



HAL
open science

Allosensitization and allograft rejection in heart transplantation: from diagnosis to outcomes

Guillaume Coutance

► **To cite this version:**

Guillaume Coutance. Allosensitization and allograft rejection in heart transplantation: from diagnosis to outcomes. Surgery. Université Paris Cité, 2019. English. NNT: 2019UNIP5084. tel-03975148

HAL Id: tel-03975148

<https://theses.hal.science/tel-03975148>

Submitted on 6 Feb 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Université de Paris

BioSPC ED 562

PARCC – UMR 970 / Équipe 12 « recherche intégrée en transplantation d'organes »

Allosensitization and allograft rejection in heart transplantation

From diagnosis to outcomes

Par Guillaume COUTANCE

Thèse de doctorat de Physiologie et Physiopathologie

Dirigée par Jean-Paul Duong Van Huyen
et par Alexandre Loupy

Présentée et soutenue publiquement à Paris le 15/10/2019

Devant un jury composé de :

Président du jury : Leprince Pascal, PUPH, Pitié-Salpêtrière, Sorbonne Université.

Rapporteurs : Analisa Angelini, Professeur de médecine, Université de Padoue, Italie et Maarten Naesens, Professeur de médecine, Université de Louvain, Belgique.

Examineurs : Shaida Varnous, PH, Pitié-Salpêtrière / Marion Rabant, MCU-PH, Hôpital Necker, Université de Paris / Patrick Bruneval, PUPH, Hôpital Européen Georges Pompidou, Université de Paris.

Membre invité : Jignesh Patel, Professeur de médecine, Cedars-Sinai medical center, David Geffen School of Medicine, Los Angeles, Etats-Unis.

Directeur de thèse : Jean-Paul Duong Van Huyen, PUPH, Hôpital Necker, Université de Paris.

Co-directeur de thèse : Alexandre Loupy, PUPH, Hôpital Necker, Université de Paris.

Titre : Allo-immunisation et rejet d'allogreffe en transplantation cardiaque : du diagnostic aux conséquences cliniques.

Introduction

L'allo-immunisation dirigée contre le greffon («*Donor-Specific Antibodies*» - DSA), et son corollaire, le rejet humoral (RH), sont des problématiques cliniques croissantes en transplantation cardiaque du fait de la morbi-mortalité qui leur est associée. Plusieurs points cruciaux de leur prise en charge demeurent non consensuels : #1-le diagnostic anatomopathologique du RH, #2-le rythme de dépistage des complications qui leur sont associées, actuellement non adapté au risque de développement de la maladie (médecine protocolaire en opposition à une médecine personnalisée), et #3-la prise en charge thérapeutique de sous-groupes de patients à haut risque immunologique.

Méthodes

Dans une première partie, nous avons étudié l'apport de deux techniques de biologie moléculaire comme compagnon au diagnostic anatomopathologique du RH : « Reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA) » et « microarray ». Dans une seconde partie, nous avons analysé, par une approche en population (études internationales multicentriques) et appliquant des outils statistiques innovants (modèles mixtes à classes latentes), deux complications majeures de l'allo-immunisation : la maladie coronaire du greffon (n=1301 patients) et les rejets d'allogreffes (n=1053 patients). Notre approche a pour but d'améliorer la modélisation et la stratification du risque de développement de la maladie, première étape vers une individualisation du suivi. Enfin, nous avons étudié l'impact de l'allo-immunisation et du RH dans 2 populations à risque : les patients greffés avec DSA pré-formés et les patients en choc cardiogénique secondaire à un RH.

Résultats

En appliquant la RT-MLPA à 183 biopsies endomyocardiques, nous avons démontré l'applicabilité de cette technique aux biopsies fixées en paraffine et développé des profils d'expression génique spécifiques des RH (surexpression de CCL4, PLA1A, GNLY, CXCL9, FCGR3 et CXCL11) et des rejets cellulaires (CCL18 et ADAMdec). Nous avons ensuite montré qu'une classification en 4 grades du degré d'inflammation microvasculaire était significativement associée à l'activité moléculaire du rejet humoral et à des paramètres cliniques d'importance (présence de DSA, dysfonction du greffon).

Par une approche épidémiologique en population, nous avons montré que l'évolution de la maladie coronaire du greffon pouvait être séparée en 4 trajectoires distinctes et universelles et que les DSA de classe II étaient un facteur de risque indépendant d'apparition et d'aggravation de la maladie. Par une approche similaire, nous avons pu améliorer la modélisation de l'évolution du risque de rejet d'allogreffe au cours du temps et déterminer cinq facteurs de risque indépendant de rejet d'allogreffe au cours de la première année (présence de DSA, âge du receveur, type de greffe, type d'induction, nombre de mismatch HLA).

En rapportant les résultats d'un protocole prospectif de prise en charge péri-opératoire des patients greffés avec DSA pré-formés, nous avons montré que ceux-ci présentaient une survie après transplantation similaire aux patients à moindre risque immunologique au prix d'une augmentation de l'incidence des RH. Enfin, nous avons décrit que le pronostic des patients en choc cardiogénique secondaire à un RH était médiocre, avec une mortalité estimée à 82% à 1 an.

Conclusion

La standardisation du diagnostic anatomopathologique du RH basée sur les techniques de biologie moléculaire, l'amélioration de la stratification du risque de complications cliniques de l'allo-immunisation et de la description des événements cliniques au sein de population à haut risque immunologique représentent autant d'avancées sur la voie d'une amélioration de la prise en charge de l'allo-immunisation et du RH en transplantation cardiaque.

Mots clefs : Transplantation cardiaque, allo-immunisation, rejet d'allogreffe, maladie coronaire du greffon, biologie moléculaire, stratification pronostique

Title : Allosensitization and allograft rejection in heart transplantation: from diagnosis to outcomes.

Introduction

Allosensitization, i.e. the presence anti-HLA Donor-Specific Antibodies (DSA), and its corollary, antibody-mediated rejection (AMR), are major causes of cardiac allograft vasculopathy (CAV), allograft rejection and graft dysfunction, which represent the leading cause of death late after heart transplantation. Several key points of their management remain debated and concern particularly #1- the lack of standardization of the pathologic diagnosis of AMR, #2- the screening protocol of allosensitization-related complications not adapted to the risk of development of the disease (protocol medicine in opposition to personalized medicine), and #3- the clinical management of subgroups of patients at high immunological risk.

Methods

In a first part, we analyzed the contribution of two molecular biology techniques in the standardization of the pathologic diagnosis of AMR: a targeted-gene expression technique, the reverse transcriptase multiplex Ligation-dependent probe amplification (RT-MLPA) and a genome-wide approach using microarrays. In a second part, we analyzed at the population level (multicentric international studies) and using innovative statistical tools (latent classes mixed models), two major complications of allosensitization: CAV (n = 1,301 patients) and allograft rejections (n = 1,053 patients). Our aim was to describe their natural history, epidemiology and risk factors to enable an accurate risk stratification. Finally, in a clinical part, we studied the impact of DSA and AMR in two populations with high immunological risk: patients transplanted with pre-formed DSA and patients with AMR-related cardiogenic shock.

Results

By applying RT-MLPA to 183 endomyocardial biopsies (EMB), we demonstrated the applicability of this technique to paraffin-embedded EMB and developed specific gene expression profiles of AMR (overexpression of CCL4, PLA1A, GNLY, CXCL9, FCGR3 and CXCL11) and cellular rejection (CCL18 and ADAMdec). We then demonstrated that the severity of microvascular inflammation, based on a semi-quantitative evaluation of its extension (none, 1 to 9, 11 to 49 and $\geq 50\%$ of the capillaries and venules) had biological (association with molecular activity of AMR) and clinical meanings (pAMR grade, DSA, graft dysfunction).

In a large multicentric and highly phenotyped prospective cohort of heart transplant recipients, we identified for the first time 4 universal distinct and mutually exclusive CAV trajectories and their respective immune (class II DSA and acute cellular rejection) and non-immune determinants. Our results provide the basis for a trajectory-based assessment of heart transplant patients for early risk stratification and patient monitoring. By a similar approach, we were able to describe the epidemiology of allograft rejection and to identify five independent risk factors for rejection during the first year (DSA, recipient's age, type of transplantation, type of induction therapy, number of HLA mismatches).

Finally, by reporting the results of a prospective protocol for perioperative management of pre-formed DSA based on plasmapheresis and polyvalent immunoglobulins infusions, we showed that those high-risk patients had a similar post-transplant survival compared to other patients at the cost of an increased incidence of AMR. Finally, we reported the poor prognosis of patients with AMR-related cardiogenic shock characterized by a one-year mortality as high as 82%.

Conclusion

The standardization of pathologic diagnosis of AMR using molecular biology techniques as a companion to pathologic analysis, the improvement in the risk stratification of allosensitization-related complications and the better description of clinical events in high immunologic risk population represent so many advances in the way of an improvement in the clinical management of DSA and AMR in heart transplantation.

Keywords: Heart transplantation, allosensitization, allograft rejection, cardiac allograft vasculopathy, molecular biology, risk stratification.

A Paul, Agathe et Emmanuelle

Acknowledgements

Ces trois années de thèse auront été une expérience inoubliable au cours de laquelle j'ai pu interagir avec des personnes formidables qui m'ont permis de progresser tant dans le domaine professionnel que personnel. En plus des remerciements formels d'usage pour ce type de document, j'y ajouterai une version personnelle en exprimant à chacun ma reconnaissance de vive voix.

« Sentir de la gratitude et ne pas l'exprimer, c'est comme emballer un cadeau et ne pas le donner ».

William Arthur Ward

Merci à mes deux directeurs de thèse, Jean-Paul et Alexandre, pour m'avoir guidé au cours de trois années et offert des opportunités professionnelles incroyables.

Merci à mon chef de service, Pascal Leprince, de me faire l'honneur de diriger ce jury de thèse et de m'offrir une place dans son service auquel je suis tant attaché.

Thanks to Prof. Analisa Angelini and Prof. Maarten Naessens for agreeing to evaluate this work and for their long trip to Paris. I am deeply honored that two experts like you are part of my Ph.D. committee.

Thanks to Prof. Jignesh Patel for agreeing to be part of this committee and for his very long trip from LA. I am also very grateful to you for having welcomed me so kindly in your department. I am looking forward to coming back in November.

Merci à Patrick Bruneval et Marion Rabant, de me faire l'honneur de leur présence dans ce jury. Cela a été un plaisir d'échanger avec vous pendant ces années.

Merci à Xavier Jouven pour ses conseils et son encadrement au cours de cette thèse ainsi que de son soutien.

Merci à Shaida Varnous, dont je ne serai jamais assez reconnaissant de m'avoir tant appris dans le domaine de la transplantation et dans tant d'autres d'ailleurs.

Publications

Accepted publications:

- Favorable Outcome of an Exclusively Posttransplant Prophylactic Strategy After Heart Transplantation in Recipients With High Immunological Risk. **Coutance G**, d'Orio V, Belin L, Bréchet N, Saheb S, Lebreton G, Bouglé A, Rouvier P, Gautreau C, Ouldammam S, Chamillard X, Huot M, Amour J, Combes A, Leprince P, Varnous S. *Transplantation*. 2019 Jul;103(7):1439-1449.

- Antibody-mediated rejection induced cardiogenic shock: Too late for conventional therapy. **Coutance G**, Van Aelst L, Hékimian G, Vidal C, Rouvier P, Saheb S, Gautreau C, Leprince P, Varnous S. *Clin Transplant*. 2018 May;32(5):e13253.

Articles submitted:

- Reverse transcriptase multiplex ligation-dependent probe amplification, a targeted gene expression tool for the diagnosis of cardiac allograft rejection on formalin-fixed endomyocardial biopsies. Nicolas Adam*, **Guillaume Coutance***, Pierre-Julien Vially, Fanny Drieux, Philippe Ruminy, Ahmad Abdel Sater, Claire Toquet, Philippe Rouvier, Arnaud François, Marie-Pierre Chenard, Eric Epailly, Romain Guillemain, Sabine Pattier, Arnaud Gay, Shaida Varnous, Jean-Luc Taupin, Marion Rabant, Alexandre Loupy, Patrick Bruneval, Jean Paul Duong Van Huyen.

