearly	n/a	5 years	n/a	skin	allograft intact at 9 years
2 [7, 10]	USA, 2001	UE	n/a	0%	Basiliximab	TAC, MMF switched to rapamycin early after transplantation, steroids (weaned, then restarted)	no	7	–/+ (class I from year 7)	8 years	Mild to moderate intimal hyperplasia on UBM	Deep tissue and interosseous arteries	Allograft intact at 17 years
3 [7, 10]	USA, 2006	UE	n/a	0%	Alemtuzumab	TAC (converted to MMF at 8 years due kidney dysfunction), rapamycin, steroids (after GV)	no	3	+ (deep vessels)/–	2 years	Mild to moderate intimal hyperplasia on UBM and MRA	Deep tissue and interosseous arteries	Allograft intact at 11 years, but limited function (stiffness/ fibrosis)
4 [7, 10]	USA, 2008	UE	n/a	0%	Alemtuzumab	TAC, MMF	no	2	–/+ (after amputation)	9 months	Severeintimal hyperplasia on UBM	Arteries and veins in terminal biopsy	Allograft loss at 9 months (ischemia)
5 [7, 10]	USA, 2008	UE	n/a	0%	Alemtuzumab	TAC, MMF (after first CMR) switched to rapamycin (after GV), steroids (after GV)	no	3	–/+ (de novo class II from year 6)	6 months	Mild to moderate intimal hyperplasia on UBM	Deep tissue and interosseous arteries	Progressive vasculopathy leading to allograft loss at 9 years
6 [7, 10]	USA, 2010	UE	n/a	0%	Alemtuzumab	TAC, MMF switched to rapamycin (after GV), steroids	no	2	+ (adnexae/ vessels in adipose tissue, deep vessels in skin)/–	6 months	Moderate to severe intimal hyperplasia on UBM	Deep tissue arteries	Allograft intact at 9 months, but patient lost to follow-up after 1 year
7 [97]	France, 2000	UE	6/6	0%	Basiliximab	TAC, MMF, steroids	no	5	+ (perineural vessels only)/–	13 years	n/a	Skin and radial artery, terminal biopsy	Allograft loss at 13 years
8 [96, 105]	France, 2003	UE	4/6	n/a	ATG	TAC, MMF, steroids	no	3	n/a/+ (class II from year 6)	11 years	No signs of GV in conventional angiography at 10 years	Skin	Loss of fingers and eventually whole allograft at 11 years

Table 1. (continued)

Patient No. [ref.]	Country, year	VCA type	HLA mismatch	Pre-VCA PRA	Induction	Maintenance immunosuppression	Donor BMC infusion	Prior CMR episodes	C4d/DSA	GV diagnosis	Imaging	Biopsy	Outcome
9 [98]	Austria, 2009	UE	6/6	0%	Alemtuzumab	TAC switched to belatacept at 6 years, MMF, steroids	no	4 (had 2 episodes of AMR prior to starting belatacept)	+ (only after amputation)/+ (class II from year 3)	7 years	n/a	Not specified, terminal biopsy	Allograft loss at 7 years
10 [11]	France, 2009	face	5/6	0%	ATG	TAC, MMF, steroids	yes (on day 4)	8	–/–	4 years	Thin focal irregularities in facial arteries on MRA	Skin	Allograft intact at 4 years, but limited function (stiffness fibrosis)
11 [2]	France, 2005	face	1/6	0%	ATG	TAC switched to rapamycin after 11 months, MMF, steroids	yes (on day 4 and 11)	2	+ (dermal vessels)/+ (de novo class II from year 7.5)	10 years	Decrease in flow of the right facial artery at distal level on MRA	SSG Nutrient vessel and facial arteries	Necrosis and partial allograft loss at 10 years, autologous reconstruction

Adapted from Ng et al. [9], with permission from John Wiley and Sons. AMR, antibody-mediated rejection; ATG, antithymocyte globulin; BMC, bone marrow cell; CMR, cell-mediated rejection; DSA, donor-specific antibody; GV, graft vasculopathy; HLA, human leukocyte antigen; MMF, mycophenolate mofetil; MRA, magnetic resonance angiography; n/a, not available; PRA, panel reactive antibody; SSG, sentinel skin graft; TAC, tacrolimus; UBM, ultrasound biomicroscopy; UE, upper extremity; VCA, vascularized composite allotransplantation.

