Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE MEDICHE SPECIALISTICHE

Ciclo XXIX

Settore Concorsuale di afferenza: 06/D1 Malattie dell'apparato cardiovascolare e malattie dell'apparato respiratorio

Settore Scientifico disciplinare: MED/ 11 Malattie dell'apparato cardiovascolare

DEFINITION OF CLINICAL AND IMMUNOLOGICAL PHENOTYPES OF GRAFT DYSFUNCTION IN HEART TRANSPLANT RECIPIENTS: PROGNOSTIC IMPLICATIONS AND ROLE OF ANTIBODY MEDIATED REJECTION

Presentata da: Dott.ssa Valentina Manfredini

Coordinatore Dottorato

Prof. Gaetano Domenico Gargiulo

Relatore

Prof. Claudio Rapezzi

Co-relatore

Dott. Francesco Grigioni

Esame finale anno 2017

Table of Contents

PART I

ANTIBODY-MEDIATED REJECTION IN HEART TRANSPLANTATION: NEW DEVELOPMENTS AND OLD UNCERTAINTIES

Review article accepted for publication on Current Opinion in Organ Transplantation in 2017

Valentina Manfredini [1], Ornella Leone [2], Valentina Agostini [2], Luciano Potena [1]

[1] Heart and Lung Transplant Program;

[2] Pathology Department Bologna University Hospital S.Orsola-Malpighi

1.1 Purpose of review

Antibody-mediated rejection (AMR) currently represents one of the main problems for clinical management of heart transplant because of its diagnostic complexity and poor evidences supporting treatments.

1.2 Recent findings

Disorder-based diagnosis is a cornerstone in defining AMR. The limitations of the current classification have been partially overcome by novel studies improving the description of the immune-pathological graft abnormalities, and by new molecular approaches allowing a better understanding of the mechanisms behind AMR and of its relationship with cellular rejection and chronic vasculopathy. In-depth characterization of donor-specific antibodies showed to provide additional prognostic information and guide for treatment. Clinical relevance of AMR is bound to appropriate detection of graft dysfunction. In addition to traditional longitudinal evaluation by echocardiogram, cardiac magnetic resonance and detection of cell-free DNA may represent novel sensitive markers for graft injury that could prompt treatment before dysfunction becomes clinically manifest.

1.3 Summary

Despite improvements in the diagnostic process, therapeutic strategies made little progress in addition to the consolidation of practices supported by limited evidences. Novel complement inhibitors appear promising in changing this scenario. Nevertheless, collaborative multicenter studies are needed to develop standardized approaches tailored to the highly variable clinical and laboratory features of AMR.

1.4 Introduction

Although improvements in immunosuppressive therapies have significantly reduced

the frequency and clinical relevance of cell-mediated rejection (CMR) after heart transplantation, antibody-mediated rejection (AMR) has emerged as a major threat and conundrum for patient management, because of the still sizeable uncertainties beyond its pathogenesis, diagnosis and management [1▪, 2].

The reported incidence of AMR may vary widely, ranging from 3 to 85% [1▪], because of the diverse diagnostic criteria, including pathological and/or clinical findings, and variations in screening frequency. Standardized pathology diagnostic criteria have improved the characterization of AMR and have also increased the recognition of the high variability in its clinical presentation. For example, AMR has been described as an acute and early-onset process responsive to treatment, but also as a hidden phenomenon that may be responsible for late and chronic graft dysfunction in patients long after transplant, usually poorly responsive to any treatment [3▪]. In addition, antibodies – the key pathogenetic feature of AMR – may not be found in all patients with clinical and pathological evidence of AMR, whereas they may be assayed in clinically silent patients [4]. To further complicate the process definition, patients with biopsy-proven CMR may often show at least some of AMR features, leading to the diagnosis of ‘mixed’ rejection, a concept still unclear in terms of definition and treatment (e.g. is it a coexistence of two different rejections? Is it a false positive for AMR? Is a cellular infiltrate in the context of AMR?) [5▪▪, 6▪▪].

In this article, we aim to dissipate some of the ambiguities surrounding AMR by discussing recent concepts going beyond its pathogenesis, diagnosis and treatment, and at the same time to highlight the most urgent unmet needs that

require future research.

1.5 Pathogenesis

AMR develops when the recipient's allogeneic immune response triggers the production of donor-specific antibodies (DSAs) directed against human leukocyte antigens (HLAs) or other non- HLA antigens that may be expressed by the graft endothelium.

The injury mediated by HLA-directed antibodies (HLA-Abs) is caused by multiple effector mechanisms, one of the best known of which is the activation of the complement cascade. Complement activation produces chemoattractants and proinflammatory mediators and ultimately leads to the synthesis of the membrane attack complex (MAC) formation. When directed against allograft endothelium, this immune-mediated storm causes acute injury with increased vascular permeability, vasculitis and microvascular thrombosis, ultimately leading to myocyte injury and graft dysfunction [7,8▪]. Due to their local proinflammatory properties, complement early proteins appear to be more responsible for complement-associated injury than the MAC complex is.

Not all DSA-induced injury is mediated by complement activity: although IgG subclass 3 are the strongest complement activator, IgG4 usually have little or no complement activity and are often linked with IgG2 as 'noncomplement fixing' [8▪▪]. Of note, immune response against HLA epitopes is usually polyclonal, thus involving multiple IgG subclasses and multiple mechanisms of injury. In this context, the obligatory link between AMR and complement has recently become

controversial, and AMR occurrence has been recognized even in absence of complement capillary deposition [7,9]. This paradigm shift was driven by gene expression profiling analyses showing that AMR signature was present in C4d-negative specimens [10] and is mirrored by experimental studies showing that intimal thickening during antibody-induced chronic rejection occurred in complement-deficient murine recipients, suggesting that there was no requirement for complement in this process [11]. In this process, the mammalian target of rapamycin (mTOR) signaling appears to play a key role, as it is activated by the HLA-Ab cross-linking on the surface of endothelial cells [12]. In addition to activating endothelial signals, Abs function by themselves as chemoattractant for monocytes, macrophages, neutrophils and natural killer (NK) cells, which all express receptors for the Fc region This direct cross-linking between antibodies and immune cells that can be recognized as infiltrating the graft tissue in biopsies may at least in part help to elucidate the conundrum of mixed rejection.

Some patients with DSA do not show histological or clinical evidence of AMR [4], suggesting that other factors influence susceptibility to or risk of rejection in the presence of antibodies that bind the graft. Endothelial cells may be the mediators of this susceptibility and act as immune regulators by expressing of complement regulatory proteins, with variable response to injury, ranging from acute lytic injury to chronic nonlytic injury characterized by cytoskeleton disruption and expression of surface procoagulant molecules: these different responses are closely associated with different clinical presentations and response to therapy [3▪,13].

1.6 Antibody-mediated rejection diagnosis: a complex multiparametric disease

Although cellular rejection diagnosis is defined by the histologic description of the nature of the inflammatory infiltrate and myocyte damage found in endomyocardial biopsies (EMBs), the diagnosis of AMR would require clinical evidence of allograft dysfunction, pathology evidence of both morphologic and immunopathologic microvascular injury on EMB, in which the main immunopathology is the capillary deposition of complement degradation product C4d, and evidence of circulating DSA. Despite the prognostic importance of any one of the three components [14,15"], in clinical practice coexistence of at least two of them is required to plan a specific treatment [16]. In this section, we will revise the improvements and pitfalls of the current diagnostic system for AMR.

1.6.1 Antibody-mediated rejection pathology classification and beyond

A working formulation for pathologic diagnosis of AMR first appeared in 2011, and in 2013 the pathological classification and grading (pAMR) was published. This included the concept of complement- negative AMR, that is pAMR may be diagnosed when more than 10% of capillaries are found filled with CD68þ macrophages, even in absence of capillary C4d deposition [7]. Additional markers of micro- circulatory inflammation appear to further obviate any need for C4d detection, which is becoming increasingly meaningless as isolate finding [17]. In a prospective study, Tible et al. [18] found that immunostaining in capillary endothelial

cells for phosphorylated S6 kinase and 6S ribosomal protein (pS6RP and p70S60K), two targets of mTOR effectors, were correlated with microvascular inflammation and DSA. This finding has been independently replicated by Li et al. [19], who described in 107 patients that these markers of endothelial inflammation were more often associated with AMR features than C4d alone.

In addition to the markers of endothelial activation, a number of studies introduced the concept of microvascular inflammation, histologically characterized by the presence of intravascular-activated mononuclear cells, including both intravascular macrophages and swollen endothelial cells [20]. In a multiinstitutional study, Fedrigo reports how the microvascular inflammatory burden that is extravascular interstitial and intravascular inflammatory cells was a constant feature of AMR and correlated with pAMR, C4d positivity and DSA positivity. In pAMR+ specimens, equivalent numbers of T lymphocytes and macrophages were seen in the intravascular and extravascular compartments. The presence of plasma cells was associated with a higher inflammatory burden and longer time post-transplant [5▪▪]. These findings mirror a more complex report from the UTAH group, which was trying to solve the conundrum of mixed rejection. After reviewing over 28 000 EMBs, Kfoury et al. [6▪▪] found that pathology mixed features may often point to an overlap between pAMR and CMR, that relapsing CMRs are accompanied by progressive pAMR features and that mixed rejection portends a worse prognosis as compared with CMR or pAMR alone.

Although improving AMR diagnostic accuracy of EMB, the addition of further histologic and immunopathologic evaluation criteria might well increase the

likelihood of interpathologist variability in reading and interpretation a well known and documented pitfall of disorder-based diagnosis of rejection [21]. Molecular biology analysis on myocardial tissue, on the other hand, may potentially progress the knowledge on the pathogenesis of rejection process and offer novel views to improve the diagnostic potential of EMBs. Afzali et al. [22••] found that in AMR cases, endothelial injury correlated with a higher gene set expression of endothelial, NK cells and inflammatory genes and was associated also with a worse prognosis. In a further development of the study, the same group was able to identify an AMR selective gene set that discriminated patients with AMR from those without and included NK transcripts, endothelial activation transcripts, macrophage transcripts and transcripts involved in the IFN-γ response. These four gene sets showed increased expression with increasing pAMR grades [23]. Of note, a pilot analysis of gene expression profiling from our group shows that information gained from gene expression profiling are more closely related to parameters of graft function than is the diagnosis based on histology-immunohistochemistry alone [24].

1.6.2 Antibodies: obscure bystanders or active players?

By definition, antibodies are the key players in the pathogenesis of AMR. Yet, their detection is felt to be neither sufficient nor necessary to diagnose AMR and to start a specific therapy in heart recipients [16]. The introduction of multiplex-bead array assays has not only revolutionized the field of circulating HLA-Ab by greatly increasing the sensitivity in Ab detection [25], but also raised concerns regarding the specificity and clinical significance of some findings. The use of flow-cytometry

allowed detecting increased prevalence of pre-transplant allosensitized patients, thus improving the immunological characterization of heart transplant candidates [26]. These circulating HLA-Abs do have a prognostic relevance for post-transplant outcomes [27,28▪▪], but in some circumstances, such as in patients with mechanical assist devices, they do not seem to be clinically important after transplantation [29], and the risk/benefit ratio of desensitizing or avoiding forbidden antigens is still unclear [30].