Journal of Heart and Lung Transplantation - Resubmitted after major revisions.

- Identification and characterization of Trajectories Of Cardiac Allograft Vasculopathy After Heart Transplantation: A Population Based Study. A Loupy*, **G Coutance***, G Bonnet* (equal contribution), J V Keer, M Raynaud, MC Bories, M Racapé, D Yoo, JP Duong, P Bruneval, S Varnous, P Leprince, R Guillemain, Ryan Levine, Jig Patel, J Kobashigawa and X Jouven.

Table des matières

ACKNOWLEDGEMENTS.....	5
PUBLICATIONS	7
AVERTISSEMENT.....	13
INTRODUCTION.....	14
INTRO-A. ADVANCED HEART FAILURE: DEFINITION AND EPIDEMIOLOGY.....	14
INTRO-B. HEART TRANSPLANTATION	14
INTRO-C. CURRENT ISSUES IN THE MANAGEMENT OF DSA AND AMR AFTER HEART TRANSPLANTATION	18
PART I. ROLE OF MOLECULAR BIOLOGY TO IMPROVE THE DIAGNOSIS OF ANTIBODY-MEDIATED REJECTION.	22
CHAPTER I-A: PHYSIOPATHOLOGY OF ANTIBODY-MEDIATED REJECTION AND DEVELOPMENTS OF MOLECULAR BIOLOGY IN HEART TRANSPLANTATION.....	23
I-A-1. PATHOPHYSIOLOGY OF ANTIBODY-MEDIATED REJECTION	23
I-A-2. MOLECULAR BIOLOGY IN HEART ALLOGRAFT REJECTION.....	26
I-A-3. OBJECTIVES	30
CHAPTER I-B: APPLICATION OF MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION TO ENDOMYOCARDIAL BIOPSIES.....	31
I-B-1. MOLECULAR BIOLOGY APPLIED TO THE DIAGNOSIS OF HEART ALLOGRAFT REJECTION	31
I-B-2. MATERIAL AND METHODS	33
I-B-3. RESULTS.....	38
I-B-4. DISCUSSION.....	44
I-B-5. CONCLUSION	46
CHAPTER I-C: EXTENSION OF INTRAVASCULAR ACTIVATED MONONUCLEAR CELLS CORRELATES WITH ANTIBODY-MEDIATED REJECTION MOLECULAR ACTIVITY, DONOR-SPECIFIC ANTIBODIES AND GRAFT FUNCTION.....	47
I-C-1. INTRODUCTION	47
I-C-2. PATIENTS AND METHODS	48
I-C-3. RESULTS.....	51
I-C-4. DISCUSSION	57

I-C-4. CONCLUSION	59
CHAPTER I-D: SYNTHESIS OF PART 1 – MOLECULAR BIOLOGY IN HEART TRANSPLANTATION.	60
<u>PART 2. RISK STRATIFICATION OF DONOR-SPECIFIED ANTIBODIES AND ANTIBODY-MEDIATED REJECTION-RELATED COMPLICATIONS</u>	<u>61</u>
CHAPTER II-A: RISK STRATIFICATION	63
II-A-1. IDENTIFICATION OF PROGNOSTIC FACTORS USING COX REGRESSION MODEL.....	64
II-A-2. INTRODUCTION TO THE INTEREST IN MIXED MODELS IN HEART TRANSPLANTATION	67
II-A-3. RISK FACTORS FOR ALLOGRAFT REJECTION AFTER HEART TRANSPLANTATION	67
II-A-4. RISK FACTORS FOR CAV.....	69
CHAPTER II-B: EPIDEMIOLOGY AND RISK FACTORS OF BIOPSY-PROVEN REJECTION DURING THE FIRST-YEAR POST-TRANSPLANT.	71
II-B-1. INTRODUCTION	71
II-B-2. MATERIALS AND METHODS	72
II-B-3. RESULTS.....	74
II-B-4. DISCUSSION.....	82
CHAPTER II-C: IDENTIFICATION AND CHARACTERIZATION OF TRAJECTORIES OF CARDIAC ALLOGRAFT VASCULOPATHY AFTER HEART TRANSPLANTATION: A POPULATION BASED STUDY.	85
II-C-1. INTRODUCTION	85
II-C-2. PATIENTS AND METHODS	86
II-C-3. RESULTS.....	90
II-C-4. DISCUSSION.....	102
II-C-5. CONCLUSION	106
CHAPTER II-D: SYNTHESIS OF EPIDEMIOLOGICAL STUDIES.	107
<u>PART 3. CLINICAL STUDIES TO BETTER CHARACTERIZE THE OUTCOMES OF AT-RISK POPULATIONS</u>	<u>108</u>
CHAPTER III-A: FAVORABLE OUTCOME OF AN EXCLUSIVELY POST-TRANSPLANT PROPHYLACTIC STRATEGY AFTER HEART TRANSPLANTATION IN RECIPIENTS WITH HIGH IMMUNOLOGICAL RISK.	109
III-A-1. HEART TRANSPLANTATION WITH PRE-FORMED ANTI-HLA DONOR-SPECIFIC ANTIBODIES.....	109
III-A-2. PATIENTS AND METHODS	110
III-A-3. RESULTS	113
III-A-4. DISCUSSION.....	121

III-A-5. CONCLUSION	125
CHAPTER III-B: ANTIBODY-MEDIATED REJECTION INDUCED CARDIOGENIC SHOCK: TOO LATE FOR CONVENTIONAL THERAPY.	126
III-B-1. INTRODUCTION	126
III-B-2. METHODS	126
III-B-3. RESULTS.....	129
III-B-4. DISCUSSION.....	134
III-B-5. CONCLUSION	136
CHAPTER III-C: CONCLUSION OF THE CLINICAL STUDIES.	137
<u>CONCLUSION</u>	<u>138</u>
DISCUSSION OF RESULTS AND FUTURE DIRECTIONS.....	139
<u>BIBLIOGRAPHY.....</u>	<u>142</u>
<u>SUPPLEMENTARY APPENDIX</u>	<u>158</u>

Table des illustrations

FIGURE 1: EVOLUTION OF THE NUMBER OF HEART TRANSPLANTATIONS. (A) ISHLT DATA, (B) “AGENCE DE LA BIOMÉDECINE”.....	15
FIGURE 2: POST-TRANSPLANT SURVIVAL. (A) ISHLT DATA, (B) “AGENCE DE LA BIOMÉDECINE”.	15
FIGURE 3: DYNAMIC OF CAUSES OF DEATH AFTER HEART TRANSPLANTATION.....	16
FIGURE 4. ALLOSENSITIZATION AND ALLOGRAFT REJECTION IN HEART TRANSPLANTATION: FROM DIAGNOSIS TO OUTCOMES.	20
FIGURE 5: PATHOPHYSIOLOGY OF ANTIBODY-MEDIATED REJECTION (25)	25
FIGURE 6: T-CELL MEDIATED REJECTION AND ANTIBODY-MEDIATED REJECTION-SELECTIVE TRANSCRIPTS (51).	27
FIGURE 7: LACK OF STANDARDIZATION IN THE DIAGNOSIS OF ANTIBODY-MEDIATED REJECTION: STRATEGIES BASED ON MOLECULAR BIOLOGY TECHNIQUES TO REFINE AMR DIAGNOSIS.	30
FIGURE 8: PRINCIPLE OF MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION.....	32
FIGURE 9: FLOWCHART OF THE RT-MLPA STUDY.....	33
FIGURE 10: HISTOPATHOLOGY AND RT-MLPA PROFILES.....	35
FIGURE 11: EXPRESSION OF TRANSCRIPTS ACCORDING TO HISTOLOGICAL DIAGNOSIS....	40
FIGURE 12: UNSUPERVISED HIERARCHICAL AND CLASS PREDICTION ANALYSIS.....	41
FIGURE 13: CLASS PREDICTION ANALYSIS	42
FIGURE 14: SEMI-QUANTITATIVE ASSESSMENT OF MICROVASCULAR INFLAMMATION USING THE MVI SCORE.....	50
FIGURE 15: MOLECULAR CORRELATES OF EXTENSION OF MICROVASCULAR INFLAMMATION MVI SCORE.....	53
FIGURE 16: RELATIONSHIP BETWEEN EXTENSION OF MICROVASCULAR INFLAMMATION (MVI SCORE) AND ISHLT CLASSIFICATIONS OF REJECTION, MFI OF IMMUNODOMINANT DSA AND IMPAIRED GRAFT FUNCTION AT THE TIME OF ENDOMYOCARDIAL BIOPSY.	55
FIGURE 17: SUMMARY OF RESULTS OF MOLECULAR BIOLOGY STUDIES.	60
FIGURE 18: OBJECTIVES OF THE PART 2: RISK STRATIFICATION.	62
FIGURE 19: DESIGN OF THE STUDY.....	73
FIGURE 20: REPARTITION OF (A) OVERALL EMB AND (B) REJECTING-EMB ACCORDING TO THE TIME POST-TRANSPLANT.	75
FIGURE 21: EVOLUTION OF THE MEAN NUMBER OF EMB PER PATIENT PERFORMED DURING THE FIRST-YEAR POST TRANSPLANT.....	75
FIGURE 22: EPIDEMIOLOGY OF ALLOGRAFT REJECTION DURING THE FIRST YEAR AFTER HEART TRANSPLANTATION.....	78

FIGURE 23: DISCRIMINATION OF REGRESSION MODELS. A : COX MODEL. B: LOGISTIC REGRESSION MODEL.	80
FIGURE 24. DESIGN OF THE STUDY.....	87
FIGURE 25. DISTINCT PROFILES OF CAV TRAJECTORIES IDENTIFIED POST-TRANSPLANTATION IN THE EUROPEAN DERIVATION COHORT (N=815).....	94
FIGURE 26: CARDIAC ALLOGRAFT VASCULOPATHY TRAJECTORIES: EXTERNAL VALIDATION IN THE AMERICAN COHORT.	95
FIGURE 27: DESCRIPTION OF OVERALL MORTALITY ACCORDING TO THE CAV TRAJECTORY IN THE OVERALL COHORT (N=1,301).....	101
FIGURE 28: SYNTHESIS OF THE RESULTS OF THE EPIDEMIOLOGICAL STUDIES.	107
FIGURE 29: BETTER UNDERSTANDING OF OUTCOMES OF AT-RISK POPULATIONS.	108
FIGURE 30: STUDY PROTOCOL.	111
FIGURE 31: MEAN FLUORESCENCE INTENSITY OF DONOR-SPECIFIC ANTIBODIES.....	115
FIGURE 32. EVOLUTION OF MFI AFTER PERI-OPERATIVE MANAGEMENT IN PATIENTS WITH PREFORMED DONOR-SPECIFIC ANTI-HLA ANTIBODY (PFDSA) MEAN FLUORESCENCE INTENSITY (MFI) LEVELS >1000.....	116
FIGURE 33. PRIMARY AND SECONDARY OUTCOMES.	117
FIGURE 34. INCIDENCE OF BIOPSY-PROVEN ANTIBODY-MEDIATED REJECTION (ABMR) (DEATHS NOT SHOWN).	117
FIGURE 35. EXPLORATORY ANALYSIS IN THE GROUP OF PATIENTS WITH PREFORMED DONOR-SPECIFIC ANTI-HLA ANTIBODY (PFDSA) MEAN FLUORESCENCE INTENSITY (MFI) LEVELS >1000.	121
FIGURE 36: SURVIVAL AFTER ANTIBODY-MEDIATED REJECTION WITH CARDIOGENIC SHOCK.	133
FIGURE 37. SUMMARY OF THE RESULTS OF CLINICAL STUDIES.	137

Avertissement

Introduction

Intro-A. Advanced heart failure: definition and epidemiology

Intro-A-1. Heart failure

Heart failure is a clinical syndrome characterized by typical symptoms (e.g. breathlessness, ankle swelling and fatigue) that may be accompanied by signs caused by a structural and/or functional cardiac abnormality, resulting in a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress (1). The prevalence of heart failure in industrialized countries is estimated to range between 1% and 2%, making this disease a real public health problem. The therapeutic management of heart failure has been revolutionized over the last twenty years. However, some patients are affected by this disease to such an extent that these treatments remain insufficient.