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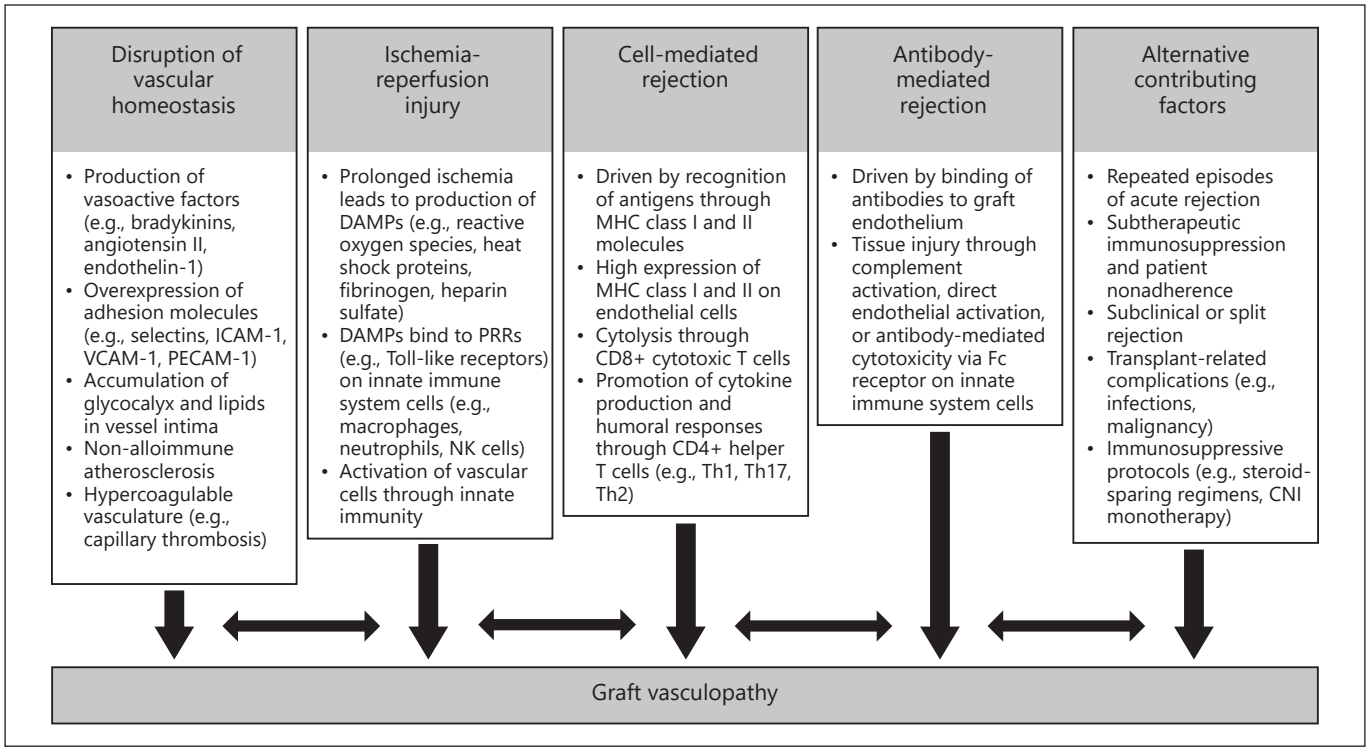

Fig. 2. An overview of possible mechanisms contributing to graft vasculopathy. CNI, calcineurin inhibitor; DAMPs, damage-associated molecular patterns; ICAM-1, intercellular adhesion molecule 1; MHC, major histocompatibility complex; NK, natural killer; PECAM-1, platelet endothelial cell adhesion molecule 1; PRRs, pattern recognition receptors; VCAM-1, vascular cell adhesion molecule 1.

[25]. Administration of statins reduced the 1-year mortality in heart transplantation by over 11% according to a meta-analysis by Mehra and Raval [26]. Furthermore, Yi et al. [27] reported amelioration of human allograft arterial injury by statins in an animal model. In this study, C.B.-17 severe combined immunodeficiency-beige mice received human artery segments as aortic interposition grafts. Upon inoculation with allogeneic human peripheral blood mononuclear cells, the occurrence of GV was reduced by simvastatin or atorvastatin, correlating to a decrease of graft-infiltrating CD3+ T cells. Another study showed the immunosuppressive effect of statin in heterotopic limb allografts in rats via attenuation of T cell activation and proliferation [28]. It is thus clear that statins have both lipid-lowering and immunomodulatory effects in allografts. Therefore, evidence for the contribution of atherosclerosis to GV in VCA may be important, but the mechanism is not clear and requires further study.

In SOT patients, the vasculature is in an activated, hypercoagulable state. The etiology for this condition is multifactorial including age, drugs, surgical procedure, diabetes mellitus, vascular anomalies, and heredity [29]. In case of cardiac transplantation hypercoagulable vasculature, defined by fibrin deposits and binding of antithrombin, has been shown to be directly associated with initiation and progression of vasculopathy [30]. Although the first clinical evidence from VCA is emerging [31], the occurrence and significance of hypercoagulable vasculature in VCAGs needs to be further established.