Several new reports show the prognostic relevance of de novo DSA in predicting the onset of clinical AMR [31], progression of cardiac allograft vasculopathy (CAV) [32,33] and overall survival [4,14]. However, it should be noted that not all DSAs are equal. Although HLA class I DSAs seem to be more important in assessing presensitization [25,34] and appear early after transplant [31], class II de novo DSA are more relevant in predicting pAMR onset and long-term mortality [4,31]. As outlined earlier, not all antibodies are complement fixing, thus when de novo DSA are found, clinicians should consider that the injury pathway elicited on the graft endothelial layer is variable and may have different time onset [3▪, 8▪]. Assay of the complement-fixing ability of DSA [35] may help to identify patients more likely to develop pathological evidence of complement-mediated injury in the EMB [36,37]. Current recommendations reasonably suggest active surveillance for de novo DSA after heart transplantation [25], although it is unclear how information gained from scheduled HLA-Ab assays should be handled. Given the controversial evidences regarding the risk/benefit balance of DSA management, to standardize our clinical practice we developed a multistep algorithm for the decision- making triggered by the detection of a de novo DSA, centered on differentiating the kind of antibody

involved, the clinical presentation and the time from transplant (Fig. 1).

Clinical and pathological evidence of AMR may be found even in absence of HLA-Ab, which may be at least partially explained either by the prozone effect or by presence of non-HLA-Ab. The prozone effect is caused by a high titer of circulating HLA-Ab, which may not revealed by the standard assay because the antigen beads become saturated and the HLA-Abs do not expose the Fc subunit to the secondary antibody. Prozone effect has to be ruled out either by diluting the serum or by adding Ethyl- enediaminetetraacetic acid (EDTA) [38]. Among the many types of non-HLA DSA, elevated levels of angiotensin-II type 1 receptor and endothelin type A receptor seem to be clinically significant in heart transplant setting, because they have been associated with early onset of CAV as well as with AMR and cellular mediated rejection. Non-HLA-Abs may be looked for when HLA-Abs are negative when AMR is highly suspected or may be used as biomarkers to stratify long-term risk of graft failure [39]. Nevertheless, current available evidence does not justify recommending active surveillance for non-HLA-Ab in heart transplant recipients.

1.6.3 Graft dysfunction: imaging, symptoms or laboratory?

AMR is a frequent cause of terminal cardiac graft failure [40], and it is reportedly associated with graft dysfunction and hemodynamic compromise in 10 – 47% of cases [1▪]. However, the definition of cardiac allograft dysfunction is a clinical unmet need in heart transplantation medicine. Criteria for defining hemodynamic compromise have been highly variable in literature and include a decrease of left ventricular (LV) ejection fraction, elevation of intracardiac pressures with a

concomitant decrease of cardiac output (CO) and the need of inotropic therapy. Traditionally, allograft dysfunction is defined by echocardiographic criteria for right or LV systolic or diastolic dysfunction, with the latter usually preceding loss in ejection fraction [41▪]. In particular, changes in systolic or diastolic function over time may be more clinically meaningful than absolute measurements to identify asymptomatic patients with possible manifestations of AMR [41▪,42]. Right heart catheterization may help to identify increased ventricular filling pressure (i.e. diastolic dysfunction) as well as decreased CO. It has to be noted, however, that diastolic dysfunction may be a manifestation of chronic myocardial fibrosis not related to acute AMR, but to chronic coronary allograft vasculopathy (which in turn may be associated with DSA). Of note, myocardial fibrosis as assessed by late gadolinium enhancement is an important predictor of mortality, independently rom CAV, and may represent a meaningful marker of graft injury/dysfunction [43▪▪]. In the effort to identify specific and non-invasive markers of ongoing rejection, recent studies pro- posed the detection of donor-derived cell-free DNA as a highly reliable marker for graft injury, being associated with biopsy-proven CMR and AMR [44,45]. Albeit preliminary, these findings sup- port the hypothesis that cell-free DNA could serve as a novel and reliable marker to trigger specific treatment for rejection and help to rule out nonspecific pathological abnormalities.

1.7 Therapy for antiboby-mediated rejection: clinical case-based evidence

When planning therapeutic strategies for AMR, it should be considered that appropriate induction; maintenance immunosuppression therapy and

immunological risk stratification are the key strategies to prevent de novo DSA onset. In addition to drug nonadherence, withdrawal of calcineurin inhibitors (CNIs) in patients at risk has been shown to increase AMR likelihood, whereas steroid withdrawal or utilization of mTOR inhibitors with low dose of CNI appears not to increase the risk [46▪, 47].

Regarding treatment of AMR, most of the evidence supporting any intervention is limited [2]. Most of therapies were originally designed to treat hematologic diseases, malignancies and auto- immune disorders, and thus the majority of treatments are off-label [1▪]. The guiding principles for the treatment of AMR are based on targeting one or multiple steps involved in its pathological process and comprise: removal of circulating DSAs, reduction of additional DSAs, suppression T-cell and B-cell responses and inhibition of complement-mediated endothelial injury (Fig. 2). In light of the limited evidence available, the principle of caution justifies the use of aggressive strategies proportionally to the severity of clinical presentation. In particular, Intravenous immunoglobulines (IVIG) and plasma-exchange/immunoabsorption appear to be effective in reducing DSAs burden with a good safety profile and are reported as first-line therapies [48,49]. Evidence based on small case series, expert consensus and established habits in clinical practice suggest that adding drugs targeting effector cells such as thymoglobulines, rituximab or bortezomib may not only improve efficacy in AMR treatment, but also increase the risk of infectious complication, thus restricting their use to AMR with severe clinical presentation or as rescue therapy after failure of first-line approaches [2,16,48 – 50]. Of note, in recent randomized trials, rituximab added to IVIG and plasma exchange failed to demonstrate ny additional benefit in kidney

transplant recipients with AMR [51] or in lung recipients with de novo DSAs [52]. In addition, the efficacy of these treatments is limited when AMR onsets later after transplantation [53].

Compounds inhibiting complement activity raised high expectations, but randomized studies provided controversial findings. Eculizumab, a C5 complement inhibitor, failed to meet the efficacy endpoint of AMR and graft loss prevention in a randomized study in kidney transplant recipients [54▪]. Conversely, the C1 esterase inhibitor Berinert appears to effectively revert DSA-mediated injury or prevent AMR in highly sensitized recipients in phase II studies [55,56].

1.8 Conclusions

Knowledge of AMR largely improved during the latest years, contributing to shade light on most of the circumstances in which the 'biopsy negative' rejection scenario was advocated. Molecular biology

is improving pathology-based diagnosis, antibody testing is elucidating the role and significance of circulating antibodies and novel drug repurposing is improving therapeutic approach. The key message of this article is that current evidence support active surveillance for AMR, both by disorder and by serological criteria, at least as means to stratify prognosis and customize follow-up procedures. However, several unmet needs are still influencing clinical practice regarding the appropriate therapeutic approach for the different diagnostic scenarios: despite widely utilized in clinical practice, very limited evidence support current treatments for AMR. Collaborative and multiinstitutional studies are now needed to design evidence-based approaches to prevent AMR onset and improve the outcome of patients with

AMR manifestations.

1.9 Figure legends

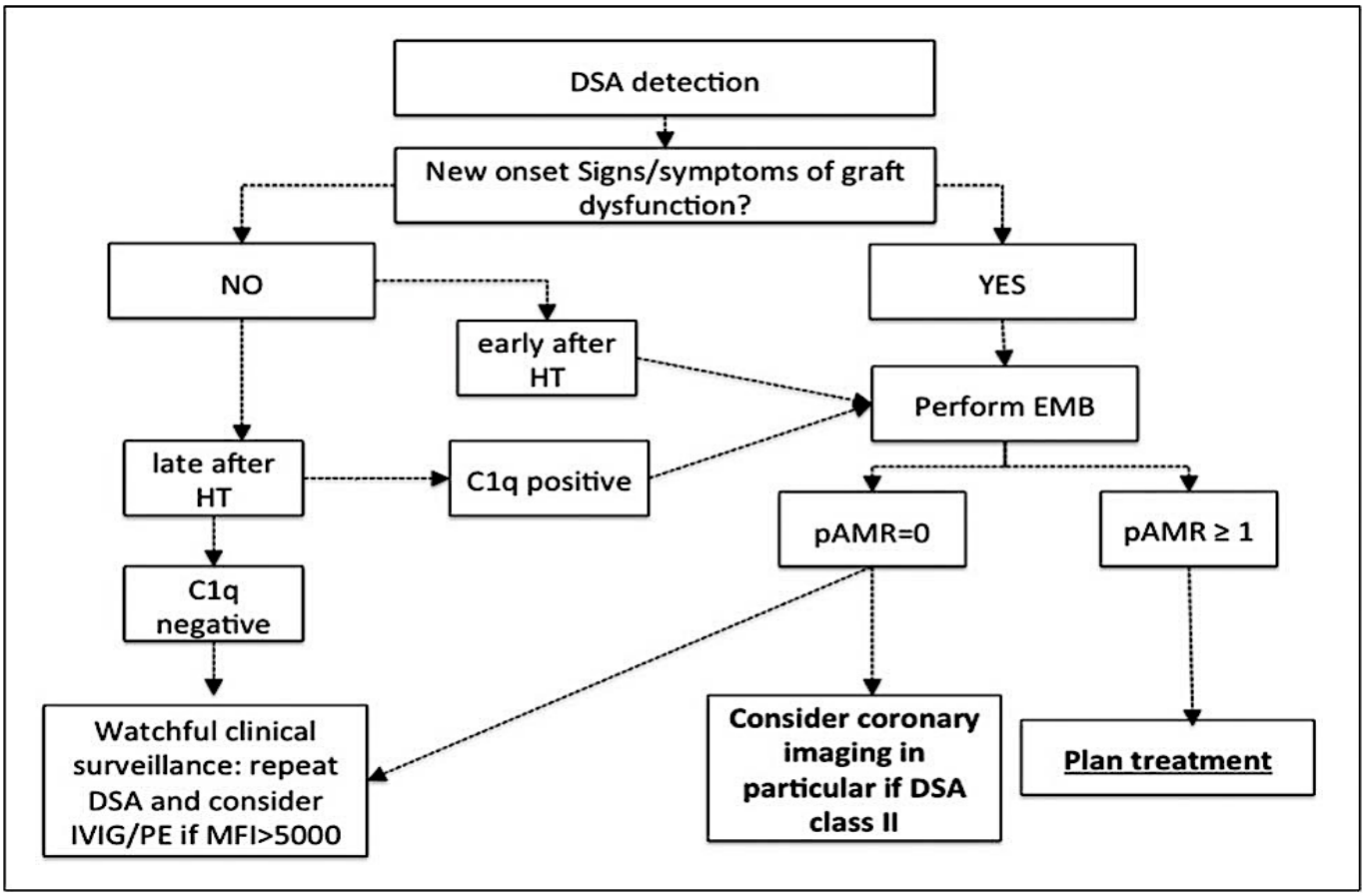


Figure 1. DSA detection represents a strong prognostic factor after heart transplant but it is not sufficient for the diagnosis of AMR and for planning a specific treatment. Indeed, there is no clear evidence of a benefit deriving from changes in patient management subsequent to information gained by DSA monitoring. Nevertheless, we believe that the possibility of DSA onset should not be ignored and thus we framed this decision-making algorithm, which takes into consideration the association of DSA with symptoms and pAMR, timing of DSA onset/detection and complement binding ability. When DSA onset is within the first years after transplantation, they are more often associated with acute rejection responding to treatment. Late onset DSA, in particular when not associated with complement binding activity, may lead to chronic injury, initially difficult to diagnose, that may express with CAV development. This may justify not performing EMB in asymptomatic patients with late onset DSA, but may support the need for a low-toxicity therapy such as intravenous immunoglobulines. The association of DSA with pAMR findings on the other hand, justifies specific treatment, in particular if associated with signs of graft dysfunction. HT: heart transplant; DSA Donor specific antibodies; EMB: endomyocardial biopsy; C1q: complement binding activity; MFI mean fluorescence intensity.