Intro-A-2. Advanced heart failure

Advanced heart failure refers to these unstable and severe patients. Its definition is based on the association of the persistence of symptoms and / or signs of heart failure despite optimal medical and electrical management and the presence of clinical, biological, electrocardiographic or ultrasound severity criteria (2,3). The prevalence of advanced heart failure is estimated at 1% of the heart failure patient population, representing several **tens of thousands of patients** in Europe or the United States. The prognosis of these patients is poor. The mortality of patients dependent on inotropic support is estimated to be as high as 75% at 1 year (4), that of INTERMACS 4 patients at 50% (5).

Intro-B. Heart transplantation

Intro-B-1. Epidemiology, post-transplant survival

Despite the development of long-term circulatory support techniques, heart transplantation (HTx) remains the reference therapeutic option for patients with

advanced heart failure (1,6). Currently, more than 5,000 heart transplants are performed each year worldwide, including nearly 500 procedures in France (Figure 1) (7,8).

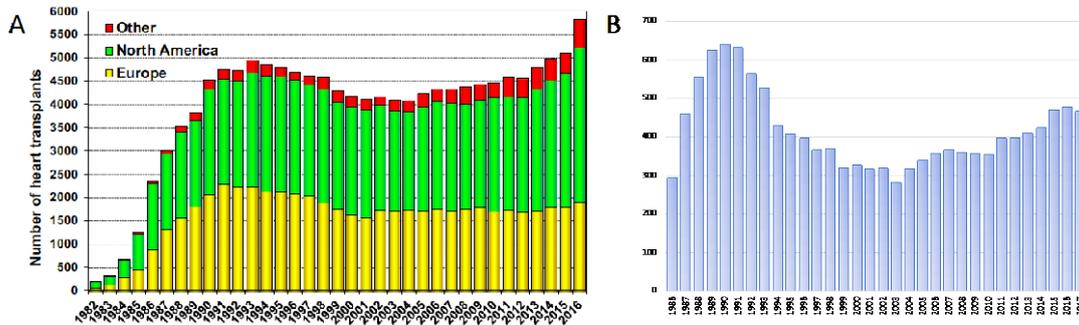


Figure 1: Evolution of the number of heart transplantations. (A) ISHLT data, (B) “Agence de la Biomédecine”.

One-year survival is about 88 % and median survival after heart transplantation now exceeds 12 years according to ISHLT data (7). In France, one-year survival is 79% for the 2013-2016 period and median survival after heart transplantation exceeds 10 years (9) (Figure 2).

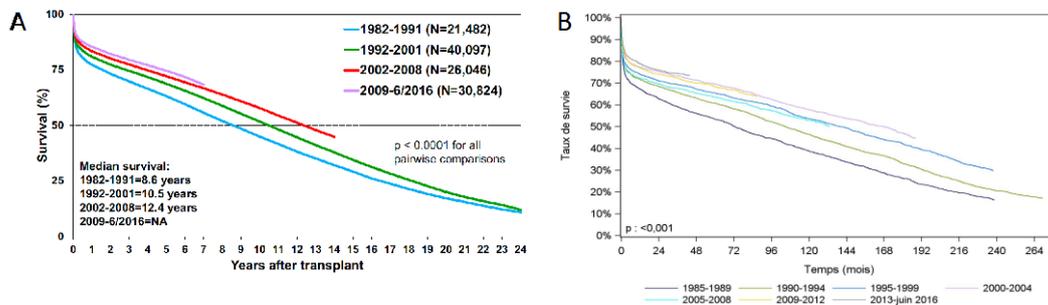


Figure 2: Post-transplant survival. (A) ISHLT data, (B) “Agence de la Biomédecine”.

The improvement of immunosuppression and short-term mechanical circulatory supports have allowed continuous improvement in survival after heart transplantation. However, only early survival has been significantly improved. The slope of survival curves beyond the first year remains stable over time, making late death causes a major challenge for improving the prognosis of heart transplant recipients.

Intro-B-2. Limits to the long-term success of transplantation: the central role of allosensitization and antibody-mediated rejection.

Beyond the first year of transplantation, two major causes account for the majority of deaths: complications of chronic immunosuppression (cancers and infections) and graft-specific diseases: cardiac allograft vasculopathy, allograft rejection and graft dysfunction (Figure 3) (7,10). The term "graft dysfunction" is a generic term used in large registers. Aside from the primary graft dysfunction occurring very early after transplantation, this entity reflects both acute dysfunctions associated with allograft rejection or undiagnosed coronary artery disease, as well as chronic dysfunction related to a restrictive evolution of heart allografts or undiagnosed coronary artery diseases.

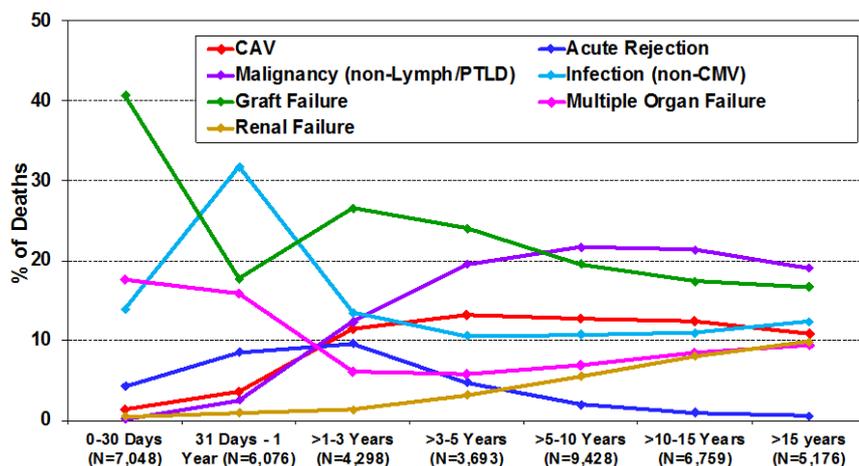


Figure 3: Dynamic of causes of death after heart transplantation

Intro-B-3.a. Acute injury: allograft rejection-related acute graft dysfunction

Allograft rejection remains an important cause of death after heart transplantation. While immunosuppressive induction or maintenance therapies are particularly effective for the prevention and treatment of acute cellular rejection, the therapeutic management of antibody-mediated rejection (AMR) remains much more complex. The incidence of acute cellular rejection has declined in the course of time (11). It now rarely brings severe graft dysfunction and is no longer associated with an increase in cardiovascular mortality, unlike AMR (12). In our experience, all the most severe cases of acute graft dysfunction responsible for a cardiogenic shock were related to an AMR

highlighting the important role of this type of rejection in the genesis of acute graft dysfunction (13).

Intro-B-3.b. Chronic injury: cardiac allograft vasculopathy

Cardiac allograft vasculopathy (CAV) is a common and severe condition following HTx. It is characterized by an intimal thickening responsible for a diffuse narrowing of the coronary arteries. Despite the progress made in terms of prevention, the incidence of cardiac allograft vasculopathy remains as high as 50% 10 years after transplantation (14). The presence of a CAV is a significant risk factor for morbidity and mortality, even at a subclinical stage diagnosed by an increase in intimal thickness on intravascular ultrasonography (15,16). In addition to the usual donors' and recipients' cardiovascular risk factors, numerous experimental and clinical studies, have established a strong link between donor-specific anti-HLA antibodies (DSA) and AMR on the one hand and the occurrence or progression of a CAV on the other hand (17–20). Antibody Dependent Cellular Cytotoxicity (ADCC) appears to have a major role in the pathophysiology of CAV (18,21).

Intro-B-3.c. Chronic injury: graft restrictive physiology

Progressive evolution to ventricular filling disorders, characterized by restrictive cardiac transplant physiology, is an important cause of late transplant dysfunction and retransplantation. These abnormalities would account for one-third of cases of heart failure beyond one year of transplantation (22) and 15% of retransplantations (23). Impairment of the coronary microcirculation is probably an important mechanism of this type of dysfunction. Several studies have established a link between DSA / AMR and restrictive physiology. The presence of DSA was associated with abnormalities of coronary microcirculation and elevation of filling pressures in a Danish study (24). Most retransplanted patients, whatever the reason, showed signs of AMR, most often subclinical, both on explanted transplants and on endomyocardial biopsies performed during usual follow-up (23).

Decreasing late mortality after heart transplantation is an unmet medical need. The graft-specific diseases (CAV, rejection, late dysfunctions, restrictive physiology) represent the first cause of death beyond the first year of transplant. Allosensitization and its corollary, the AMR, are at the center of all these graft-specific diseases due to the acute and chronic lesions, both micro and macrovascular, that they might induce (25). Improving the management of allosensitization and AMR is therefore a priority in heart transplantation.

Intro-C. Current issues in the management of DSA and AMR after heart transplantation

The current medical management, which is extremely heterogeneous from one center to another, suffers from three major limitations: i) the difficulty of pathologic diagnosis, ii) the lack of prognostic stratification tool able to help the clinical-decision making, and iii) and the lack of consensus regarding the therapeutic management, direct consequent of the previous issues (26–28).

Our objective is to increase our knowledge of these three fundamental points of the management of DSA and AMR in heart transplantation by applying innovative methodologies for cardiac pathology, epidemiology and therapeutic management.

Intro-C-1. Pathologic diagnosis of AMR

The diagnosis of AMR in heart transplantation only relies on the pathologic analysis of an endomyocardial biopsy (EMB), regardless of the presence of DSA or transplant dysfunction (29,30). Antibody-mediated rejection (AMR) is graded from pAMR0 to pAMR3 according to the presence of #1- microvascular inflammation defined by the intravascular accumulation of activated mononuclear cells (IAMC) within the myocardial capillaries (= histopathologic AMR, pAMR1-H+) and #2- signs of local activation of the complement pathway (C4d, C3d: multifocal or diffuse strong capillaries staining) and/or presence of intravascular macrophages (CD68+, ≥10% of capillaries)

defining immunopathologic AMR (pAMR1-I+). The presence of both histopathologic and immunopathologic findings defines pAMR2. The third grade, pAMR3, is reserved to the very rare forms of severe pathologic AMR (interstitial hemorrhage, capillary fragmentation) (29,30).

Although widely used, this classification suffers from a number of inaccuracies (30). One of the major limitations is the dichotomization of the diagnosis of microvascular inflammation (present / absent) without clearly defined diagnostic threshold. This directly impacts the problem of interobserver agreement, which is low for acute cellular rejection (31), and which has never been evaluated on a large scale for AMR. The large disparities in care between centers are probably due in part to these diagnostic difficulties. Molecular biology techniques have demonstrated their potential relevance in rationalizing the diagnosis of AMR, but several constraints, in particular financial constraints, are a limit to their application in clinical practice (32,33).

We hypothesize that i) a technique of molecular biology, the **RT-MLPA** (Multiplex Ligation-dependent Probe Amplification), which is inexpensive and transposable into the routine of a pathology laboratory, is suitable for the analysis of gene expression on formalin-fixed paraffin-embedded (FFPE) EMB and may be a support for the **pathologic diagnosis**, and ii) that a **semi-quantitative evaluation of microvascular inflammation** in four stages is correlated with **the molecular activity of AMR** and could have a role in the **diagnostic standardization and prognostic stratification of AMR** (Figure 4).

Intro-C-2. Screening for complications of allosensitization and AMR

Screening for CAV and allograft rejection is based on invasive, iterative and protocolized tests, coronary angiography and endomyocardial biopsy, respectively (34,35). As the natural history and the risk factors of these complications are not deeply described, the frequency of screening tests is applied in the same way to all patients, regardless of their risk of developing the disease. It results in a poor diagnostic cost-effectiveness compared to the risk of complications inherent to these invasive tests (11).