Ischemia-Reperfusion Injury

Ischemia-reperfusion injury (IRI) is a key issue in perioperative graft injury and of great importance. Ischemia is the absence of blood flow and reperfusion, the recommencement of blood perfusion into previously ischemic tissue are two phases with distinct pathophysiology. In transplantation, ischemia starts with termination of donor circulation (warm ischemia), continues during perioperative graft cold storage (cold ischemia), and ends with reperfusion after unclamping. The recovery and handling of donor tissue has a huge impact on the integrity of allograft vessels. In this manner, the transplant procedure itself, irrespective of immunological HLA-based donor and recipient disparity, initiates events that

contribute to deterioration and dysfunction [32]. Yi et al. [33] demonstrated that IRI, as part of perioperative injury, is sufficient to induce GV, which is further boosted by adaptive immune responses.

The mechanisms of IRI, including the generation of reactive oxygen species, initiation of inflammation and coagulation pathways, endothelial activation and damage, and their detrimental effects during transplantation, have been previously summarized for both SOT and VCA [34, 35]. IRI correlates with the development of transplant vasculopathy in SOT [36, 37]. Accordingly, it is plausible that IRI may trigger chronic rejection and promote GV in VCA as well. In mouse orthotopic hindlimb transplantation, prolonged cold ischemia led to increased vascular endothelial injury, vascular alteration, and eventual organ dysfunction [38]. In a rat model of vascularized allogenic skin flaps, Shimizu et al. [39] found that prolonged ischemia of 6 h duration caused earlier and stronger episodes of acute rejection than 1 h of ischemia under immunosuppression with cyclosporine A. This is in line with another study where vascularized skin flaps were transplanted from Wistar Kyoto to Fisher 344 rats after 1 or 3 h of ischemia, with no immunosuppression, revealing higher rejection grade scores in skin/muscle biopsies of animals with prolonged graft ischemia time [40]. These experimental findings correlating rejection to prolonged ischemia time and IRI need clinical verification. Owing to the low numbers in VCA, this aim might be difficult to accomplish. Nevertheless, Herzberg et al. [41] attributed prolonged ischemia time of >9 h in their bilateral hand transplant patient to inferior intrinsic muscle function recovery compared to an Austrian bilateral hand transplant patient with only 3 h of ischemia. Landin et al. [42] suspected perioperative IRI to be a cause of later flexor contracture in a forearm transplant recipient. Caterson et al. [43] did not find a correlation between ischemia time and the frequency of rejection in their face transplant series. Thus far, reports from clinical VCA are more of a speculative nature for being singular cases or small series.

These circumstances stress the importance of peri- and postoperative monitoring and quantification of ischemic injury in VCA. Immediate pathological changes after transplantation due to IRI could be assessed with biopsies over 24–48 h after ischemia [44]. Outcome indicators such as circulating and tissue lactate, nitroblue tetrazolium, tissue myeloperoxidase, CD31, and wet-to-dry weight ratio could be taken into account, but may lack specificity [38, 45–48]. Systemic effects of IRI could be measured by assessment of various cytokines and chemokines, including TNF-α, IFN-γ, IL-6, and VEGF, but are currently not the standard [49]. It remains challenging to fully understand the contribution of IRI versus peri-/postoperative inflammation or immune response in general to the changes seen in the abovementioned markers or histological signs.

Currently much effort is undertaken to establish novel preservation protocols for both preservation/perfusion fluids [50–55] and ex vivo machine perfusion [56–62] to reduce or avoid IRI. While some preservation solutions were shown to be favorable compared to others in SOT, this needs yet to be identified in VCA. Pursuing this goal, two experimental studies of syngeneic rat hindlimb transplantation compared the effects of different preservation solutions (saline, University of Wisconsin solution, HTK, HTK-N, TiProtec) and cold ischemia time (ranging from 2 to 30 h) on tissue injury [63, 64]. Interestingly, no clear superiority of any of the studied preservation solutions could be demonstrated, and the authors concluded that the length of cold ischemia time is the most significant factor determining IRI in VCA [63].

One future direction might aim at implementing and customizing preservation fluids with addition of supplements [65, 66], improved oxygenation [67], combined to controlled hypo- and (sub)normothermic machine perfusion, to reduce ischemic injury during preservation and thus improve immunological and functional outcomes. Moreover, improved preservation strategies would allow prolonged bridging time from donor to recipient and increase allograft availability and allocation potential.

Cell-Mediated Rejection

Cell-mediated rejection is the predominant mechanism during acute rejection episodes as suggested by perivascular CD3/CD4-positive infiltrates in histological specimens of VCAGs [68]. In acute cell-mediated rejection, the effector T cells execute their function mainly by two mechanisms. While CD8-positive T cells mediate cytolysis after binding of peptides to major histocompatibility complex (MHC) class I molecules, CD4-positive effector T cells respond to the binding of antigens to MHC class II molecules [69]. Endothelial cells are the primary target of effector T cells in the vasculature as they present high levels of class I and II HLA molecules, compared to graft SMCs which express minimal levels of HLA complexes [70, 71].