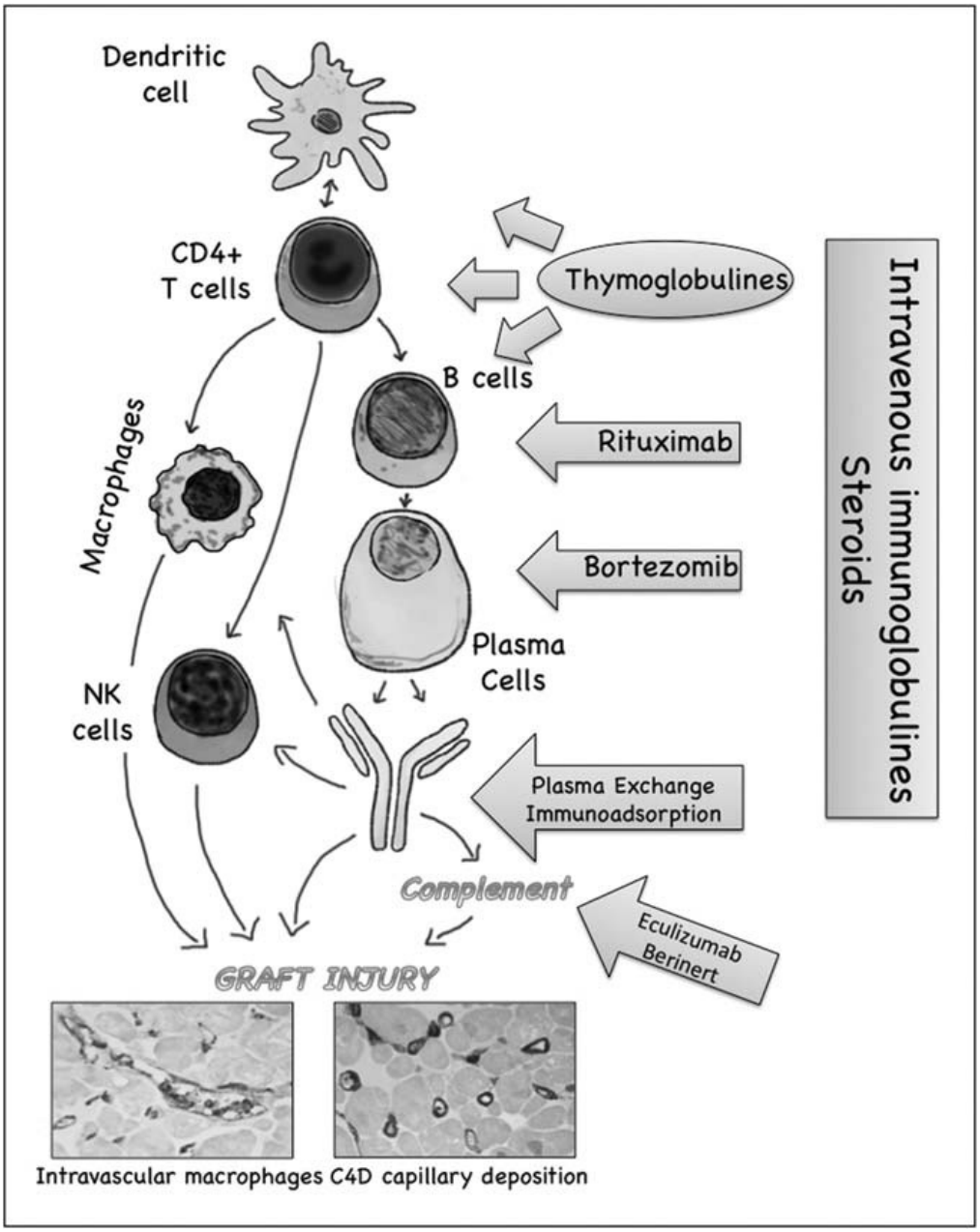


Figure 2. Antigen presenting cells, B-cells, CD4 T-Cells, plasma cells, immunoglobulines, NK cells, macrophages, and complement proteins compose a complex and interactive scenario in which graft injury may occur. NK cells and macrophages may elicit complement independent graft injury, trans-activated by DSA and by inflammatory chemochines produced by endothelial cells upon the binding of the DSA (see references 8 and 12). As indicated in the figure, each of these steps may represent a therapeutic target of one or multiple drug strategy. Intravenous immunoglobulines have multi-target action as anti-inflammatory, immune-modulators, complement inhibition and by suppressing exposure of HLA class I and II.

1.10 References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as ■ of special interest, and ■■ of outstanding interest

1. ■ Colvin M, Cook J, Chang P, et al. *Antibody-mediated rejection in cardiac & transplantation: emerging knowledge in diagnosis and management*. Circulation 2015; 131:1608 – 1639.
This position paper extensively provides the heart transplant professionals with a complete and detailed overview of the current status of the diagnosis and treatments of antibody-mediated rejection (AMR) in heart transplantation, including recommendations to facilitate evolving standardization of strategies for future studies.

2. Kobashigawa J, Crespo-Leiro MG, Ensminger SM, et al. *Report from a consensus conference on antibody-mediated rejection in heart transplantation*. J Heart Lung Transplant 2011; 30:252–269.

3. ■ Clerkin KJ, Restaino SW, Zorn E, et al. *The effect of timing and graft dysfunction on survival and cardiac allograft vasculopathy in antibody-mediated rejection*. J Heart Lung Transplant 2016; 35:1059 – 1066.
This article shows that late-onset AMR is more often associated with graft dysfunction and poor prognosis as compared with early-onset AMR. This concept is important for planning AMR surveillance and therapeutic strategies when AMR is diagnosed.

4. Clerkin KJ, Farr MA, Restaino SW, et al. *Donor-specific anti-HLA antibodies with antibody-mediated rejection and long-term outcomes following heart transplantation*. J Heart Lung Transplant 2016. [Epub ahead of print]

5. ■■ Fedrigo M, Leone O, Burke MM, et al. *Inflammatory cell burden and phenotype in endomyocardial biopsies with antibody-mediated rejection (AMR): a multi-center pilot study from the AECVP*. Am J Transplant 2015; 15:526 – 534.
In this multicenter case-controlled pilot study, the authors studied the inflammatory burden and phenotype in endomyocardial biopsy (EMB) with or without AMR. The key message of the study was that AMR is often associated with heterogeneous cellular infiltrate, the extension of which is associated with adverse prognosis.

6. ■ Kfoury AG, Miller DV, Snow GL, et al. *Mixed cellular and antibody-mediated rejection in heart transplantation: in-depth pathologic and clinical observations*. J Heart Lung Transplant 2016; 35:335–341.
In this single-center extensive review of banked biopsies, authors sought to clarify the interplay between AMR and cell-mediated rejection and the prognostic relevance of mixed rejection.

7. Berry GJ, Burke MM, Andersen C, et al*. The 2013 International Society for Heart and Lung Transplantation Working Formulation for the standardization of nomenclature in the pathologic diagnosis of antibody-mediated rejection in heart transplantation*. J Heart Lung Transplant 2013; 32:1147 – 1162.

8. ■ Thomas KA, Valenzuela NM, Reed EF. *The perfect storm: HLA antibodies, complement, FcgammaRs, and endothelium in transplant rejection*. Trends Mol Med 2015; 21:319 – 329.

Thorough review of the principal mechanisms of human leukocyte antigens (HLA) antibodies-mediated injury of the graft after organ transplant. Understanding in details every single effector function may provide new targets for more effective therapeutic strategies.

9. Haas M. *Emerging concepts and controversies in renal pathology: C4d- negative and arterial lesions as manifestations of antibody-mediated transplant rejection*. Surg Pathol Clin 2014; 7:457 – 467.

10. Sis B, Halloran PF. *Endothelial transcripts uncover a previously unknown phenotype: C4d-negative antibody-mediated rejection*. Curr Opin Organ Transplant 2010; 15:42 – 48.

11. Hirohashi T, Uehara S, Chase CM, et al. *Complement independent antibody- mediated endarteritis and transplant arteriopathy in mice*. Am J Transplant 2010; 10:510 – 517.

12. Jin YP, Valenzuela NM, Ziegler ME, et al. *Everolimus inhibits anti-HLA I antibody-mediated endothelial cell signaling, migration and proliferation more potently than sirolimus*. Am J Transplant 2014; 14:806 – 819.

13. Drachenberg CB, Papadimitriou JC. *Endothelial injury in renal antibody- mediated allograft rejection: a schematic view based on pathogenesis*. Transplantation 2013; 95:1073 – 1083.

14. Smith JD, Banner NR, Hamour IM, et al. *De novo donor HLA-specific antibodies after heart transplantation are an independent predictor of poor patient survival*. Am J Transplant 2011; 11:312 – 319.

15. ■ Tran A, Fixler D, Huang R, et al. *Donor-specific HLA alloantibodies: impact on cardiac allograft vasculopathy, rejection, and survival after pediatric heart transplantation*. J Heart Lung Transplant 2016; 35:87 – 91.
The objective of this study was to examine the development and consequences of de novo donor-specific antibodies (DSA) in pediatric heart transplant recipients. The article shows how de novo DSAs have a negative impact in cardiac vasculopathy, ejection, and graft survival in the first 12 months after transplant.

16. Chih S, Tinckam KJ, Ross HJ. *A survey of current practice for antibody-mediated rejection in heart transplantation*. Am J Transplant 2013; 13:1069–1074.

17. Zielinski T, Sobieszczanska-Malek M, Karczmarz M, et al. *Lack of impact of presence of positive C4d staining in capillaries in myocardial biopsies on long- term survival of heart transplant patients*. Transplant Proc 2016; 48:1767– 1769.

18. Tible M, Loupy A, Vernerey D, et al. *Pathologic classification of antibody- mediated rejection correlates with donor-specific antibodies and endothelial cell activation*. J Heart Lung Transplant 2013; 32:769 – 776.

19. Li F, Wei J, Valenzuela NM, et al. *Phosphorylated S6 kinase and S6 ribosomal protein are diagnostic markers of antibody-mediated rejection in heart allografts*. J Heart Lung Transplant 2015; 34:580 – 587.

20. Bruneval P, Angelini A, Miller D, et al. *The XIIIth Banff conference on allograft pathology: the Banff 2015 heart meeting report: improving antibody-mediated rejection diagnostics: strengths, unmet needs, and future directions*. Am J Transplant 2017; 17:42 – 53.