We hypothesize that a better knowledge of CAV's and AMR's **epidemiology, natural history and risk factors**, based on the use of **innovative statistical tools** and on a

population-based approach, would allow a better prognostic stratification of these diseases; a first step toward the rationalization of invasive screening tests (Figure 4).

Intro-C-3. Therapeutic management of populations at high immunological risk.

Therapeutic managements of allosensitization and AMR are highly heterogeneous between centers (26). Latest guidelines on these topics highlight the lack of consensus and the inability to recommend an accurate medical management in most situations due to the lack of clinical data (27,28). The prognosis and therapeutic management of certain populations at immunological risk have been subject to poor evaluation, especially concerning heart transplantation with pre-formed DSA. Most centers avoid this type of transplantation given the increased risk of post-transplant morbidity and mortality (36–39).

We hypothesize that a **desensitization** protocol, based on plasmapheresis and intravenous polyvalent immunoglobulins (IVIg) infusions, implemented through a **prospective** protocol and applied at the **time of transplantation** can enable to **minimize the impact of pre-formed DSA** on post-transplant morbidity and mortality.

Advances in diagnosis, prognostic stratification and therapeutics concerning DSA and AMR may have a real clinical impact on the future of cardiac transplant patients and thus constitute a research field of primary importance. We will apply innovative methodologies, in terms of cardiac pathology, epidemiology, patient's care (Figure 4).

↓ *Figure 4. Allosensitization and allograft rejection in heart transplantation: from diagnosis to outcomes.*

Part I: role of molecular biology to improve the diagnosis of antibody-mediated rejection.

Part II: Epidemiologic approaches to enhance prognostic stratification of CAV and allograft rejections.

Part III: Clinical studies to improve the understanding of at-risk subpopulation – transplantation with pre-formed DSA & AMR-induced cardiogenic shock.

AMR means Antibody-mediated rejection, CAV: cardiac allograft vasculopathy, DSA: donor-specific antibodies, IAMC: intravascular activated mononuclear cells, RT-MLPA: Retro-Transcription Multiplex Ligation-dependent Probe Amplification.

ALLOSENSITIZATION AND ALLOGRAFT REJECTION IN HEART TRANSPLANTATION: FROM DIAGNOSIS TO OUTCOMES

To enhance patients' care by improving the:

PURPOSE

DIAGNOSIS OF ALLOGRAFT REJECTION

RISK STRATIFICATION

DESCRIPTION OF PATIENTS' OUTCOMES

METHODS



MOLECULAR BIOLOGY

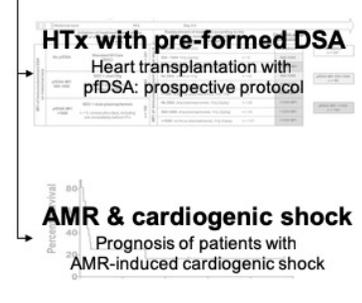
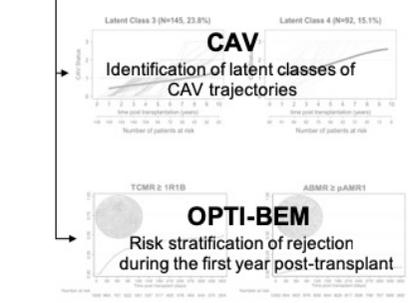
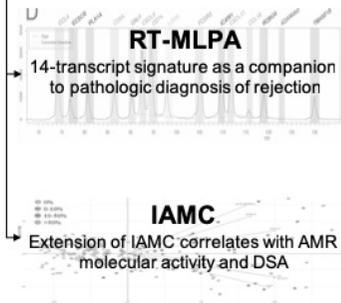


EPIDEMIOLOGICAL APPROACH



CLINICAL STUDIES

STUDIES



Part I.

Role of molecular biology to improve the diagnosis of antibody-mediated rejection.

Whereas the **degree of microvascular inflammation** is graded according to a semiquantitative **histologic scale** in kidney transplantation (40,41), the threshold for which IAMC are considered significant for the possibility of AMR in hematoxylin & eosin stained sections **has not been formally established in heart transplantation** (30).

This absence of a clear threshold for the diagnosis of AMR might explain in part the **important variability of AMR diagnosis and treatment** across centers (26). **Gene expression analysis** of the allograft tissue has been increasingly recognized as a powerful tool for the understanding of the pathophysiological pathways of rejection after heart transplantation (10,11) but their applicability in clinical practice is limited by several flaws.

After a brief review of the **physiopathology** of AMR and the developments of **molecular biology** in the field of cardiac allograft rejection, we will detail **our results** concerning the application of two molecular biology techniques to the diagnosis of AMR.

Chapter I-A: Physiopathology of antibody-mediated rejection and developments of molecular biology in heart transplantation.

I-A-1. Pathophysiology of antibody-mediated rejection

The donor vasculature present at the interface between donor tissue and the recipient immune system is the primary target of the alloimmune response. The complex pathophysiology of AMR is more and more understood and implies i) the production of donor-specific antibodies (DSA), mostly anti-HLA antibodies, as an alloimmune response to organ transplantation or to pre-transplant sensitizing events, ii) the binding of these DSA on the donor's endothelial cells, iii) responsible for the activation of both complement-dependent and complement-independent pathways leading to cells damage and allograft rejection.

I-A-2-a. Complement-dependent pathway (Figure 5)

Ever since the discovery by Terasaki et al. of the importance of complement-dependent cytotoxicity (CDC) crossmatch to predict hyperacute rejection of allograft, evidence of the central role of the complement activation in the pathophysiology of AMR has been growing (42).

Classical pathway activation is initiated when plasma C1q binds to the Fc segments of IgM and IgG. The extent of complement activation depends on the antibody isotype, the abundance of the target antigen and density of immunoglobulins, the local concentration of complement regulatory proteins, and the influence of antibody-targeting therapies (25). Activation of the complement cascade results in the formation of a number of biologically active complement fragments. C3b is covalently fixed to cell surfaces and acts as an opsonin. The C3a and C5a fragments act as powerful inducers of the local inflammatory response. Cellular signaling pathways activated after binding of these fragments to endothelial cells (through G protein-coupled receptors) results in

the release of pro-inflammatory cytokines, von Willebrand factor and heparan sulfate (43). The complement activation cascade leads to the assembly of the C5b-9 molecular complex, also called membrane attack complex (MAC). This results from the interaction between C5b, C6 and C7 which anchors this first complex to the membrane and causes the recruitment of C8. The molecular tetrad then leads to the recruitment and polymerization C9 of which forms a pore in the membrane causing osmotic cell lysis. This lytic cellular effect requires a high density of MAC at the membrane level and is considered nowadays as a rare event during AMR. MAC's contribution to complement-mediated endothelial dysfunction is thought to be mediated by its so-called sub lytic activity resulting in endothelial activation via the non-canonical NF-Kb signaling pathway leading to the expression of pro-inflammatory proteins, in particular IL-6 (43,44). Finally, complement activation and MAC binding to endothelial cells also stimulate the adaptive immune system, which increase interferon production. γ (IF- γ).

I-A-2-b. Complement-independent pathway (Figure 5)

The cellular component also plays a major role in AMR. Thus, similarly to acute cellular rejection, of which "immunological synapse" between the T cell and the antigen presenting cell in the interstitial sector is the cornerstone of the pathologic process, an "immunological synapse" is also one of the fundamental mechanisms of the AMR, though in the vascular sector, between the *Natural Killer* (NK) cell and the endothelial cells via FCGR3A (Fc gamma receptor, CD16A) and the DSA (45). Activation of the NK cell will result in:

- the production and release of interferon-gamma (IFNG), *tumor necrosis factor* (TNF), and granulocyte-macrophage colony-stimulating factor (CSF2), increasing i) the cytotoxicity of surrounding monocytes (TNF- α , IL-1, IL-6) and ii) the expressions of CXCL9 and CXCL10, which facilitates the recruitment of new NK cells,
- the production and release of pro-inflammatory chemokines supporting monocytes recruitment (CCL3 and CCL4),
- the release of the content of NK cells granules (perforin, granulysin granzymes A and B) responsible for endothelial lesions via the antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism.

Figure 5 summarizes current knowledge of the pathophysiology of AMR. In the era in which molecular tools play an important role in alloimmune pathology, knowledge of the different cellular and soluble effectors involved in AMR, shown below, is essential to understand the interpretation of transcriptomics data.

« élément sous droit, diffusion non autorisée »

Figure 5: Pathophysiology of antibody-mediated rejection (25)

This figure summarizes the pathophysiology of antibody-mediated rejection in solid organ transplantation. Left side: complement-independent pathways; right side: complement-dependent pathways.

I-A-2. Molecular biology in heart allograft rejection

The complexity of the histological diagnosis of rejection and the limitations of the pathologic analysis of the EMB led to the development of new tools in order to improve and standardize the usual diagnostic classification. The application of different molecular biology techniques to allograft rejection has resulted in a scientifically valuable production that has allowed progress both in understanding pathophysiology and in clinical applications. Several techniques have been applied, from micro-array genome-wide techniques to the most recent "targeted-gene expression" techniques such as NanoString technology, through targeted analysis of individual transcripts by qPCR. In the remainder of this chapter, we will only develop messenger RNA (mRNA) expression assays, although many high quality studies have assessed the diagnostic value of analysis of small non-coding RNA expression in tissues (microRNA) (46–48).

I-A-2-a. Genome-wide expression

I-A-2-a-1. Description

Microarray technology has made possible to improve our understanding of the pathophysiology of allograft rejection, by allowing large and a priori-free analyzes of the expression of several tens of thousands of transcripts simultaneously. Our understanding of AMR has been particularly impacted by these techniques (45). Each DNA microarray contains several tens of thousands of probes, which are synthetic DNA sequences specific for the gene whose expression is to be analyzed. After extraction of mRNAs and their retro-transcription into labeled complementary DNAs (cDNAs), these cDNAs are to be put in contact with the chip. During the hybridization step, the cDNAs are to hybridize to the specific sequences of the probe attached to the array. After washing off the unbound cDNAs, the array is then analyzed by a high-resolution scanner at the excitation wavelength of the fluorochrome.

I-A-2-a-3. Pathogenesis-based transcripts (PBT)

The comparison of molecular expression profiles between acute cellular rejections, AMR and non-rejection biopsies, allowed the detection of several tens of differentially expressed transcripts (45,49–51). For example, CD8A (a coreceptor expressed on the surface of the cytotoxic T lymphocyte and allowing interaction with HLA class I molecules), ADAMDEC, CTLA4 (costimulatory signal) are particularly expressed in

acute cellular rejection. Conversely, molecules such as CXCL9 and 10 (chemokines promoting the recruitment of NK cells), CCL4, PLA1, are more expressed in AMR (Figure 6). Interestingly, these molecular profiles specific of rejections are comparable between renal and heart transplantations, suggesting the presence of common underlying pathophysiological mechanisms. (32,45).

« élément sous droit, diffusion non autorisée »

Figure 6: T-cell mediated rejection and antibody-mediated rejection-selective transcripts (51).

The dots represent individual microarray probe sets. The x axis illustrates the false discovery rate-adjusted P value for the association of each transcript with the diagnosis of rejection, with the fold change on the y axis. Red: top 30 TCMR-selective, blue: top 30 ABMR-selective, black: top 30 rejection-associated.

A first step for the integration of these molecular results is the consideration of the underlying pathophysiological processes. The Pathogenesis PBTs represent defined major biological pathways of the underlying disease. Applied to the diagnosis of allograft rejection, several PBT have been defined: infiltration of macrophages (QCMAT), infiltration of NK cells (NKB), infiltration of T cells (TCB), gamma-interferon (GRIT1), endothelial transcripts (ENDATs) and Donor-Specific Antibody transcripts (DSAST) (32).