Although T cell function has been extensively investigated, their involvement and contribution in the pathogenesis of vascular changes in VCA remains unclear. Contrary to the mechanisms mentioned above, a report by Lian et al. [72] suggests that donor effector T cells were

responsible for tissue damage in the face of VCAG. The targets of donor T cells and their role in vascular injury should therefore be further addressed. Other than that, there is also evidence indicating that chronic CAV can be mediated by B cells independently of antibody production, as found by Zeng et al. [73] in a murine model.

Immunosuppressive drugs such as calcineurin inhibitors, mTOR inhibitors, mycophenolate mofetil (MMF), or steroids target T cell function and can thus efficiently control acute T cell-mediated rejection. Nevertheless, GV can develop under conventional immunosuppression and clinical unapparent rejection in both SOT and VCA and is a major cause of late progressive graft failure [2]. This suggests that current immunosuppressive drug regimens are insufficient to prevent long-term vascular damage, and additional mechanisms may be involved.

Antibody-Mediated Rejection

In SOT, vascular rejection is traditionally associated with cell-mediated rejection, but the humoral involvement is increasingly recognized to be part of this process [74, 75]. Despite most rejection episodes being cell-mediated in VCA, cases of acute antibody-mediated rejection (AMR) in face- or hand-transplanted patients have also been reported [76, 77]. During AMR, donor-specific antibodies (DSA) trigger rejection [78]. DSA typically activate complement deposition in the endothelium (C4d) via the classical pathway [76, 79, 80], even though complement-deposition free rejection has been reported [81]. AMR usually manifests as chronic rejection and slow, progressive graft deterioration, favoring development of GV. Indeed, in renal or cardiac transplantation, AMR is believed to drive chronic rejection and promote GV [82, 83]. A supposed mechanism of action is complement-mediated injury of the allograft vessels and persistent or progressive vascular inflammation with intimal hyperplasia [84, 85]. Additional evidence also suggests that DSA binding direct to endothelial cells can lead to GV, bypassing activation of the complement system [86]. However, the exact mechanism by which these antibodies, being more prone to endothelial cells than SMCs, promote proliferation of the latter and migration into the hyperplastic intima is not yet clear [13]. AMR may be more likely in the presence of C4d deposition and occurrence of DSA [87]. However, graft complement deposition may also occur independently from DSA, e.g., through binding of autoantibodies after IRI [88, 89]. Reports from both SOT and VCA suggest that also non-HLA-related antibodies, like those directed against angiotensin II type 1 receptor, may play a role in vasculopathy [90–93]. Given these aspects, the accurate diagnosis of AMR remains a challenge [94, 95].

Indeed, in clinical VCA, the diagnostic criteria for AMR are not included in the current Banff classification [4]. Therefore, the role of AMR in chronic rejection and GV still has to be clarified. The first face transplant patient (No. 11, Table 1) who partially lost the allograft as late as 10 years after transplantation developed progressively increasing de novo class II DSA, C4d deposits in the endothelium, as well as GV [2]. On the other hand, experience with upper extremity transplantation from the same group showed that GV can occur in patients with low-level transient DSA (No. 8, Table 1) [96] and even without DSA (No. 7, Table 1) [97]. Additionally, Kaufman et al. [7] reported on 2 patients with severe GV. In the first case, strong progressive vasculopathy eventually resulted in graft loss 9 months postoperatively (No. 4, Table 1). No DSA were detected, nor did the explanted tissue show any evidence of C4d deposition. Strikingly, DSA were detected 3 days after hand amputation and cessation of immunosuppression. The second patient (No. 6, Table 1) with severe GV showed C4d deposits in deep vessels, but no circulating DSA could be detected [7]. According to a follow-up report from the same center, one patient (No. 5, Table 1) developed de novo class II DSA 6 years after transplantation and ultimately lost his allograft due to GV 3 years later [10]. Interestingly, in a bilateral hand transplant patient in Austria, acute AMR due to de novo DSA was reported by Weissenbacher et al. [77]. The same group also added costimulatory blockade drug belatacept into maintenance immunosuppression of 4 upper extremity transplant recipients [98]. Unfortunately, in 1 patient (No. 9, Table 1) with high levels of class II DSA at the time of belatacept initiation, acute rejection occurred and eventually led to allograft amputation. The biopsies from the amputated hand revealed severe GV in association with positive C4d stain [98].

In summary, 100% of patients with GV and detectable class II DSA lost their allograft within 2.5–5 years from first detection of class II DSA (Table 1). Occurrence of GV in absence of DSA in some other patients, however, suggests that both cell- and antibody-mediated mechanisms are involved in the pathophysiology of GV.

Repeated and Subclinical Rejection

The relationship between number of acute rejection episodes, development of chronic rejection, and GV is not fully understood to date. Reports from experimental models of rat and nonhuman primate (NHP) VCAs showed that repetitive acute rejection combined with

subtherapeutic treatment [99], reduction of maintenance immunosuppression [100], or complete discontinuation of immunosuppressive drugs [101] can induce vascular changes resembling those of GV. A couple of reports in clinical VCA suggest a similar course in patients with decrease or discontinuation of immunosuppression due to nonadherence [6, 97, 102] or complications [2, 11]. On the other hand, there is also evidence that GV can develop in patients compliant with the immunosuppressive regimen [7, 103].