21. Angelini A, Andersen CB, Bartoloni G, et al. *A web-based pilot study of inter-pathologist reproducibility using the ISHLT 2004 working formulation for biopsy diagnosis of cardiac allograft rejection: the European experience*. J Heart Lung Transplant 2011; 30:1214 – 1220.

22. ■■ Afzali B, Chapman E, Racape M, et al. *Molecular assessment of microcirculation injury in formalin-fixed human cardiac allograft biopsies with antibody- mediated rejection*. Am J Transplant 2017; 17:496 – 505.
This study assessed molecular diagnostics in EMBs of patients with AMR, quantifying a set of endothelial, natural killer and other inflammatory genes. Subsequently, the gene set expression was compared between ISHLT grading and correlated with DSA, endothelial injury by electron microscopy and prognosis.

23. Loupy A, Duong Van Huyen JP, Hidalgo L, et al. *Gene expression profiling for the identification and classification of antibody-mediated heart rejection*. Circulation 2017; 135:917 – 935.

24. Borgese L, Gaudenzi A, Reeve J, et al. *Preliminary insights from the interheart study: prospective validation and clinical correlates of the molecular micro- scope diagnostic system in heart transplant biopsies*. Transpl Int 2016; 29:5. (Abstract).

25. Tait BD, Susal C, Gebel HM, et al. *Consensus guidelines on the testing and clinical management issues associated with HLA and non-HLA antibodies in transplantation*. Transplantation 2013; 95:19 – 47.

26. O'Connor MJ, Keeshan BC, Lin KY, et al. *Changes in the methodology of preheart transplant human leukocyte antibody assessment: an analysis of the United Network for Organ Sharing database*. Clin Transplant 2015; 29:842– 850.

27. Potena L, Bontadini A, Iannelli S, et al. *Occurrence of fatal and nonfatal adverse outcomes after heart transplantation in patients with pretransplant noncytotoxic HLA antibodies*. J Transplant 2013; 2013:519680.

28. ■■ Svobodova E, Gazdic T, Kubanek M, et al. *Novel insights into pretransplant allosensitization in heart transplant recipients in the contemporary era of immunosuppression and rejection surveillance*. Transplant Int 2016; 29:63–72.
The authors retrospectively analyzed retransplant era, panel-reactive antibodies, retransplant cross-match and clinical data to study the clinical consequences and the potential utility of these new assays in predicting AMR episodes.

29. Arnaoutakis GJ, George TJ, Kilic A, et al. *Effect of sensitization in US heart transplant recipients bridged with a ventricular assist device: update in a modern cohort.* J Thorac Cardiovasc Surg 2011; 142:1236 – 1245; 1245.e1.

30. Feingold B, Webber SA, Bryce CL, et al. *Comparison of listing strategies for allosensitized heart transplant candidates requiring transplant at high urgency: a decision model analysis*. Am J Transplant 2015; 15:427– 435.

31. Frank R, Molina MR, Wald JW, et al. *Correlation of circulating donor-specific anti-HLA antibodies and presence of C4d in endomyocardial biopsy with heart allograft outcomes: a single-center, retrospective study*. J Heart Lung Transplant 2013; 32:410 – 417.

32. Frank R, Molina MR, Goldberg LR, et al. *Circulating donor-specific antihuman leukocyte antigen antibodies and complement C4d deposition are associated with the development of cardiac allograft vasculopathy*. Am J Clin Pathol 2014; 142:809 – 815.

33. Coutance G, Ouldamar S, Rouvier P, et al. *Late antibody-mediated rejection after heart transplantation: mortality, graft function, and fulminant cardiac allograft vasculopathy*. J Heart Lung Transplant 2015; 34:1050 – 1057.

34. Raess M, Frohlich G, Roos M, et al. *Donor-specific anti-HLA antibodies detected by Luminex: predictive for short-term but not long-term survival after heart transplantation*. Transpl Int 2013; 26:1097 – 1107.

35. Zeevi A, Lunz J, Feingold B, et al. *Persistent strong anti-HLA antibody at high titer is complement binding and associated with increased risk of antibody- mediated rejection in heart transplant recipients*. J Heart Lung Transplant
2013; 32:98 – 105.

36. Frank R, Lal P, Kearns J, et al. *Correlation of circulating complement-fixing donor-specific antibodies identified by the C1q assay and presence of C4d in endomyocardial biopsy specimens*. Am J Clin Pathol 2016; 145:62 – 68.

37. Viglietti D, Loupy A, Vernerey D, et al. *Value of donor-specific anti-HLA antibody monitoring and characterization for risk stratification of kidney allograft loss*. J Am Soc Nephrol 2016; 28:702 – 715.

38. ■ Anani WQ, Zeevi A, Lunz JG. *EDTA treatment of serum unmasks complement-mediated prozone inhibition in human leukocyte antigen antibody testing*. Am J Clin Pathol 2016; 146:346 – 352.
This study shows how serum pre-treatment with EDTA can mitigate prozone effect and improve accuracy in HLAab detection.

39. Hiemann NE, Meyer R, Wellnhofer E, et al. *Non-HLA antibodies targeting vascular receptors enhance alloimmune response and microvasculopathy after heart transplantation*. Transplantation 2012; 94:919 – 924.

40. ■■ Loupy A, Toquet C, Rouvier P, et al. *Late failing heart allografts: pathology of cardiac allograft vasculopathy and association with antibody-mediated rejection.* Am J Transplantat 2016; 16:111 – 120.
Amazing series of pathology assessment of failing hearts explanted after retransplantation. In this study, the authors explore the link between evidence of AMR and subsequent failing grafts and CAV development.

41. ■ Badano LP, Miglioranza MH, Edvardsen T, et al. *European Association of Cardiovascular Imaging/Cardiovascular Imaging Department of the Brazilian Society of Cardiology recommendations for the use of cardiac imaging to assess and follow patients after heart transplantation*. Eur Heart J Cardiovasc Imaging 2015; 16:919 – 948.
Comprehensive recommendations about ultrasound diagnostics in heart trans- plantation.

42. Frea S, Iacovino C, Botta M, et al. *Does asymptomatic recurrent diffuse capillary C4d complement deposition impair cardiac allograft function?* Clin Transplant 2016; 30:1314 – 1323.

43. ■■ Pedrotti P, Vittori C, Facchetti R, et al. *Prognostic impact of late gadolinium enhancement in the risk stratification of heart transplant patients.* Eur Heart J Cardiovasc Imaging 2017; 18:130 – 137; In press.
In this article, authors prospectively assess the extension of myocardial fibrosis by cardiac MRI describing the association among fibrosis, CAV and prognosis. Fibrosis assessment may become a surrogate indicator for graft function.

44. De Vlaminck I, Valantine HA, Snyder TM, et al. *Circulating cell-free DNA enables noninvasive diagnosis of heart transplant rejection*. Sci Transl Med 2014; 6:241ra77.

45. Hidestrand M, Tomita-Mitchell A, Hidestrand PM, et al. *Highly sensitive noninvasive cardiac transplant rejection monitoring using targeted quantification of donor-specific cell-free deoxyribonucleic acid*. J Am Coll Cardiol 2014; 63:1224 – 1226.

46. ■ O'Leary JG, Samaniego M, Barrio MC, et al. *The influence of immunosuppressive agents on the risk of de novo donor-specific HLA antibody production in solid organ transplant recipients.* Transplantation 2016; 100:39 – 53.
Comprehensive review about the effect of immunosuppression on the onset of de novo DSAs. Induction and maintenance agents are assessed with important implications for clinical practice.

47. Dobbels F, De Geest S, van Cleemput J, et al. *Effect of late medication noncompliance on outcome after heart transplantation: a 5-year follow-up*. J Heart Lung Transplant 2004; 23:1245 – 1251.

48. Thrush PT, Pahl E, Naftel DC, et al. *A multi-institutional evaluation of antibody-mediated rejection utilizing the pediatric heart transplant study database: incidence, therapies and outcomes*. J Heart Lung Transplant 2016; 35:1497– 1504.

49. Gazdic T, Svobodova E, Kubanek M, et al. *Bortezomib-containing regimen for primary treatment of early antibody-mediated cardiac allograft rejection: a case report*. Prog Transplant 2015; 25:147 – 152.

50. Baum C, Reichenspurner H, Deuse T. *Bortezomib rescue therapy in a patient with recurrent antibody-mediated rejection after lung transplantation*. J Heart Lung Transplant 2013; 32:1270 – 1271.

51. Sautenet B, Blancho G, Buchler M, et al. *One-year results of the effects of rituximab on acute antibody-mediated rejection in renal transplantation: RITUX ERAH, a multicenter double-blind randomized placebo-controlled trial*. Trans- plantation 2016; 100:391 – 399.

52. Ius F, Sommer W, Tudorache I, et al. *Preemptive treatment with therapeutic plasma exchange and rituximab for early donor-specific antibodies after lung transplantation*. J Heart Lung Transplant 2015; 34:50 – 58.

53. Hodges AM, Lyster H, McDermott A, et al. *Late antibody-mediated rejection after heart transplantation following the development of de novo donor-specific human leukocyte antigen antibody.* Transplantation 2012; 93:650 – 656.

54. ■ Alexion Provides Update on Phase 2 *Clinical Trial with Eculizumab in Antibody & Mediated Rejection (AMR) in Living-Donor Kidney Transplant Recipients.* Press Release, 2015. [Accessed 20 January 2017], at http://news.alexion-pharma.com/press-release/company-news/alexion-provides-update-phase-2-clinical-trial-eculizumab-antibody-mediat.
This is the press release from the study sponsor acknowledging that the study did not meet the primary endpoint at a pre-specified interim analysis. No further data have been published in peer-review journals since then.

55. Viglietti D, Gosset C, Loupy A, et al. *C1 inhibitor in acute antibody-mediated rejection nonresponsive to conventional therapy in kidney transplant recipients: a pilot study*. Am J Transplant 2016; 16:1596 – 1603.

56. Vo AA, Zeevi A, Choi J, et al. *A phase I/II placebo-controlled trial of C1- inhibitor for prevention of antibody-mediated rejection in HLA sensitized patients*. Transplantation 2015; 99:299 – 308.

PART II
DEFINITION OF CLINICAL AND IMMUNOLOGICAL PHENOTYPES OF ANTIBODY MEDIATED REJECTION IN HEART TRANSPLANT RECIPIENTS: PROGNOSTIC AND THERAPEUTIC IMPLICATIONS

"CLIN-Heart project" Interventional non-pharmacological prospective study

2.1 Introduction

Heart failure is a chronic medical condition that affects more than 6.5 million people in Europe and is one of the most frequent causes of hospitalization in adult population. In a select group of end-stage patients, heart transplant is considered the treatment that provides the best benefit in terms of survival. During the least 4 decades short- and long-term mortality was significantly reduced with a median survival that actually exceeds 11 years. In particular this improvement is correlated with an increased survival during the first year after surgery, together with a modest change in the annual risk from that point on, and it is mainly related with both improvements in perioperative management and new immunosuppressive regimens which have decreased the prevalence of acute rejection in the early period.[1,2]

Despite these impressive results, data from the ISHLT (International Society for Heart and Lung Transplantation) Registry reported that the graft dysfunction (GD), which includes both coronary graft vasculopathy (CAV) and episodes of rejection (cell-mediated (CMR) antibody-mediated (AMR) and mixed), causes approximately 40% of deaths after the first 5 years after surgery [3]. (Figure 1)

Late post-transplantation acute graft dysfunction is less frequent than chronic presentation which is usually characterized by a slow and progressive onset of diastolic abnormalities accompanied by slight decrease in ejection fraction and worsening heart failure symptoms.