I-A-2-a-3. Molecular assessment of disease states

Since no transcript is either perfectly sensitive or specific to a type of rejection, it is necessary to compare averaged expressions of groups of transcripts to obtain practical information about rejection classification. This is the second step of integration. Several statistical tools are available, ranging from unsupervised statistical analysis methods such as "archetype analysis" to supervised techniques for creating binary "classifiers" based on the use of the most differentially expressed transcripts for each type of rejection through principal component analysis. These different statistical methods make it possible to translate the huge sum of molecular data into a simplified information on molecular diagnostic probabilities (32,52).

I-A-2-b. Targeted-gene expression analysis

Various targeted-gene expression techniques aimed at analyzing the expression of the genes involved in rejection have been applied for the diagnosis of heart allograft rejection. Although they only involve a limited number of pre-selected transcripts of interest, these techniques remain robust, even in case of degradation of the mRNA quality, and can therefore be applied to FFPE biopsies. This approach has many advantages in the context of an application in clinical practice: i) the extra-byte required for genome-wide techniques is no longer mandatory, ii) the high number of available biopsies would allow a rapid validation of the targeted molecular signatures, and iii) the concomitant routine pathologic analysis of the same fragment analyzed by molecular biology is now possible (limiting the effect of sampling bias).

I-A-2-b-1. q-PCR

The qPCR technique was applied in the 2000s for the diagnosis of graft rejection. (53–55). Although this have helped us to begin to understand the pathophysiology of AMR as never before, these isolated PCR techniques failed to transition into transplantation clinical practices, both due to the important prerequisites necessary for the selection of a very limited number of transcripts of interest (one hypothesis and the analysis of one pathophysiological pathway), and also due to the clinical, pathologic and molecular variability of allograft rejections, which explains why a single transcript is neither sufficiently sensitive nor specific enough to establish a diagnosis of rejection. It is therefore essential to be able to simultaneously analyze several transcripts

corresponding to several pathophysiological pathways in order to gain both sensitivity and specificity.

I-A-2-b-2. NanoString

NanoString's "nCounter Analysis System" is based on a digital molecular barcoding technology based on direct multiplexed measurement of gene expression. Each color-coded barcode is attached to a single target-specific probe corresponding to a single gene which can be individually counted without amplification (56). The lack of amplification step, sources of potential errors and variability between samples is an important asset of this technology.

This molecular biology technique was applied to the diagnosis of AMR on FFPE EMB by the Edmonton team. On the basis of the results of genome-wide studies and after selection of pathophysiological pathways of interest, a set of 38 transcripts was selected comprising 18 endothelial transcripts, 10 inflammation / interferon- γ transcripts, 6 NK transcripts and 4 housekeeping genes. AMR-gene set expression was significantly higher in the AMR compared with the ACR and the control biopsies, higher in patients with DSA compared with patients without DSA, strongly associated with the degree of endothelial swelling, and showed greater accuracy for the diagnosis of AMR than for DSA or C4d (33). Although promising, the costs inherent in this technology, both at the time of the initial investment and for the reagents used in routine, might be a barrier to its use in clinical practice.

I-A-2-c. Retro-Transcription Multiplex Ligation-dependent Probe Amplification (RT-MLPA)

RT-MLPA is a multiplex PCR technique able to analyze relative gene expression profiles. This molecular biology technique has never been applied to the diagnosis of allograft rejection but presents various points of interest in comparison with other molecular approaches that could make it a suitable technique for this indication. Its reliability has already been demonstrated by comparing its use on FFPE tissue with genome-wide techniques on fresh tissue, particularly in the diagnosis of hematological malignancies (57,58). It is an inexpensive technique applicable in a routine practice of a pathology laboratory without major investments. In addition to its rapidity and low cost, MLPA offers the following advantages: i) only ligated oligonucleotides, i.e., those bound to the cDNA strand, are likely to be amplified, thus avoiding the amplification of

oligonucleotides that have not bound to the target cDNA; ii) the use of a single pair of primers facilitates the amplification and detection of multiple targets, thus avoiding the problems caused by the presence of a large quantity and number of different primers; and iii) this technique can amplify up to 40 different probes simultaneously. We will apply this molecular biology technique to the diagnosis of allograft rejection in a first study.

I-A-3. Objectives

The aim of the two following studies was to assess whether a molecular biology approach might help standardizing the diagnosis of AMR in heart transplantation (Figure 7).

We hypothesize i) that **RT-MLPA**, is applicable to paraffin-fixed EMB and might be a support for the **pathologic diagnosis** of allograft **rejection** by identifying a **rejection-specific molecular signature** (no rejection, acute cellular rejection, AMR), and ii) that a **semi-quantitative evaluation of microvascular cardiac inflammation** in four stages is correlated with **the molecular activity of AMR** and could have a role in the **diagnostic standardization and prognostic stratification of AMR** (Figure 7).

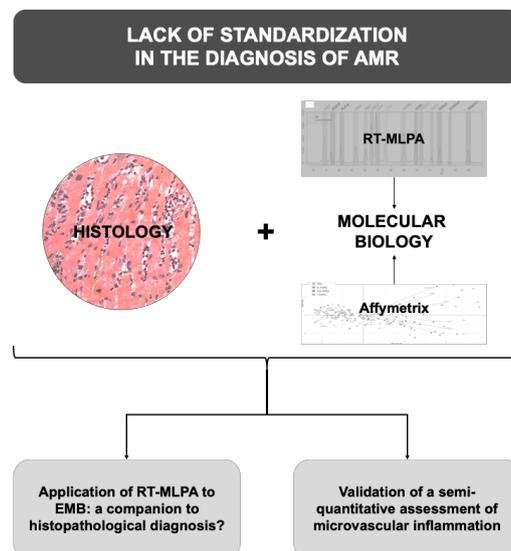


Figure 7: Lack of standardization in the diagnosis of antibody-mediated rejection: strategies based on molecular biology techniques to refine AMR diagnosis.

This figure summarizes the development of two techniques of molecular biology applied in our studies aimed at standardizing the diagnosis of AMR in heart transplantation.

AMR means antibody-mediated rejection, EMB: endomyocardial biopsy, RT-MLPA: Reverse Transcriptase-Multiplex Ligase-dependent Probe Amplification.

Chapter I-B: Application of Multiplex Ligation-dependent Probe Amplification to endomyocardial biopsies.

Article in revision in the Journal of Heart and Lung Transplantation.

I-B-1. Molecular biology applied to the diagnosis of heart allograft rejection

Protocol endomyocardial biopsies are a key element of the follow-up of heart transplant recipients (34). The histological analysis of myocardial samples is the gold standard for the diagnosis of heart allograft rejections and guides therapies (29,59). However, this histology-based strategy is limited by several flaws. The reliability of histological analysis is nowadays questioned as inter-observer reproducibility has been reported to be as low as 28% for the diagnosis of acute cellular rejection (31). Besides, the histological grading may be particularly challenging for microvascular inflammation, a key criterion for the diagnosis of antibody-mediated rejection (AMR) (27). Reliable, objective and disease-specific (i.e., rejection versus non-rejection and acute cellular rejection – ACR – versus AMR) markers of rejection are an important unmet clinical need (30).

Gene expression analysis of the allograft tissue based on pan-genomic approaches has been increasingly recognized as a powerful tool for the understanding of the pathophysiological pathways of rejection after heart transplantation (32,45). However, several flaws may limit the impact of these techniques in everyday practice and their widespread use to improve the diagnosis of rejection. Targeted gene expression analysis has emerged as a potentially rapid, easy-to-use, and cost-effective alternative to pan-genomic approaches (60,61). Reverse Transcriptase-Multiplex Ligase-dependent Probe Amplification (RT-MLPA) is an efficient technique of targeted gene relative expression analysis suitable for formalin-fixed paraffin-embedded (FFPE) tissue (57,62).

The underlying principle of MLPA is to make the amplification step of the hybridized probes dependent on a ligation between the two probes specific for the transcript of

interest (Figure 8). In practice, for each transcript of interest, a pair of specific probes must be designed. These two probes must be complementary to two adjacent sequences of the cDNA to be analyzed. Each probe must comprise at one end a PCR primer sequence common to the other pairs of probes, allowing a common amplification step at the end of the reaction using a single primer pair. Spacers are to be added to the 3' end of the probe to obtain lengths of final amplicon specific to each transcript of interest. Finally, the amplification products are analyzed by fragment analysis using an ABI 3130 XL capillary electrophoresis analyzer makes it possible to obtain gene expression profiles.

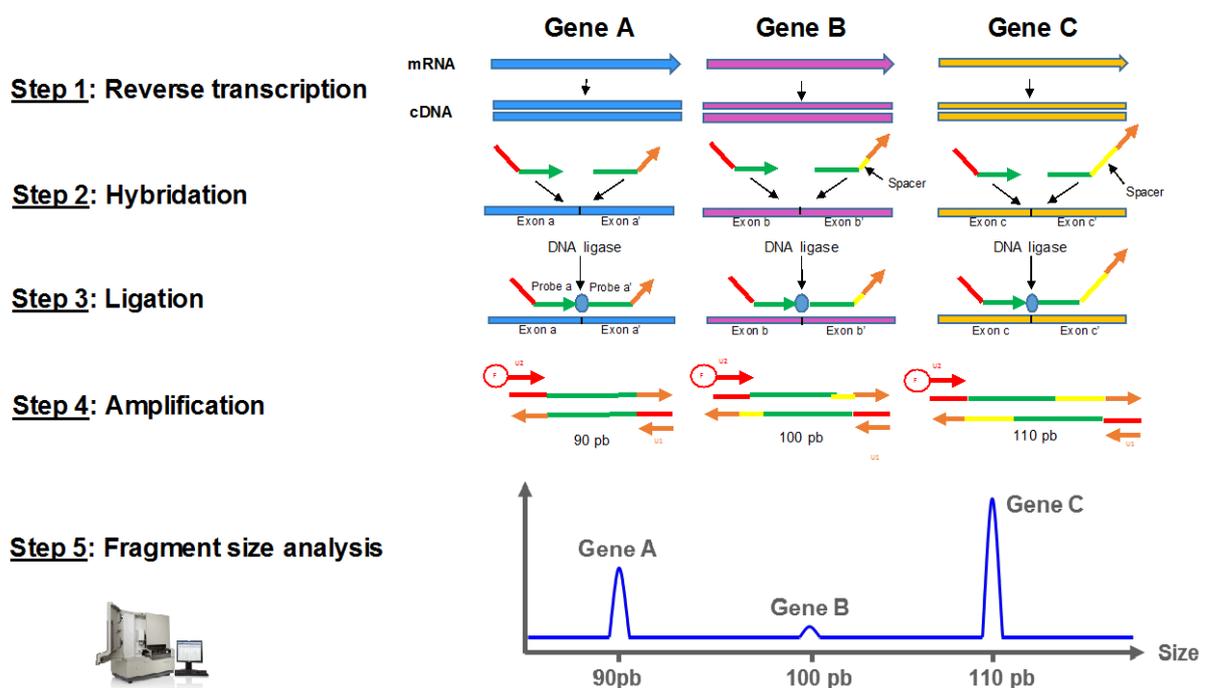


Figure 8: Principle of Multiplex Ligation-dependent Probe Amplification.

Synthesis of the 5 steps of RT-MLPA : reverse transcription, hybridation, ligation, amplification and fragment size analysis. Example of application of RT-MLPA to the analysis of the expression of a 3-transcript set. Each couple of probes contains a specific sequence of the gene to analyze (green), a common primer sequence (red and orange) and a spacer of variable length (yellow).

Our aim was to assess RT-MLPA for the diagnosis of allograft rejection in heart transplantation. Using a 14-transcript RT-MLPA signature, we built a two-step class prediction analysis and compared molecular diagnosis (non-rejection, AMR, and ACR) to histopathology.