Alternative Pathways

Immunosuppression is currently required to prevent graft rejection in clinical VCA recipients, and serious adverse effects should be considered. In the literature the focus is on metabolic, infectious, or malignant adverse effects of immunosuppressive regimen in VCA [2, 104, 105], but the pathophysiological link to GV has not yet been studied.

A multitude of induction and maintenance immunosuppression protocols is reported in the clinical VCA literature. The most commonly used induction agent is antithymocyte globulin. Alternative induction regimens have been reported, e.g., the use of basiliximab [106, 107], alemtuzumab [7, 108, 109], or rituximab [110]. Other induction strategies included allograft preradiation [106], extracorporeal photopheresis [104], plasmapheresis with intravenous immunoglobulin in a sensitized recipient [76], or infusion of donor-derived bone marrow cells [109, 111]. Maintenance immunosuppression usually consists of triple therapy with tacrolimus, MMF, and steroids. Tacrolimus target levels have been reported somewhere between 6 and 15 ng/mL [109, 112, 113], and MMF daily doses have ranged from 720 mg [114] to 3 g [111]. Furthermore, attempts to withdraw MMF [115] or steroids [7, 116] as well as tacrolimus monotherapy [109] have been described. Progressive decrease of renal function has led to conversion of tacrolimus to mTOR inhibitor and/or belatacept in a few upper extremity and face transplant recipients [98, 113, 117, 118]. As of now, it remains unclear which abovementioned induction and maintenance protocols are beneficial against the development of GV.

Endothelial dysfunction in renal transplantation might be related to cyclosporine A [119] and tacrolimus medication [120]. TLR4 signaling in endothelial cells may play a role in this process according to Rodrigues-Diez et al. [121]. Indeed, all patients in Table 1 received tacrolimus, but evidence for pathophysiological association with GV is difficult to prove owing the limited number of cases. The same accounts for steroid-sparing immunosuppression or use of mTOR inhibitors, since it appears that GV can develop under any currently used immunosuppressive regimen.

Another potential trigger for vascular alterations in allografts might be of infectious origin, despite weak evidence. In atherosclerosis, concomitant bacterial infection such as gastritis, gingivitis, or respiratory infection might influence disease development [122]. Moreover, cytomegalovirus infection is considered a risk factor for CAV [123]; however, the exact mechanism has not yet been clarified.

Diagnosis and Monitoring of Vascular Alterations in VCA

Histopathological Markers

Markers for the detection or prediction of vascular changes or acute/chronic rejection are of great interest for both VCA and SOT (Table 2) [124, 125]. Although a decent amount of research has been carried out in SOT, little is known, and there is a paucity of knowledge for VCA. Hautz et al. [68] provided an extensive histopathological analysis for different markers during rejection in hand transplant recipients. Interestingly, many of these markers were directly or indirectly associated with GV: cellular infiltration included CD3, CD4, CD8, CD20, CD68, and HLA class II-DR expression in tissue samples; AMR was associated with CD20 and C4d; IDO and Foxp3 were regarded as markers for tolerance; LFA-1, ICAM-1, E-selectin, P-selectin, VE-cadherin, and Psoriasin were summarized as adhesion molecules. Of these markers, CD68, Foxp3, and IDO expression as well as CD4/CD8 ratio correlated well with the grade of rejection. Moreover, lymphocyte adhesion molecules, especially ICAM-1 and E-selectin, also strongly correlated with the severity of rejection. In this context, the role of CD68 as an adhesion molecule could also be taken into consideration, although its exact role remains unclear [126]. In a follow-up study of the same group, tissue specimens from NHP and rats were added to the histopathological assessment [127]. In this work, peripheral node addressin (PNAd) as a marker of lymphatic neoangiogenesis was evaluated during rejection. Its expression significantly increased in the intragraft endothelium upon rejection in human skin compared to naïve skin and correlated with B and T lymphocyte infiltration and LFA-1 expression in the tissue. Of interest, PNAd expression was clearly higher in biopsies at 5 or more postoperative years compared to early fol-

Table 2. Potential detection and monitoring of vascular alterations in experimental and clinical VCA