In literature the graft damage mediated by AMR is one of the main mechanisms leading to graft dysfunction in the post-transplant but, while for the CMR diagnosis is established on the basis of histological staging on endomyocardial biopsy (EMBs) and it is typically treated with the common immunosuppressive drugs, the diagnosis of AMR is much more

complex. According to ISHLT guidelines, the latter is based on the presence of the following criteria: signs / clinical symptoms of cardiac dysfunction, evidence of anti-HLA antibodies against the donor (donor specific antibodies, DSAs), and immunohistochemical and pathological findings on biopsy [3].

It has been show that AMR is followed by increased graft loss, cardiac allograft vasculopathy (CAV), and death.[4–6] A consensus report from an ISHLT task force references previous works where, while early AMR was associated to graft dysfunction in 68% of patients, in late AMR, only 13% was hemodynamically significant. Moreover, a pair of recent single-centre studies demonstrated that late AMR (>1 year after transplant) featured a 1-year mortality of 50% to 53%.[7,8]

This complexity is also underlined by the fact that, in some cases, the presence of typical pathological findings of CMR and AMR in the same sample biopsy has been reported. This suggests the possible correlation between the humoral and cellular branch of the immune system.

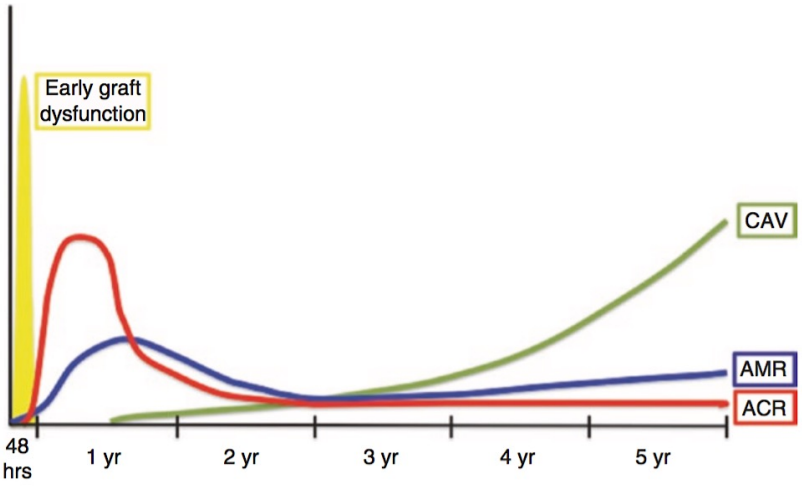
Finally another common and typical cause of GD is CAV, which can be considered as a form of chronic vascular rejection. Typically, CAV development is triggered by metabolic risk factors – as well as native coronary atherosclerosis – and by immune-mediated injury, including antibody-mediated endothelial injury (indeed, CAV may be a chronic phenotype of AMR). CAV is characterized by diffuse intimal hyperplasia arising from peripheral vessels and spreading to proximal tracts of arteries and veins, thus determining diffuse luminal narrowing, often without focal luminal stenoses.[9]

In this study we aimed to characterize clinical phenotypes of patients with GD, either acute or chronic expression, comparing their outcomes with stable patients. In addition, we explored the risk factors for outcome in GD patients focusing our attention on the allo-

immune response of the graft.

Figure 1 from The Pathology of Cardiac Transplantation (page 156)

Fig. 10.1 The graph shows the trend of the main causes of acute and chronic graft dysfunction according with time post-transplantation. Primary and secondary early graft dysfunction occurs within the 24–48 h after surgery. (ACR: acute cellular rejection; AMR: antibody-mediated rejection; CAV: cardiac allograft vasculopathy)



2.2 Aim of the study

Despite its clinical relevance, there is a lack of consensus regarding the definition of graft dysfunction (GD) in heart transplant (HT). This project is based on a prospective non-interventional clinical study in which we aimed to characterize clinical phenotypes of patients with GD, either acute or chronic, comparing their outcomes with stable patients.

Primary objective of the study:

- Identify the clinical and subclinical phenotypes of patients that define the presence of graft dysfunction and correlate these characteristics with subsequent cardiovascular and non-cardiovascular events and mortality.

Secondary objectives of the study:

- Understanding the most significant risk factor that predict mortality and cardiovascular

events in the subgroup of heart transplant patients with signs or symptoms of graft dysfunction, focusing our attention on the allo-imune response and the presence of donor specific antibodies (DSAs) or humoral rejection.

2.3 Study design

The project was set up as single centre, non-interventional controlled study, in patients who underwent heart transplant. All consecutive patients enrolled have been divided in three different groups.

- GROUP A: Patients who underwent heart transplantation within less than five years, who received endomyocardial biopsies (EBMs) according to the clinical practice followed at our Centre (the current clinical monitoring protocol involves performing EBMs for monitoring of cellular rejection until the fifth year post-transplant)

- GROUP B: Patients with clinical and instrumental signs of graft dysfunction, regardless of the distance of the transplant. GD was defined as at least one of the following: left ventricular ejection fraction (LVEF)<55%, symptomatic cardiac allograft vasculopathy (CAV), or new onset symptoms of heart failure. These patients may be subjected to an EBM, only if deemed clinically necessary, in order to exclude the presence of rejection.
- GROUP C: Stable patients with a period free of rejection events exceeding 5 years and / or which do not require high therapeutic regimens with immunosuppressive drugs in post-transplant maintenance. It is expected that this group of patients have no clinical need for control EBMs.

2.4 Methods

2.4.1 Study population

Inclusion criteria:

- Heart transplanted patients included in the following categories:

A) Patients who had received a control EBM according to the protocol applied in our Centre

Or

B) Patients who presented clinical signs or symptoms of graft dysfunction

Or

C) Patients immunologically "stable" for over 5 years, with normal function of the graft

- Signature of informed consent
- Age ≥ 18 years

Exclusion criteria:

- Multiorgan transplant

2.4.2 Clinical assessments

V1- Baseline evaluation

In Group A, the “per-protocol” EBM represented our index evaluation. For Group B and Group C patients, V1 coincided with one of the outpatient clinical controls, when the patient will sign informed consent to participate in the protocol.

Biopsies and clinical visits in accordance with the guidelines approved at our transplant Centre respect the following protocol:

- 1 month 1 biopsy / week 2 visits / week
- 2-3 months 1 biopsy / 15 days 1 view / week
- 3-6 months biopsy 1 / month 1 view / 15 days
- 6-12 months biopsy 1/2 months 1 visit / month
- 1-2 years biopsy 1/4 months 1 view / 2 months
- 2-5 years 1 biopsy / 1 view 6 months / 3 months
- > 5 years 1 view / 3-4 months

During the baseline evaluation every patients underwent a complete clinical and instrumental assessment which included: the collection of demographic data and the previous medical history, physical examination, resting ECG, transthoracic echocardiography, standard laboratory tests and right heart catheterization (only in patients who underwent EBMs).

Finally the evaluation of the humoral response against the graft was performed by using specific laboratory tests that dose the titre of anti-HLA antibodies (DSAs or non DSAs) in serum samples.

Follow-up visits:

Clinical follow-up controls were organized at intervals of 6 months (+/- 2 weeks). During each visit we collected: physical examination data, resting ECG, echocardiography, blood tests, antibodies HLA and non-HLA dosages. We also performed additional laboratory test on blood samples at 1 month (M1), 3 months (M3) and 9 months (M9) (See table 1). Biopsies will be repeated for patients in group A on the basis of our protocol (see above).

Table 1

follow up (every 6 months (+/- 2weeks)	**V1** **Baseline**	**M1**	**M3**	**V2** **M6**	**M9**	**V3** **M12**	**V4** **M18**	**V5** **M24**
Informed Consent	X							
Right Heart Catheterization/ EBMs	X			X		X	X	X
Clinical Evaluation	X			X		X	X	X
ECG	X			X		X	X	X
Ultrasounds	X			X		X	X	X
Laboratory tests	X	X	X	X	X	X	X	X
HLA-antibodies	X			X		X	X	X

* Group A patients received a EBMS on the basis of our standard clinical assessment, while group B and C patients, EBMs have been performed only for clinical indications to rule out the presence of rejection.

2.4.3 Standard Procedures

- Laboratory tests: we performed normal routine blood tests (i.e. complete blood count, renal and liver function, lipid profile, sodium, potassium, glucose etc.)
- Evaluation of CAV: The presence of CAV is routinely detected by intracoronary ultrasound (IVUS) and angiography at 1 month, 1 year, and 5 years after heart transplant. Thereafter the evaluation is repeated at 5-year intervals when there are not controindications.
- Evaluation of EBMs specimens and histological grading: Serial sections of endomyocardial samples, are usually stained with hematoxylin-eosin to assess the

degree CMR according to the Working Formulation of ISHLT 2005. According to the ISHLT 2011 recommendations, AMR pathological grading is established basing to histopathology findings of microvascular inflammation (intravascular mononuclear cells and endothelial cells) and immunohistochemical findings (capillary C4d deposits in more than 50% of vessels).

AMR pathological grading:

pAMR 0 = absence of histopathological and immunohistochemical findings.

pAMR 1 = positive histopathological findings or positive immunohistochemical

pAMR 2 = histopathological and immunohistochemical both positive

pAMR 3 = evidence of histopathological findings of severe damage (e.i. Interstitial haemorrhage, extensive fragmentation, mixed inflammatory infiltrate)

2.4.4 Specific study procedures

- HLA antibodies detection in venous blood samples (every 6 months) IgG anti-HLA reactivity in the sera was tested with a bead-based screening assay (LABScreen mixed kit -One Lambda) which simultaneously detects class I and class II antibodies with microbeads coated with purified class I and class II HLA antigens. In case of positivity the serum have been analysed on the Luminex platform with the same LABScreen Single Antigen test, which employs beads labelled with a specific antigen, both class I and class II. The technique and software used are the same. The results will be interpreted using the MFI values (Normalized Mean Fluorescence Intensity). The cut-off in positive test is represented by a value higher than 1000 MFI. These analyses allow detecting the presence of anti-HLA immunoglobulins, regardless of their ability to fix complement.

2.4.5 Evaluation of results

Outcomes Mesures

- Overall mortality in group A, B and C.

- Hospitalizations for cardiovascular events (CV hospitalizations)

- Hospitalization for non-cardiovascular events (non-CV hospitalizations)

Combined Endpoints

- Death or/and CV hospitalizations

2.4.6 Statistical Analysis

Statistical analyses were performed using JMP 7.0 (SAS Institute, Cary, NC, USA). Baseline characteristics in the three groups were compared using, as appropriate, Student's t-test, ANOVA or Chi-square test. Outcome data and survival were calculated using Kaplan-Meier method with the Long-rank test.