I-B-2. Material and methods

I-B-2-a. Study design

We performed a retrospective, case-control, multicenter study. A total of 206 EMB were selected (allograft rejection: n=148, no rejection: n=58) corresponding to 206 adult heart transplant recipients monitored from five French heart transplant centers (Georges Pompidou European hospital - Paris, La Pitié-Salpêtrière - Paris, Rouen, Strasbourg and Nantes University Hospitals) between November 2005 and April 2017. All RNAs were extracted from FFPE tissues previously used for routine histopathologic diagnosis of rejection. Twenty-three biopsies (11.2 %) failed to pass RNA quality control and were excluded. The remaining 183 patients were randomized into a training series (n=113) and a validation series (n=70). A two-step class prediction analysis (Rejection versus non-rejection; AMR versus ACR) was developed from the training series and applied to the validation series. Our principal objective was to define the agreement between pathology and RT-MLPA. The study design is summarized in Figure 9.

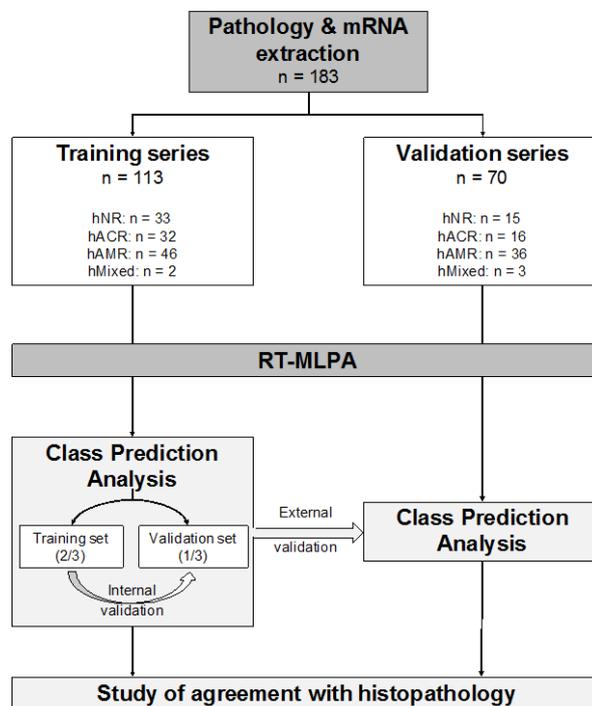


Figure 9: Flowchart of the RT-MLPA study

183 endomyocardial biopsies corresponding to 183 heart allograft recipients were selected for the study and randomized into a training (n=113) and a validation series (n=70). hACR: histological diagnosis of acute cellular rejection, hAMR: histological diagnosis of antibody-mediated rejection, hMixed: histological diagnosis of mixed rejection and hNR: histological diagnosis of non-rejection.

I-B-2-b. Patients

Our study complies with the Declaration of Helsinki and the institutional review board approved the protocol. Written informed consent for data collection and gene expression analysis of a stored EMB was obtained at the time of enrolment in the study. Baseline donor and recipient characteristics were collected from the prospective national registry CRISTAL database (French National Agency for Organ Procurement). Immunological and immunosuppressive regimen data were retrospectively collected from the patients' charts. Codes were used to ensure strict donor and recipient anonymity.

I-B-2-c. Histopathology

A review of all the EMB was performed by two pathologists, independently of the initial pathologic diagnosis, clinical, and molecular data. EMB specimens were formalin-fixed, paraffin-embedded, and routinely stained with hematoxylin and eosin. Immunohistochemistry was performed in tissue sections with rabbit monoclonal rabbit anti-C4d and mouse monoclonal anti-CD68 antibodies using the immunoperoxidase method, as previously described (63). EMB were evaluated for rejection according to the last ISHLT classification for acute cellular rejection (ACR), and to the last recommendations for the diagnosis of AMR (29,30,59). Mixed rejection was defined by the presence of ACR $\geq 2R$ and pAMR $\geq pAMR2$. Histological low-grade rejections were defined as ACR 1R and/or pAMR1(I+) rejections. Histopathology is illustrated in Figure 10-A/C.

I-B-2-d. Detection of antibodies against donor-specific HLA molecules

HLA typing of transplant recipients was performed by molecular biology using the Innolipa HLA typing kit (Innogenetics, Belgium). Heart transplant donors, HLA-A, -B, -DR, and -DQ tissue-typing was performed using the microlymphocytotoxicity technique with tissue-typing trays (One-Lambda Inc, Canoga Park, CA). Briefly, identification of anti-HLA donor-specific antibodies (DSA) was performed by Luminex SA (LuminexLABScreen Single Antigen, One-Lambda Inc) as previously described (64). The value of 500 Mean Fluorescence Intensity (MFI) in our laboratory was based on the criteria of validation of negative control sera.

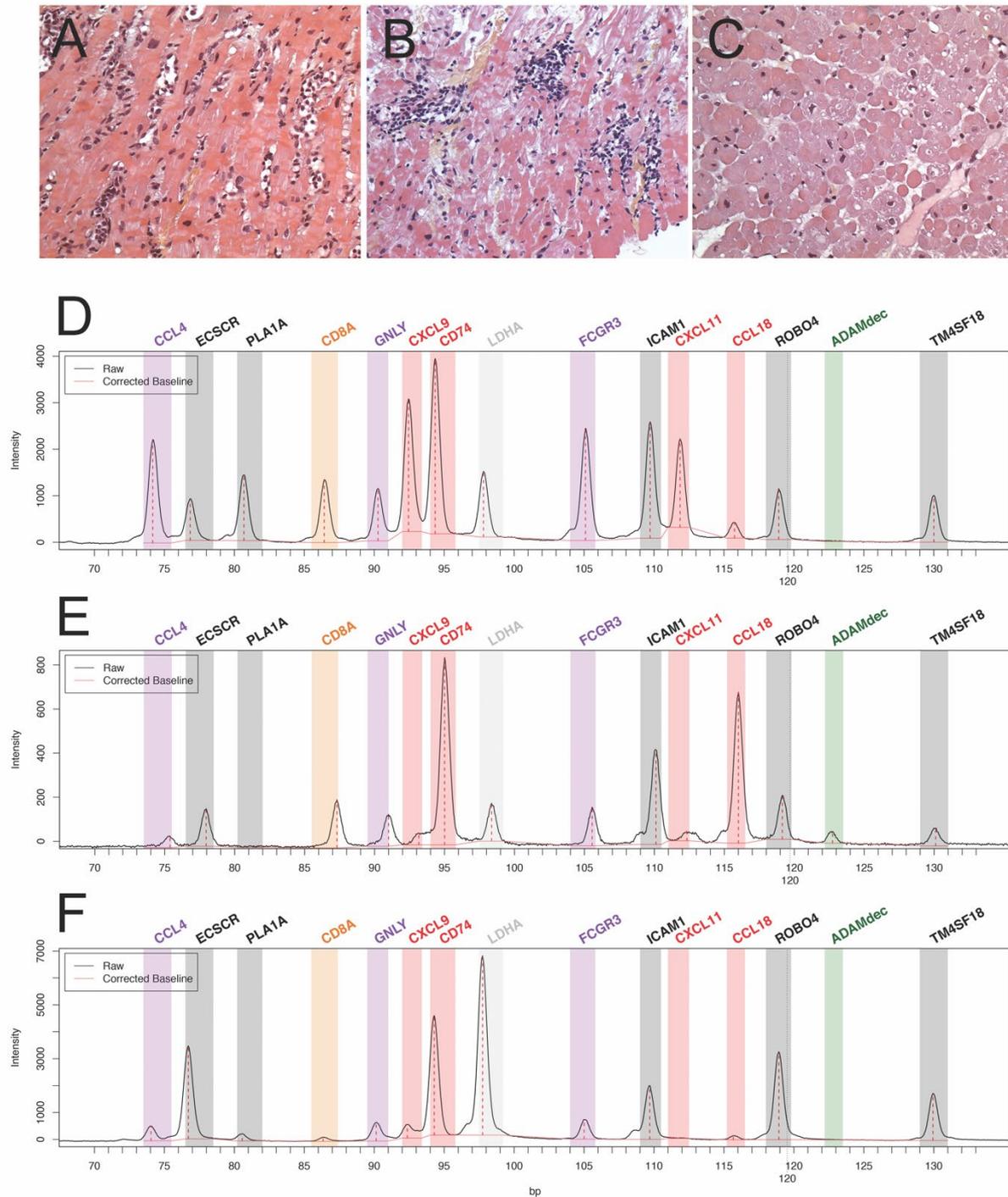


Figure 10: Histopathology and RT-MLPA profiles

A-C. Histopathology.

A- Antibody-mediated rejection: Microcirculation inflammation defined by intravascular activated mononuclear cells accumulation in capillaries.

B- Acute cellular rejection: perivascular lymphocytic infiltrates.

C- Non-rejection EMB with no myocardial inflammation.

D-F. RT-MLPA profiles.

Molecular profile of antibody-mediated rejection (D) with high expression of CCL4, PLA1A, GNLY, CXCL9, FCGR3 and CXCL11 and of acute cellular rejection (E) with high expression of ADAMdec and CCL18 as compared to non-rejecting control EMB (F).

I-B-2-e. RT-MLPA probes design

Fourteen transcripts were included in the RT-MLPA assay. Genes were selected from the Affymetrix and NanoString literature for their performance to diagnose rejection and to distinguish between AMR and ACR (32,45,33,50). They included interferon-induced genes (CXCL11, CXCL9), endothelial activation related genes (PLA1A, ROBO4, ECSCR, TM4SF18, ICAM-1), NK related genes (FCGR3, CCL4, GNLY), cytotoxic T lymphocytes (CD3, CCL18), and antigen presentation (CD74 and ADAMDEC1). A housekeeper gene was added to evaluate the global gene expression level (LDHA). The probe's design has been described by Bobée et al (57). Briefly, all 5' probes presented a common 5' end tail corresponding to the fluorescent labelled PCR probe illu2-FAM. In addition to the specific region, 3' probes presented a common sequence at their 3' end, a phosphorylated end for the ligation reaction at their 5' extremity and spacer sequences between the specific and common regions to allow the separation of the amplicons during the fragment size analysis. The common sequences were used to amplify all ligation products within a single PCR reaction. Probes used in the mix are detailed in Table 1.

I-B-2-f. RT-MLPA procedure

The protocol and data processing of the RT-MLPA procedure were used as previously described, using MLPA reagents from MRC-Holland (Amsterdam, The Netherlands) (57). RNA extraction: All RNAs were extracted from FFPE tissues previously used for routine histopathologic diagnosis of rejection. RNA isolation from paraffin blocks was performed using the 16 LEV FFPE Maxwell kit and the Maxwell 16 automation system (Promega, Madison WI) using the manufacturer's instructions. Reverse transcription: 2 mL of total RNA from the FFPE EMB were added to 3.75 mL of reverse transcription mix containing random hexamer primers. Next, 0.5 mL of Moloney murine leukemia virus reverse transcriptase was added, and the samples were incubated for 15 minutes at 37°C for cDNA synthesis, heated for 2 minutes at 98 °C, and cooled at 4 °C. Ligation: 3 mL of RT-MLPA probe mix was added (1.5 mL of SALSA-MLPA buffer and 1.5 mL of final dilution probe mix) before denaturation at 95°C for 2 minutes and hybridization at 60 °C for one hour. Ligation of the annealed oligonucleotides was performed at 54 °C for 15 minutes, adding 32 mL of ligation mix, and heated for 5 minutes at 98 °C. PCR: 2.5 mL of the ligation mixture was added to 7.5 mL of Salsa PCR master mix

I-B-2-g. Statistical methods

We report means and standard deviations to describe continuous variables, and numbers and percentages for categorical variables. We compared the means and counts with the Student t-test or ANOVA and χ^2 test, respectively. Unsupervised clustering analysis and dendrograms of the molecular profiles obtained after RT-MLPA procedures were performed with the hcluster module of the amap package in the R software. A class prediction analysis was developed to assign each molecular profile obtained by RT-MLPA with a molecular diagnosis: mNR (molecular non-rejecting), mACR (molecular ACR) and mAMR (molecular AMR). A two-step linear predictor score (LPS) method was applied to train two consecutive Bayesian predictors. The first (LPS1) was built to identify rejection cases (rejection vs mNR) and the second (LPS2) to discriminate mAMR from mACR cases. Each LPS was first developed in the training series that was randomly divided into two subgroups. The first subgroup ($\frac{2}{3}$ of the profiles = training set) and the second subgroup ($\frac{1}{3}$ of the profiles = validation set) were used to create the model and to assess its internal validation, respectively. External validation was then performed using the validation series (Figure 9). Statistical significance was set at $p \leq 0.05$. All tests were two-sided. Statistical analyses were performed using STATA 15.0 software (StataCorp LP, College Station, TX) and R software.