Type	Markers	Invasiveness	Sample	Application	Positive attributes	Negative attributes	References
Histopathological							
Cell infiltration	CD3, CD4, CD8, CD20, CD68, HLA class II-DR	High	Tissue	Experimental/ clinical VCA	Gold standard, best resolution, multiple stainings are possible	Graft injury/ dysfunction, rejection triggering, sampling bias, not desirable for frequent use	68, 72, 127
AMR	CD20, C4d						68, 127
Graft tolerance	IDO, Foxp3						68, 72, 127
Endothelial activation/ cell adhesion	LFA-1, ICAM-1, E-selectin, P-selectin, VE-cadherin, Psoriasin						68, 127
SMC proliferation	Alpha smooth muscle actin						129
Lymphatic neoangiogenesis	PNAd						127
Noninvasive							
Proteomics	HLA and non-HLA antibodies (e.g., AGTR1-Ab), multiple protein panel, VEGF-C, VEGF-A, PF-4	Low	Blood	Clinical VCA/ clinical SOT			90–93, 136, 137
Gene expression profiles	Multiple gene panel	Low	Blood	Clinical SOT	No graft injury	Not widely established in clinical routine, sensitivity and specificity concerns	134, 135
Cell-free DNA	Calculation of donor-derived DNA fraction	Low	Blood				138, 139
B cell repertoire	Sequencing of antibody heavy chain transcript	Low	Blood				140
mRNA	Perforin, granzyme B	Very low	Urine				141
Oxidative stress	Methylated alkane contour	Very low	Breath				142
Imaging							
Intravascular ultrasound	Vessel wall thickness (intima/ media), occlusion degree	High	Image	Clinical SOT	Sensitive to early vascular changes	Invasiveness	144
Optical coherence tomography		High (intravascular)/ very low (transdermal)		Clinical SOT	No graft injury in transdermal application, high resolution	Low tissue penetration depth in transdermal application	146, 147
Ultrasound biomicroscopy		Very low		Clinical VCA	No graft injury, suitable for frequent use	Sensitivity and specificity concerns	7, 143
Reflectance confocal microscopy		Very low		Experimental VCA	No graft injury	Expensive, low tissue penetration depth	148
Multiphoton microscopy		Very low		Experimental	No graft injury	Expensive, low tissue penetration depth	149, 150

AGTR1-Ab, angiotensin II type 1 receptor antibody; AMR, antibody-mediated rejection; HLA, human leukocyte antigen; ICAM-1, intercellular adhesion molecule 1; PNAd, peripheral node addressin; SMC, smooth muscle cell; SOT, solid organ transplantation; VCA, vascularized composite allotransplantation.

low-up after transplantation. Earlier, PNAd had also been linked to lymphoid neoangiogenesis in tertiary lymphoid organs of murine cardiac transplantation [128]. An additional marker helping in the detection of transplant vasculopathy might be alpha smooth muscle actin as it is linked to intimal SMC proliferation [129].

An inherent disadvantage of the abovementioned markers is the invasive nature of the tissue biopsy. In addition, allograft vessels can be concomitantly affected by different pathologies, and it might be difficult to differentiate between inflammatory, infectious, and neoplastic dermatoses in skin biopsies of VCAGs [130]. Wolfram et al. suggest novel approaches to address this issue by using gene expression analysis [131] or computational modeling of cytokine profiles [132].

In summary, there are no predictive markers yet for transplant vasculopathy/chronic rejection in VCA.

Noninvasive Markers

Given the fact that detection of class II de novo DSA is almost inevitably associated with GV and allograft loss in VCA (Table 1) [2, 10, 98], regular monitoring of circulating MHC antibodies appears to be of utmost relevance. Indeed, a recent retrospective multicenter study revealed that from 44 analyzed upper extremity recipients, 14 (32%) developed de novo DSA [133]. However, a strong correlation between DSA and graft survival could not be found, suggesting a relatively high variability between the involved centers with regard to antibody development as well as sampling differences [133].

Additional noninvasive markers are evaluated as alternatives to graft biopsies in the field of transplantation, with potential implications for VCA (Table 2). These might be based on gene expression profiling [134, 135], proteomic analysis [136, 137], cell-free DNA [138, 139], or B cell repertoire sequencing [140] analyzed from peripheral blood samples or skin patch tests. Other studies point out at the possibility of marker analysis from urine samples or even breath [141, 142]. Even though all of these markers might be promising, it is important to note that their sensitivity and specificity have not been assessed in VCA yet.

Clinical Monitoring of GV

Conventional graft biopsy is associated with donor site morbidity and scarring, especially relevant in face transplant recipients, or could even trigger episodes of acute rejection. While noninvasive markers are not established yet, noninvasive monitoring of rejection and vasculopathy in VCAGs is desired.

To overcome this problem, noninvasive imaging techniques are currently being evaluated in VCA recipients (Table 2) [7, 143]. Computed tomography and magnetic resonance angiography seem to fail the scope of monitoring transplant vasculopathy owing to limited spatial resolution and low sensitivity for early stages of the disease. Ultrasound biomicroscopy (UBM) might be a useful alternative for this purpose. Indeed, a study by Kaufman et al. [7] provides the first report about the use of UBM for imaging and monitoring of transplant vasculopathy in larger and smaller arteries in clinical VCA. Interestingly, in the Louisville series of 6 hand transplant patients with follow-ups from 9 months to 12 years, all presented with some degree of vasculopathy [7]. However, the 2 subjects with the longest follow-up (>10 years) showed the least vascular changes with minimal luminal occlusion [7]. UBM has also been evaluated as a diagnostic tool in a series of face transplant recipients, proving its applicability for repetitive postoperative vessel monitoring. Kueckelhaus et al. [143] found that even if the intima was thicker in all sites in face transplant patients than healthy controls, the ratio between intimal thickness of facial and radial arteries was similar in transplanted patients and controls. To confirm UBM as a proper diagnostic tool for GV in face transplants, a long-term follow-up ideally including tissue biopsies will have to clarify its sensitivity and reliability. UBM as a monitoring tool in VCA is in its infancy, and to our best knowledge comparisons to vascular changes during other procedures than VCA such as replantations or free tissue transfers are lacking.