Risk factors, associated with clinical events, have been analysed by Cox analysis and statistical regression models.

All statistical analyses were conducted at significance level of 0.05. All continuous variables were reported by mean and standard deviation, or by median and interquartile range in case of skewed distribution. Nominal variables were expressed as a number an percentages.

2.5 Results

2.5.1 Baseline comparison between group A, B and C

We enrolled a total of 134 consecutive heart transplant (HT) patients followed in our department between November 24th 2014 and April 28th 2016. 62 patients have been included in group A (46%), 32 (24%) in group B and 40 (30%) patients in group C.

All clinical and laboratories data were collected during the scheduled visits and our date of last follow-up was December 1st 2016.

The median age of our total population was 60 years [50 - 68], 31% were female and the most frequent causes for heart transplant were ischemic disease and dilated cardiomyopathy which respectively occurred in 26% and 37.4% of cases.

In table 2 we show some clinical variables to highlight the principal baseline differences between the three groups of enrolment. Patients in group B and in group C had a longer time distance from heart transplant and were older respect group A (respectively 146 [88 - 206] months and 202 [150 - 234] months vs. 11.98 [0.43-42.5]; p<0.01). The median age was 65.6 [58.6- 72.5] in group B, 67.4 [56.8 – 74.5] in group C vs. 55.1 [44.6 -60.9] in group A (p<0.01). Moreover in Group B, defined as the group of patients with signs or symptoms of graft dysfunction, there was an significantly higher percentage of patients with a class NYHA > II (71.9%, p<0.01) and showed a significantly lower LVEF (59.4% in group B had a LVEF<55%; p<0.01) respect to the other two groups.

Moreover, in group B we found a significantly higher percentage of patients with positive Donor Specific Antibodies (DSAs) (p=0.05) at the time of enrolment but no differences were showed for non-DSAs HLA-antibodies. A higher prevalence of CAV (59.4%) were detected in group B, even if for 18 patients the angiographic evaluation was not available

(p<0.01).

The number of cases of non-CV-hospitalization and of CV hospitalizations, during the first year before the time of enrolment (V1), in group B was higher respect group A and C (respectively p=0.03, p<0.001).

Finally ECG performed at V1 showed in group B had a longer duration of QRS and QTc, and a higher number of cases of patients with low-voltage ECG.

Table 2 Baseline characteristics in group a, B and C.

All (N= 134)	**GROUP A** (N= 62)	**GROUP B** (N= 32)	**GROUP C** (N= 40)	P
Aetiology:				
CAD	19 (30.7%)	10 (31.3%)	6 (15%)	0.2
DCM	18 (29%)	13 (40.6%)	19 (47.5%)	
Others	25 (40.3%)	9 (28.13%)	15 (37.5%)	
Time after HTx (months)	11.98 [0.43-42.5]	146 [88 - 206]	202 [150 - 234]	**<0.001 ***
Female sex	23 (37.1%)	8 (25%)	11 (27.5%)	0.4
Age (years)	55.1 [44.6 -60.9]	65.6 [58.6- 72.5]	67.4 [56.8 – 74.5]	**< 0.001 ***
NYHA >II	9 (14.5%)	23 (71.9%)	11 (27.5%)	**< 0.001**
LVEF<55%	0	19 (59.4 %)	0	**< 0.001**
Positive DSAs	4 (6.5%)	7 (21.9%)	3 (7.5%)	**0.05**
Positive non- DSAs	9 (14.5%)	5 (15.6%)	2 (5%)	0.28
CAV	11 (17.7%)	19 (59.4%)	8 (20%)	**<0.01**

Non-CV Events pre-V1	2 (3.2%)	5 (15.6%)	1 (2.5%)	**0.03**
CV Events pre V1	11 (17.7%)	16 (50%)	3 (7.5%)	**< 0.001**
QRS (msec)	103 ± 21	131 ± 34	106 ±19	**<0.001**
QTc (msec)	448 ± 19.7	474 ± 30	445 ± 25	**<0.001**
Low-voltage ECG (mVolt)	6 (12%)	9 (36%)	2 (6%)	**<0.001**

CAD: Coronary Artery Disease, DCM: Dilated Cardiomyopathy, Others: Hypertrophic Cardiomyopathy, Restrictive cardiomyopathy, Right ventricular arritmogenic cardiomyopathy, Post-myocarditis cardiomyopathy, Post-chemotherapy cardiomyopathy, LVEF: Left Ventricular Ejection Fraction, DSAs: Donor Specific Antibodies, non-DSAs: non Donor Specific HLA antibodies, CAV: coronary graft vasculopathy, Events pre V1: non CV hospitalizations pre V1, CV Events: CV hospitalizations pre V1

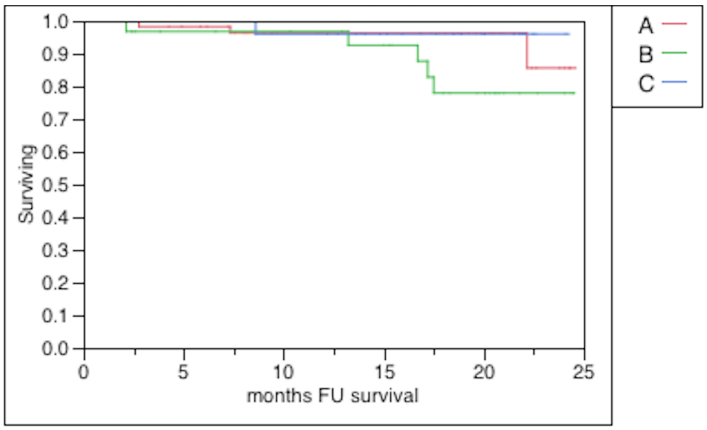
2.5.2 Outcomes

Patients with graft dysfunction (group B) during the 2 years of follow up had a worse prognosis. As it is shown in Figure 1, the mortality rate after 24 months was 23% while in Group A and C were 15% and 13% respectively (p=0.05).

Moreover Group B patients had a higher incidence of CV hospitalizations and non-CV hospitalizations compared with the other two groups (Figure 3 and 5).

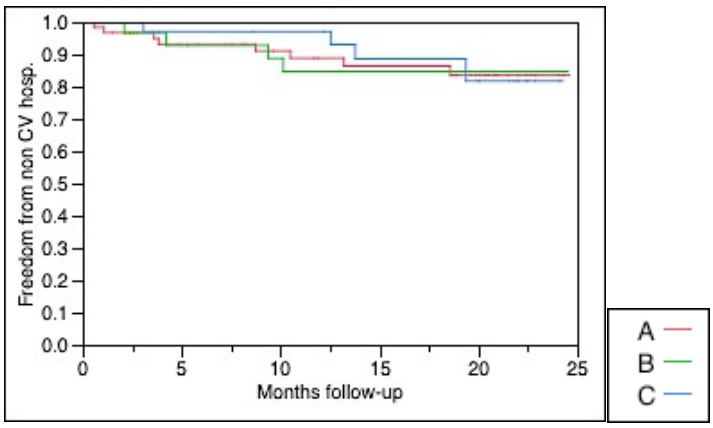
The most common cause of CV hospitalization was graft rejection in the 30.7% of the cases (2 cases were diagnosed as AMR in group B and 2 cases were episodes of ACR one in group A and one in group B).(Figure 4.a, 4.b,4.c)

Figure 1 :Overall survival in Group A, B and C



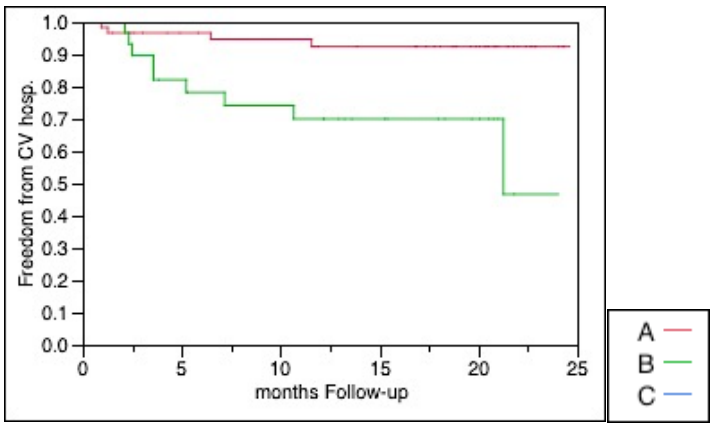
p= 0.05

Figure 2: Freedom from non-CV hospitaliaztion



p= 0.83

Figure 3: Freedom from CV hospitalizations



p <0.001

Figure 4.a: Causes of CV hospitalizations

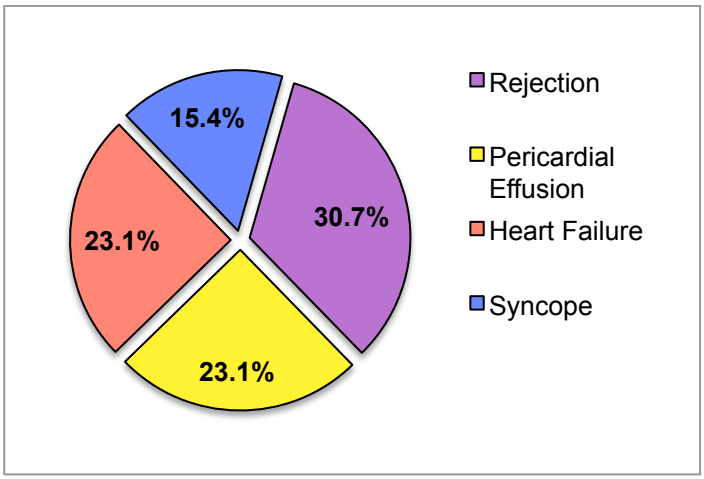


Figure 4.b: Causes of CV hospitalizations in group B

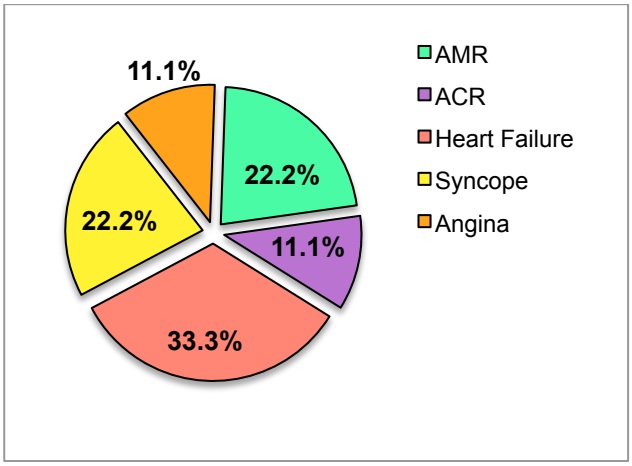
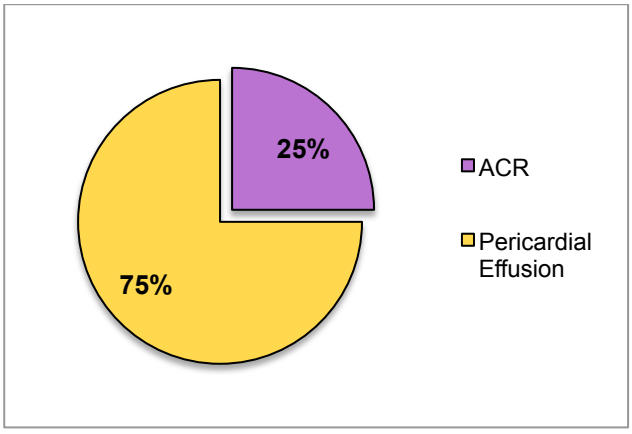
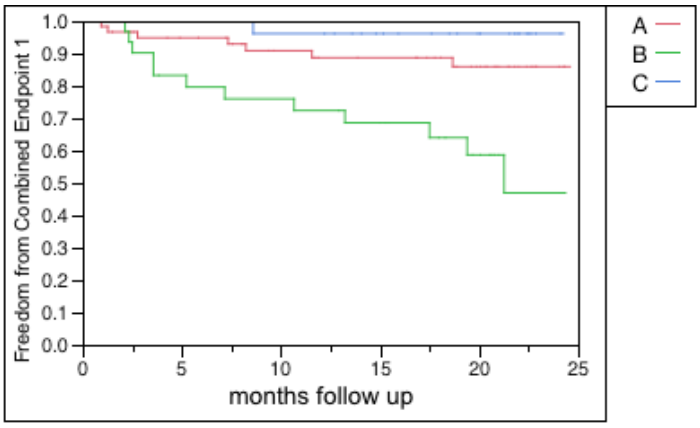