I-B-3. Results

I-B-3-a. Patients characteristics and histopathology

The clinical and histopathologic characteristics of the 183 included patients are summarized in Table 2. In brief, patients were mostly males (66%) and mean age at transplant was 46.7 ± 14 years old. Almost three-quarters of the patients received an induction therapy based on anti-thymocyte globulin (ATG) infusions. The median time between heart transplantation and EMB was 60.7 ± 15 months, and 16% of EMB were performed in the six weeks following heart transplantation. Most of the patients were on triple immunosuppressive therapy at the time of EMB (calcineurin inhibitors, mycophenolate mofetil, and corticosteroids) and an anti-HLA DSA was present in 41.5% of the cases. Baseline characteristics were similar between the training and the validation series.

	Training series	Validation series	p value
Donor age (years)	40.9±14	41.6±13.3	0.76
Donor gender (male: n, %)	75 (67.6)	46 (65.7)	0.48
Recipient age (years)	47.5±14.3	45.6±13.4	0.37
Recipient gender (male: n, %)	85 (75.2)	51 (72.9)	0.72
Cold ischemic time (min)	180±59	187±61	0.48
Primary Heart disease			
- Dilated cardiomyopathy (n,%)	60 (53.1)	33 (47.1)	0.68
- Ischemic cardiomyopathy (n,%)	33 (29.2)	24 (34.3)	
- Other (n,%)	20 (17.7)	13 (18.6)	
Induction regimen			
IS regimen at biopsy			
Time post-transplant (months)	48±64	34±55	0.12
Pathology			
No rejection (n,%)	33 (29.2)	16 (22.9)	0.81
hAMR (n,%)	46 (40.7)	31 (44.3)	
- pAMR1(I+) (n,%)	7 (6.2)	6 (8.6)	
- pAMR1 (H+) (n,%)	8 (7.1)	3 (4.3)	
- pAMR2-3 (n,%)	31 (27.4)	22 (31.4)	
hACR (n,%)	32 (28.3)	22 (31.4)	
- 1R (n,%)	5 (4.4)	13 (18.6)	
- 2R (n,%)	15 (13.3)	7 (10)	
- 3R (n,%)	12 (10.6)	2 (2.8)	
hMixed rejection* (n,%)	2 (1.8)	1 (1.4)	

Table 2: Characteristics of patients and endomyocardial biopsies

hACR means histological diagnosis of acute cellular rejection, mAMR: histological diagnosis of antibody-mediated rejection, hMixed rejection: histological diagnosis of mixed rejection, IS regimen: immunosuppressive regimen.

I-B-3-b. RT-MLPA profiles on FFPE endomyocardial biopsies

Examples of RT-MLPA profiles in EMB with a pathologic diagnosis of AMR, ACR and no rejection are given in Figure 10-D/F. AMR was characterized by high expression of CCL4, PLA1A, GNLY, CXCL9, FCGR3, and CXCL11. ACR was characterized by high relative expression of CCL18 and ADAMdec (Figure 11).

Unsupervised hierarchical analysis of these profiles is given in Figure 12-A. Three molecular clusters were delineated, that corresponded to the pathologic diagnosis of the non-rejection, ACR, and AMR, respectively.

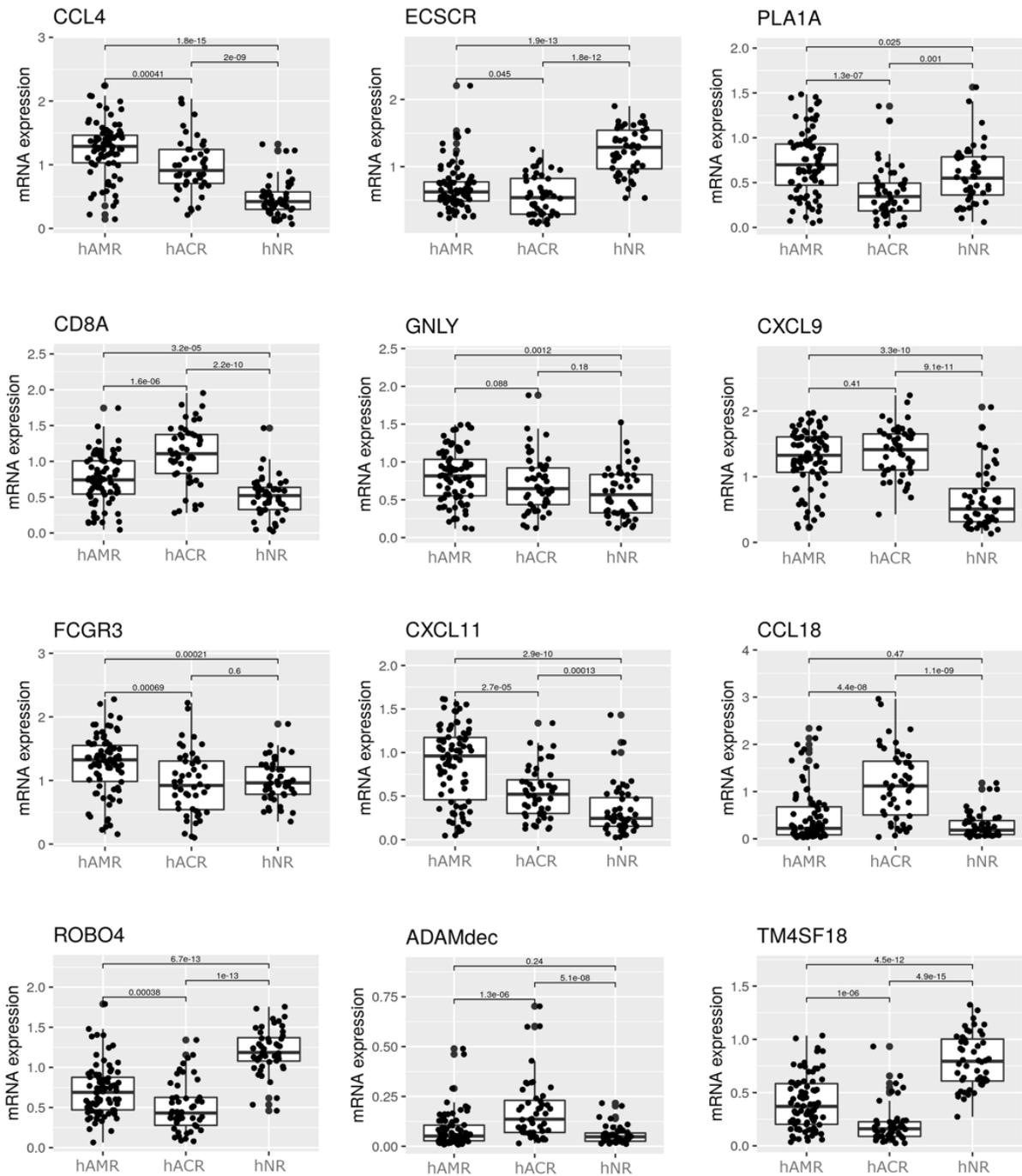


Figure 11: Expression of transcripts according to histological diagnosis

CCL4, PLA1A, CXCL9, FCGR3, CXCL11, and TN4SF18 were overexpressed in hAMR biopsies compared to histological non-rejection. CD8A, CXCL9, CCL18, and ADMAdec were overexpressed in hACR biopsies compared to histological non-rejection. ECSCR, ROBO4, and TM4SF18 were under expressed in histological rejection, either AMR or ACR, compared to histological non-rejection. hAMR: histological antibody-mediated rejection (n=82), hACR: histological cellular rejection (n=48), hNR: histological non-rejection (n=48). Mixed rejection (n=5) is not shown.

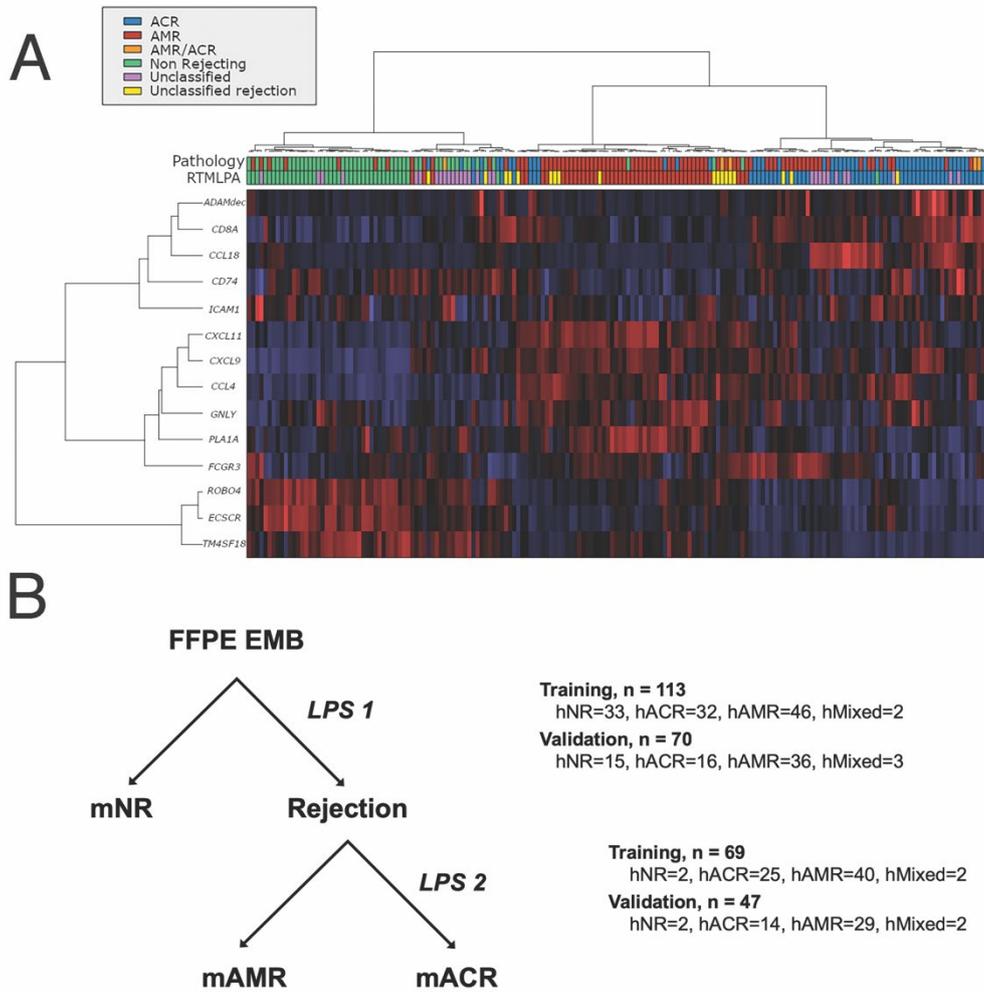


Figure 12: Unsupervised hierarchical and class prediction analysis

A. Unsupervised hierarchical analysis of RT-MLPA analysis from the 183 EMB. Histological diagnosis is provided on the upper line (Pathology). Three clusters of molecular patterns are delimited by the relative expression of the 14 selected transcripts. The lower line (RTMLPA) provides the molecular diagnosis according to the two steps in the class prediction analysis. Unclassified: Cases unclassified by LPS1. Unclassified rejection: cases classified as rejection by LPS1 but unclassified by LPS2 (see below).

B. The principle of the RT-MLPA prediction model for rejection. The RT-MLPA assay consists of two consecutive linear prediction scores (LPS). The first LPS is designed to identify rejection cases among all EMB, whereas the second is designed to discriminate mAMR from mACR.