In clinical heart transplantation, intravascular ultrasound is routinely used for the assessment of CAV and associated perivascular proliferation [144]. As further refinement of intravascular monitoring, attention is drawn to another interesting imaging technique called optical coherence tomography (OCT). This technique, in contrast to ultrasound, works with near-infrared electromagnetic waves and provides the advantage of high-resolution quantitative vessel imaging [145]. It has been utilized to visualize initial vascular alterations of CAV heart transplantation [146]. In VCA, the transdermal application of OCT could provide noninvasive monitoring of the vasculature. Indeed, one group evaluated OCT for imaging of superficial temporal arteries by transdermal OCT in giant cell arteritis with mixed results for limited penetration depth [147]. Whether the actual depth of penetration is sufficient to image the vessels of interest in VCA needs therefore to be investigated.

The field of VCA could potentially benefit from several other advanced imaging techniques. In a study by Zor

et al. [148], vascularized groin flaps were transplanted between Sprague-Dawley rats and noninvasively monitored for signs of acute rejection by the means of reflectance confocal microscopy, and a significant correlation between histopathological grades and reflectance confocal microscopy scores could be established. Multiphoton microscopy is another technique that enables three-dimensional, sub-micrometer-resolved imaging of unstained living tissue, potentially filling the resolution gap between OCT and scanning electron microscopy [149]. This could have significant implications in clinical research, including detection of vascular anatomy. Low depth of tissue penetration, device bulkiness, and immobility as well as high costs typically limit the use of such technologies [150].

Experimental Models of Vasculopathy in VCAGs

Experimental models of chronic rejection and GV are crucial for the understanding of these pathophysiological processes and development of preventive and therapeutic strategies. As mentioned above, three basic animal models of chronic rejection have been studied for this purpose. In a rat hindlimb transplantation model, grafts underwent repetitive cycles of acute rejection-remission, trying to arbitrarily imitate chronic rejection. Upon signs of acute rejection, cyclosporine A and dexamethasone were administered until remission and then discontinued. The grafts showed significant myointimal proliferation at 90 days in H&E-stained specimens, resulting in concentric luminal occlusion and perivascular and muscular fibrosis [99].

Mundinger et al. [100] used an NHP fibula flap model in mismatched cynomolgus macaques. The animals received either initial high-dose tacrolimus monotherapy ($n = 3$) or low-dose tacrolimus with anti-CD28 costimulatory blocking antibody ($n = 2$). The tacrolimus dose was reduced to maintenance levels in both groups after 28 days, and all animals reached the endpoint of 180 postoperative days. Histological analysis at endpoint revealed GV in both animals in the low-dose group; unfortunately, this was not investigated in the first group [100].

In another work from the same group, chronic rejection in an NHP heterotopic face transplant model was reported [101]. Five animals with long-term graft survival (>200 days) and detectable macrochimerism were completely weaned of immunosuppression consisting of tacrolimus/MMF ($n = 4$) or tacrolimus/anti-CD28 costimulatory blocking antibody ($n = 1$). All five grafts were rejected after immunosuppression withdrawal, and biopsies from all grafts revealed neointimal hyperplasia and arterial luminal narrowing consistent with vasculopathy [101]. As early biopsies were unavailable, it remains speculative whether the observed vascular changes were of acute or chronic origin.

Although the three abovementioned models are capable of producing vascular changes consistent with GV, it is questionable how appropriately these models reflect chronic rejection. In the first model, repetitive episodes of acute rejection resulted in GV, potentially reflecting the clinical VCA situation, with a majority of patients undergoing multiple acute rejection episodes in the first 2 years after transplantation. Long-term vascular changes in the NHP model may, however, better reflect chronic graft deterioration under subtherapeutic immunosuppression.

Further models for chronic rejection in VCA could be derived from established SOT models [151]. The Fisher to Lewis rat model is another interesting model of chronic rejection in kidney transplantation [152]. Compared to the well-established Brown Norway to Lewis full mismatch VCA model, the Fischer/Lewis model is a minor mismatch model with no need for immunosuppression. Similarly, taking advantage of minor genetic mismatches, the WF.1L/Lewis rat model could also be utilized in VCA. WF.1L is a congenic rat strain derived from backcrossing Lewis to Wistar Furth rats. In a study by Forbes et al. [153], hearts from WF.1L rats were transplanted into Lewis rats without the need for maintenance immunosuppression. While the grafts clinically showed no rejection and an indefinite survival, they developed vasculitis with myointimal thickening. Such models could be of high interest for experimental VCA with a focus on GV.