Figure 4.c: Causes of CV hospitalizations in group A



Freedom from Combined Endpoint 1 (death or/and CV hospitalizations)



p< 0.001

2.5.3 Sub group Analysis: Risk factor for death or/and CV hospitalizatios (Combined Endpoint)

Clinical presentation of GD in group B was highly heterogeneous: 19% of patients had an acute presentation (3 for acute rejection, and 3 for acute coronary syndromes); 66% had chronic presentation: 17(53%) associated with CAV, and 4(13%) as chronic dysfunction after antibody-mediated rejection. 5 patients had acute symptoms but no-graft related cause emerged.

Low EF, time from HT, and chronic clinical presentation (p=0.04, p=0.05 and p<0.01) were risk factors for the combined endpoint, in particular no patients with an acute GD died or was hospitalized for CV in the follow up. (Table 3)

Table 3 Risk factors for death and or CV hospitalizations

Cox regression model

Group B (n=32)	**RR**	**Lower 95%- upper 95%**	**p**
Years from HT	1.08	1.00-1.18	**0.05**
LVEF<55%	4.19	1.09-27.45	**0.04**
NYHA>II	2.70	0.7-17.66	0.15
CAV	0.38	0.10 – 1.22	0.1
Chronic GD	n.a.*	n.a.	**<0.001**
DSAs	1.34	0.35-4.28	0.64

* None of the patients with acute presentation of GD had any endpoint event, vs. 40% of those with chronic presentation of GD. Lack of events in the acute GD group does not allow to calculate relative risk.

2.6 Discussion

This prospective study tried to investigate the clinical impact of GD in patients who underwent heart transplant in terms of survival and cardiovascular outcomes.

The prevalence of GD in our population was 24%. The 18.8% of patients with GD had a GD within 5 years after transplant; in the half of these cases they had an acute rejection. The rest of patients had a GD more than 5 years after transplant, and the major cause of GD was related to CAV (65.4%), while rejections in the late post-operative period represented the 19% and were more frequently due to a chronic AMR.

Moreover GD had two different clinical presentations. 21.8% patients had an acute expression related to acute rejections or acute coronary syndrome (5 patients had acute symptoms but no-graft related cause emerged).

After acute GD, patients recovered completely or with minimal clinical consequences, no combined endpoint was recorded.

The majority had a chronic presentation, with a persistent reduction LVEF, and the most frequent cause was CAV (53%) while the 13% had a GD related to chronic AMR (3 patients) and in only one case had a mixed rejection (1 patient). Ten of these patients showed at least one combined enpoint (death or /and CV hospitalization).

It should be noted that it was not possible to analyse separately the causes of death because in some of the cases the cause of death was unclear.

Only 5 patients of our total population had a clear episode of antibody-mediated rejection (AMR) and 4 of them had a chronic dysfunction and were not transplanted recently (<5 years). All 4 of them belonged to group B: only two out of these 4 patients had the combined endpoint 2.

Other 3 patients in group A (< 5 years from heart transplant) who underwent EBMs showed a positive pAMR and positive DSAs but without hemodynamic compromise. All of them didn't present our Combined endpoint 2.

These data seems to confirm the findings of Kevin J. et al in retrospective study published in 2016. In their article, they observe that late AMR is frequently associated with graft dysfunction, and when graft dysfunction is present with late AMR, there is a significantly increased mortality and rapid development of CAV despite aggressive treatment compared with all other groups of AMR [1].

2.7 Conclusions

GD after HT is correlated with a worse outcome and survival, in particular when the GD had a chronic clinical presentation. AMR had a low incidence in our population, with a

selective clinical impact. DSAs did not seem to be a good predictor of death or cardiovascular events in the group of patients with GD.

2.8 References

1.Kevin J. Clerkin, et al. *The effect of timing and graft dysfunction on survival and cardiac allograft vasculopathy in antibody-mediated rejection* J of Heart and Lung Transplant, 2016; 35: 1055-1066

2 .Lund LH, Edwards LB, Kucheryavaya AY, et al. *The Registry of the International Society for Heart and Lung Transplantation: Thirty- second Official Adult Heart Transplantation Report-2015; Focus Theme: Early Graft Failure*. J Heart Lung Transplant 2015;34:1244-54.

3. Kobashigawa J, Crespo-Leiro MG, Ensminger SM, et al*. Report from a consensus conference on antibody-mediated rejection in heart transplantation.* J Heart Lung Transplant 2011;30:252-69.

4.Michaels PJ, Espejo ML, Kobashigawa J, et al. *Humoral rejection in cardiac transplantation: risk factors, hemodynamic consequences and relationship to transplant coronary artery disease.* J Heart Lung Transplant 2003;22:58-69.

5. Reed EF, Demetris AJ, Hammond E, et al. *Acute antibody-mediated rejection of cardiac transplants.* J Heart Lung Transplant 2006;25:153-9.

6.Kfoury AG, Renlund DG, Snow GL, et al*. A clinical correlation study of severity of antibody-mediated rejection and cardiovascular mortality in heart transplantation.* The Journal of heart and lung transplantation: the official publication of the International Society for Heart Trans- plantation 2009;28:51-7.

7.Coutance G, Ouldamar S, Rouvier P, et al*. Late antibody-mediated rejection after heart transplantation: Mortality, graft function, and fulminant cardiac allograft vasculopathy*. The Journal of Heart and Lung Transplantation 2015;34:1050-7.

8.Hodges AM, Lyster H, McDermott A, et al. *Late antibody-mediated rejection after heart transplantation following the development of de novo donor-specific human leukocyte antigen antibody*. Trans- plantation 2012;93:650-6.

9. Mehra MR, Crespo-Leiro MG, Dipchand A, et al*. International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for cardiac allograft vasculopathy—2010*. The Journal of heart and lung transplantation: the official publication of the International Society for Heart Transplantation 2010;29:717-27.

PART III

QUANTIFERON MONITOR (QFM) PROJECT

3.1 Introduction

The achievement of an optimal balance between adequate protection from rejection and the adverse consequences of over-immunosuppression (i.e. infections and metabolic toxicities of drugs) is a pivotal, yet fully unmet need in clinical practice after heart transplant. Note that according to ISHLT registry infectious death is more frequent than rejection death (Figure 1)

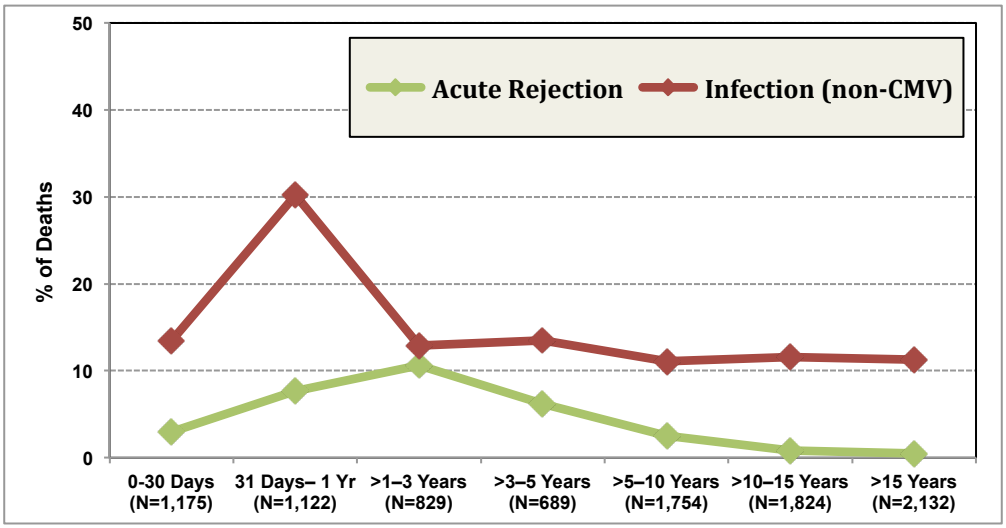
In recent years, several laboratory tests have been used in medical research in order to assess the state of immune activation in patients undergoing solid organ transplant, one of the most used is the Cylex ImmunoKnow test. This method measures the amount of adenosine triphosphate produced by activated CD4 + T lymphocytes after stimulation with some reagents (phytohemagglutinin).

A strong response implies that transplant immune-compromised patients have a high risk of rejection, and a low response reflects a high level of immunosuppression with an increased risk of infections. Different studies confirm these results, but the method lacks high sensitivity since it cannot quantify the magnitude of the innate immune response.

Lately, a new laboratory tests, the QuantiFERON Monitor (QFM), has been introduced. It measures the plasma production of interferon-gamma after incubation of heparinized blood sample by stimulating both the innate immune system (R848) and the acquired immune system (CD3). However, to date, this test has never been studied in the context of heart transplantation, and it is not known whether it is able to provide additional information respect to the consolidated "Cylex".

Thus, we tested the clinical applicability of a novel immune monitoring test – QFM - developed to detect conditions of over- or under- immunosuppression after heart transplant, focusing our analysis on infectious risk.

Figure 1: ISHLT registry: adult heart transplants relative incidence of leading cause of death



3.2 Aim of the study

In this study we used the QFM test in a group of patients enrolled in the CLIN-heart protocol to analyse the impact of this novel immune monitoring essay on the risk of infection in the 3-6 months subsequent to the essay.

3.3 Methods

3.3.1 Population

We enrolled a group of heart transplanted patients who were already part of one of the three enrolment groups (A, B, C), previously described in the CLIN-heart Protocol (Part II). These patients had to fit the sub-sequent criteria.

Inclusion criteria:

- Patients aged ≥ 18 years
- Signing of informed consent

Exclusion criteria:

- Patients not compliant to immunosuppressive regimens.

- Patients treated with interferon-gamma.
- Combined transplantation.