Potential Treatment Options and Perspectives

There are several potential novel therapeutic approaches that might reduce or mitigate chronic rejection and GV. First, drug-based induction and maintenance therapy is of particular interest. Costimulatory blockade can induce allograft tolerance and reduce GV in experimental models of SOT [154–156]. In experimental VCA, reports of tolerance induction in small [157] and large [158] animals sound promising. However, clinical studies specifically assessing GV are lacking. Furthermore, studies in heart transplantation suggested that mTOR inhibitors could prove beneficial in preventing GV [159, 160]. In clinical VCA, the significance of mTOR inhibi-

tors is still unclear. Reports of a face and hand recipient suggest that the use of mTOR inhibitors could not prevent changes resembling chronic rejection and GV [2, 96]. Second, cell-based therapies could help reduce GV. In a humanized mouse model, two distinct populations of expanded regulatory T cells were utilized for this purpose [161]. The authors found that both CD127-positive and -negative regulatory T cell-expanded populations administered systemically immediately after transplantation were capable of inhibiting intimal hyperplasia and GV [161]. The immunomodulatory potential of mesenchymal stem cells is also in the spotlight for VCA in terms of increasing graft acceptance [162–165]. Mesenchymal stem cells are known to reduce endothelial activation [166] and to attenuate inflammatory intimal thickening in rodent arteries [167]. Recently, our group has shown in a rat hindlimb transplant model that repetitive administration of adipose-derived mesenchymal stem cells in the first 2 weeks after transplantation drastically reduced intimal thickening in large vessels of long-term surviving animals [168].

Additional potential therapeutic strategies against GV may include optimized preservation systems. Machine perfusion is superior to cold storage, with improved survival and function of the transplanted grafts in SOT [169, 170], and a similar effect might be expected for VCA. Indeed, Werner et al. [57] showed that 24 h of ex vivo perfusion of human limbs preserved neuromuscular function. In terms of minimizing IRI and its associated vascular damage, a variety of additives to the current cold storage solutions could be beneficial [171]. In this context, polyethylene glycol, hydroxide sulfide, and simvastatin seem promising [65, 66, 172]. Additionally, enteral as well as parenteral administration of simvastatin may have an alleviating effect on IRI. A study by Zhao et al. [173] in a mouse hindlimb ischemic model showed that intraperitoneal administration of simvastatin parallel to ischemic insult of 2 h followed by reperfusion can reduce IRI by reduction of cytokine formation, downregulating adhesion molecule expression and reducing leukocyte accumulation. In a setting of rat cardiac transplantation, the work by Tuuminen et al. [174] provided evidence that donor oral pretreatment with simvastatin could counteract IRI, endothelial dysfunction, and chronic rejection.

In summary, there is no clinically applicable causal treatment of transplant vasculopathy in VCA to date. The first chronic graft failure in a face transplant patient could not be reversed [2] and underscores the serious threat of chronic rejection and GV for VCA [175]. To our best knowledge, prevention seems to be the best strategy.

Conclusions and Outlook

GV appears to be a multifactorial entity shared between acute and chronic rejection. However, the true mechanisms of GV are unknown and with good long-term results in many VCA patients without GV, the exact mechanisms have still to be elucidated. Advances made in reconstructive transplantation over the last two decades in both peri- and postoperative management allowed for very satisfying short- and mid-term results, which, however, were short-lived in some patients owing to progressive long-term graft deterioration. Chronic rejection and GV are often referred to as similar phenomena, but with the current state of knowledge and in light of different pathophysiological pathways potentially resulting in vascular changes, their relationship and shared characteristics remain to be clarified.

Proper diagnostic markers and tools must be implemented to detect GV in the early phase, and the exact underlying pathways need to be better understood to evaluate novel drug- and cell-based therapies for the prevention, attenuation, or reversal of GV.

Although limited to a low number of cases, the strong association of class II de novo DSA with AMR, GV, and ultimately allograft loss calls for attention. If AMR is suspected, deep tissue biopsy should be favored since standard skin biopsy might not be sufficient for diagnosis of GV. Accordingly, with the aim of stopping progression of GV at an early stage, the threshold for antibody-targeted therapies during suspected AMR should be lowered, even in the absence of strong clinical signs of rejection.

Noninvasive diagnostic tools such as UBM are gaining relevance in VCA because they allow for repetitive monitoring of the zone of interest without morbidity, but these applications are in their infancy and need to be further evaluated. Detection of GV by peripheral blood gene or marker analysis are additional promising alternatives.

At present, the best "therapy" for multifactorial GV is, perhaps, prevention. Evidence points at better outcomes and reduced GV by reducing IRI through optimized preservation protocols, concomitant cell therapies, and novel drug-based induction therapies, or a combination of all three.

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

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Author Contributions

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