3.3.2 QFM test

This method measures interferon (IFN)-gamma production in plasma, after incubation of heparinized blood, with innate (R848) and adaptive (CD3) stimulants. High doses of INF-line reflect a more intense immune response.

The first QFM test detection coincided with the baseline assessment (V1) of CLIN-heart protocol; subsequently patients were followed for 3 to 6 months and the incidence of infection was assessed.

3.3.3 Endpoints

- Any infection as recorded clinically during the visit or reported by the patient including:
    - High temperature with clinical symptoms
    - CMV isolates needing treatment (>10,000 DNA copies/ml)
    - Gastroenteritis with systemic implications in the context of epidemic season, even with no fever
- Infection etiology is supposed based on a microbial isolate, when available, or on the most likely outcome of the clinical course (I.e. a respiratory syndrome with fever resolving with antibiotics is regarded as bacterial; a flu-like syndrome with gastroenteritis self resolving is regarded as viral). Weakness: overlapping syndromes cannot be definitely elucidated

3.3.4 Statistical analysis

Statistical analyses were performed using JMP 7.0 (SAS Institute, Cary, NC, USA). QFM results are reported as median [25th-75th percentile]. To identify a cut-off value we used ROC analysis and median values were compared using Student's t-test.

3.4 Results

We enrolled 128 patients, whose main characteristics were: 57 ±14 y old, 67% males, with 60±10% ejection fraction, with a median time from transplant of 5 [1-16] years after heart transplant.

For 106 patients with at least 3-months follow-up, we recorded 32 (30%) infectious episode in the subsequent 3 to 6 months. QFM results showed a wide variability in the study patients, with a median (range) value of 104 [30-517] IU/ml of IFN-g.

Patients developing clinical manifestations of infections had QFM result significantly lower than those without infections (45[12-84] vs.176 [43-664] IU/ml; P<0.01). Figure 2 shows that patients with viral infections had a lower QFM value with respect to patients with bacterial infections. Of note, all the 3 infections in the high QFM group were related to self-resolving viral syndromes.

Figure 2: Baseline QFM and subsequent infection (N= 106 patients): QFM and infection 3-6 months later.

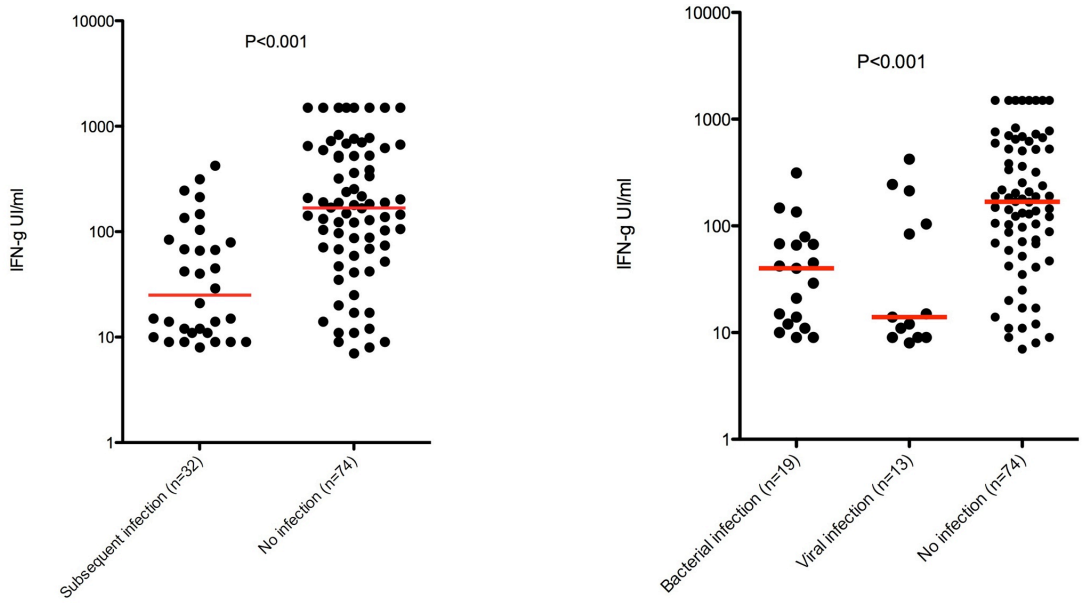
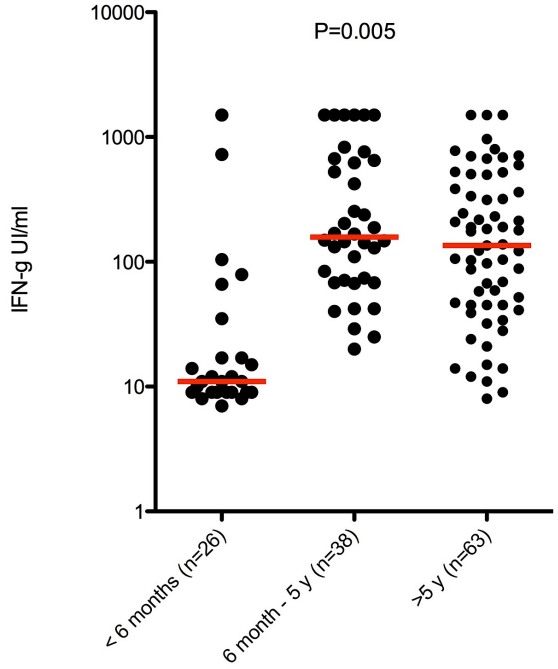


Figure 3: Time from heart transplant and QFM (n=128 patients)



Although patients at less than one year after HT had significantly lower QFM test than

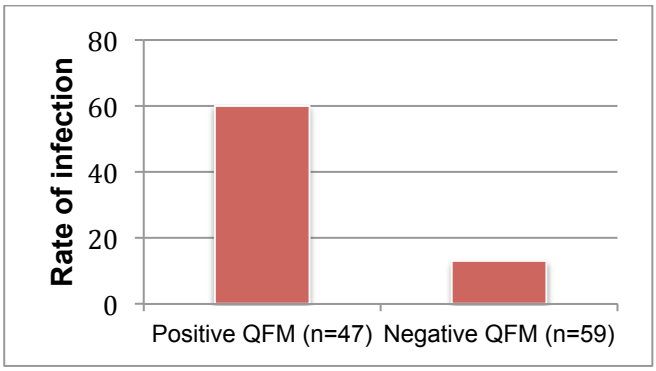
those at longer follow-up, the predictive ability of QFM on the risk of infection persisted significantly after adjusting for post-HT follow-up (Table 1).

Table 1: QFM>100 adjusted for possible confounders

	OR	95% C.I.	p
QFM > 100mU/ml	6.76	1.87 – 32.5	<0.01
Time from HTX (per y)	1.02	0.94 – 1.09	0.9
Lymph count	2.18	0.68 – 7.92	0.4

By ROC analysis, we identified a cut-off of 100 IU/ml to discriminate the subgroup at higher risk of infection: 58.9% of patients with QFM<100 developed infections vs. 15.3% of those with QFM ≥100 IU/ml (P<0.05). (Figure 4)

Figure 4:Era specific QFM threshold (QFM≥ 100 IU/ml) and rate of subsequent infection

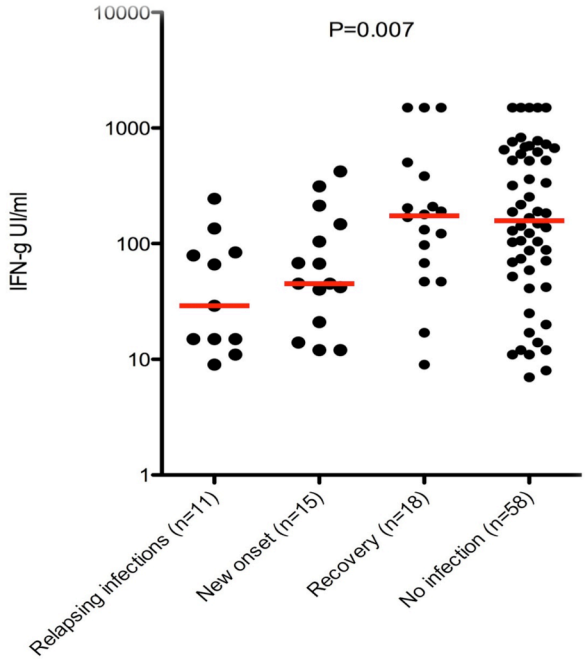


3.4.1 Infection recurrence and QFM

We performed a second detection of QFM recording subsequent infective episodes. We found that patients with a “relapsing episode of infection” (with an infection both before and after the second QFM) and patients with a new onset infection (only post the second QFM test) showed lower values with respect to the group free from infections and the group who

had a previous (pre-second QFM) infection but recovered completely (p<0.01) (Figure 5)

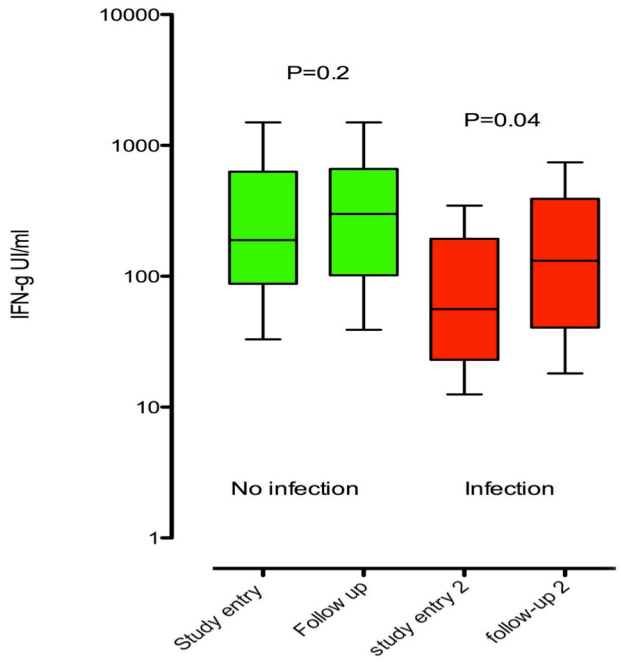
Figure 5: infection recurrence and QFM



As it is shown in the sequent Figure 6, infection events may boost immunity at follow-up assay: in fact we have higher values of QFM in particular in patients who recovered from infection.

The change of QFM doesn't seem to predict subsequent infection. (p=0.3)

Figure 6: infections boost QFM



3.5 Discussion and conclusions

This study provides first suggestive evidence that a novel immune-monitoring method of IFN-g assay after stimulation of innate and adaptive immunity may identify HT recipients with low responsiveness of immune system and high risk of infection (more reliable after early period) and it seem possible to identify a definite threshold of risk which could be different in early and late post- transplant recipients. Moreover QFM essay seems to be useful in detecting progressive immune reconstitution after transplantation. We need of a large number of cases to assess if it is a true representation of the wide variability in immune response ability or if it is al limit of the assay.

Further analysis is needed to assess the effect of therapy modulation on QFM and the relationship with acute rejection episodes and the sensitivity of the assay to changes in immunosuppressive drugs. Finally it could be interesting improving the knowledge of the immune-mechanisms beyond the response of the assay in correlation with T-cells subpopulations.