LPS means Linear prediction score, mACR: molecular acute cellular rejection, mAMR: molecular antibody-mediated rejection, mNR: molecular non-rejection.

I-B-3-c. Class Prediction Analysis

The steps of class prediction analysis are summarized in Figure 12-B. A two-step linear predictor score (LPS) method was applied to train two consecutive Bayesian predictors. The first (LPS1) was built to identify rejection cases (Rejection vs. mNR) and the second (LPS2) to discriminate mAMR from mACR cases. Each LPS was first developed in the training series and applied in the validation series. The details of the performance of LPS1 and LPS2 in the training series is given in Figure 13. LPS1 identified 28 (24.8%) and 10 (14.3%) mNR, 69 (61.1%) and 47 (67.1%) Rejection, in the training and validation series, respectively. LPS2 identified 31 (44.9%) and 15 (31.9%) mAMR, 31 (44.9%) and 21 (44.7%) mACR, in the training and validation series, respectively. Overall 29 cases (15.8%) and 18 (15.5%) cases were left unclassified by LPS1 and LPS2, respectively.

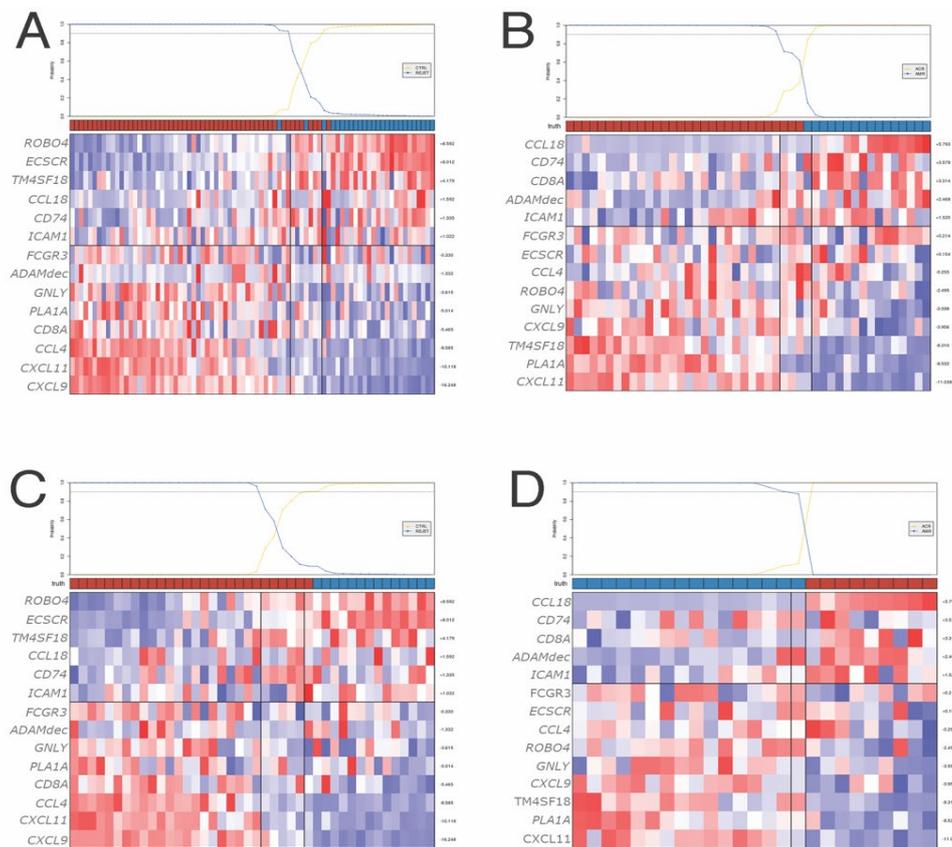


Figure 13: Class Prediction Analysis

A and B: Development and internal validation of LPS1. The most discriminant transcripts between mNR and rejection were ROBO4, ECSCR, TM4SF18, CCL4, CXCL11, and CXCL9.

C and D: Development and internal validation of LPS2. The most discriminant transcripts between mAMR and mACR were CCL18, CD74, CD8A, ADAMDEC1, PLA1A, TM4SF18, and CXCL11.

The confidence threshold to classify a sample was set at 90 percent.

I-B-3-d. Agreement between histology and RT-MLPA

Table 3 details the results of the agreement between the histopathology and RT-MLPA in the training series (n=97), validation series (n=57), and overall cohort (n=154) after exclusion of the cases left unclassified by the class prediction analysis. RT-MLPA and histopathology agreed for the diagnosis of rejection in 91.8%, 82.9%, and 92.2% of the EMB in the training, validation, and overall cohort, respectively. In rejecting cases, an agreement for the type of rejection (AMR or ACR) was achieved in 86.7%, 66.7%, and 79.2% of the EMB in the test (n=60), validation (n=36), and overall cohort (n=96), respectively. Finally, RT-MLPA and histopathology agreed for a final diagnosis (No rejection, AMR, or ACR) in 82.2%, 66.7%, and 76.8% of the EMB in test, validation and overall cohort, respectively.

Agreement for the diagnosis of rejection	
- Test series (n=97)	89 (91.8%)
- Validation series (n=57)	53 (82.9%)
- Overall (n=154)	142 (92.2%)
Agreement for the type of rejection	
- Test series (n=60)	52 (86.7%)
- Validation series (n=36)	24 (66.7%)
- Overall (n=96)	76 (79.2%)
Agreement for the final diagnosis	
- Test series (n=90)	74 (82.2%)
- Validation series (n=48)	32 (66.7%)
- Overall (n=138)	106 (76.8%)

Table 3: Agreement between histopathology and RT-MLPA

I-B-3-e. Disagreement between histology and RT-MLPA

A summary of the disagreements between histopathology and RT-MLPA for the diagnosis of rejection (n=12) and for the type of rejection (n=20) is given in Table 4. Twelve cases (6.5%) were discordant between histology and molecular diagnosis by LPS1 including eight cases of mNR diagnosis with histological rejection (mostly low-grade rejection, n = 5 (62.5%): pAMR1(I+): n=4; ACR 1R: n=1) and four cases of hNR with molecular rejection. Interestingly, graft function was severely impaired at the time of EMB in two out of four of the hNR cases with molecular rejection. The histological grade of rejection was significantly associated with the risk of discordance by LPS1, which was higher in case of the histological low-grade rejection ($p < 0.01$).

Twenty cases (17.2%) were discordant between the histology and molecular diagnosis by LPS2 including 13 cases (65%) of hAMR classified as mACR. The time post-transplant was significantly associated with the risk of discordance by LPS2, which was higher in patients with EMB < six weeks post-transplant ($p = 0.02$).

Pathology	RT-MLPA	n (%)
For the diagnosis of rejection (n=12)		
No rejection	mAMR	2 (16.7%)
No rejection	mACR	0 (0%)
No rejection	Rejection	2 (16.7%)
pAMR1(I+)	mNR	4 (33.3%)
pAMR1(H+)	mNR	1 (8.3%)
pAMR2-3	mNR	1 (8.3%)
ACR 1R	mNR	1 (8.3%)
ACR 2R	mNR	1 (8.3%)
ACR 3R	mNR	0 (0%)
Mixed	mNR	0 (0%)
For the type of rejection (n=20)		
pAMR1(I+)	mACR	2 (10%)
pAMR1(H+)	mACR	2 (10%)
pAMR2-3	mACR	13 (65%)
ACR 1R	mAMR	2 (10%)
ACR 2R	mAMR	1 (5%)
ACR 3R	mAMR	0 (0%)

Table 4: Details of disagreement between histopathology and RT-MLPA

ACR means acute cellular rejection, AMR: antibody-mediated rejection, mACR: molecular diagnosis of acute cellular rejection, mAMR: molecular diagnosis of antibody-mediated rejection, mNR: molecular diagnosis of non-rejection.

I-B-4. Discussion

We described a first evaluation of the diagnostic performance of RT-MLPA, a technique of targeted gene expression analysis suitable for FFPE EMB to detect biopsy-proven rejection in heart transplantation. By studying the relative expression of 14 selected transcripts (endothelial burden, NK cells, interferon- γ pathway, effector T-cell and antigen presentation) on 183 EMB, the unsupervised analysis identified three clusters of gene expression. A two-step class prediction analysis was developed to assign each molecular profile obtained by RT-MLPA with a molecular diagnosis: molecular non-rejection (mNR), mACR and mAMR. Overall agreement between RT-MLPA (mNR, mACR and mAMR) and histopathology (hNR, hACR and hAMR) were

92.2%, 79.2% and 76.8% for the diagnosis or rejection, the type of rejection and the final diagnosis, respectively.

Reliable, objective and disease-specific markers of rejection are an important unmet clinical need in the field of heart transplantation. Gene expression analysis of the allograft tissue based on pan-genomic approaches has been increasingly recognized as a powerful tool for the discovery and the understanding of the pathophysiological pathways of rejection in solid organ transplant (51,65,66). Recently, the analysis of the expression of a 34-gene set using the NanoString nCounter demonstrated the efficiency of this technology in the field of AMR in heart transplantation (33). As underlined by the Banff 2017 meeting report, the molecular assessment using new methods of targeted-gene expression of a selected gene-set from FFPE routine biopsies is the next step forward for a multicenter validation of the contribution of molecular pathology in the daily clinical practice (40). Within this context, we evaluated the diagnostic performance of RT-MLPA, a new cheap, robust and easy-to-use technique of multiplex PCR able to analyze the profiles of expression of a selected-gene set from FFPE-biopsies (57). Our aim was to develop a tool able to bring additional value to the gold-standard pathologic assessment of EMB and to help both pathologists and clinicians in case of uncertainty. We wanted to introduce a two-step approach allowing us to discriminate non-rejection from rejection and ACR from AMR. We believe this approach fits clinical practice. The selection of our 14-transcript set was based on the Affimetrix and NanoString literature. A major interest of our technique is its applicability to FFPE EMB. First, this allows histopathologic assessment of the myocardial samples analyzed, excluding the non-specific lesions such as fibrosis, fibrin clot, previous biopsy site or pericardial tissue. Second, this technique can be included in a standard of care procedure since an extra-bite for a fresh-frozen sample is not required, thus limiting the risk of potential procedural complications. Third, we can analyze simultaneously all myocardial samples of a paraffin block. The focal distribution of the rejection accounts for the high variability of myocardial samples and the need for at least three samples to accurately assess rejection (29,59). Finally, RT-MLPA is a cost-effective, strong and easy-to-use technique based on common instruments and reagents and could thus easily be implemented into routine diagnosis workflows. Using the RT-MLPA procedure and class prediction analysis, the agreement between molecular and pathology diagnoses was high (92.2%, 79.2% and

76.8% for the diagnosis of rejection, the type of rejection and the final diagnosis, respectively). However, we observed several discordant cases. At the level of diagnosis of rejection (LPS1), discordant cases included mainly cases with low-grade of rejection (pAMR1(I+), ACR1R) classified as non-rejection by RT-MLPA. Such low-grade rejections have previously been identified as molecularly quiescent according to Affimetrix and NanoString nCounter studies (33,45,49). Moreover, the clinical relevance of isolated subclinical pAMR1(I+) and ACR 1R is debated and international guidelines currently do not recommend their treatment (27,34). Other discordant cases included cases with normal histology (hNR) that were classified as molecular rejection. Interestingly, two of these cases had severe graft dysfunction at the time of biopsy and could correspond to “biopsy-negative” rejections (67). Disagreements between histology and RT-MLPA for the diagnosis of the type of rejection corresponded mostly to hAMR being classified as mACR. Early post-transplant EMB were at higher risk of disagreement at the LPS2 level. Induction therapy, ischemia-reperfusion lesions, inflammatory post-transplant burden might modify gene expression and make it more difficult to distinguish AMR from ACR.

The present study should be interpreted within the context of its limitations. Our study was not designed to assess the performance of RT-MLPA as compared to the molecular gold standard (microarray analysis). However, the molecular expression comparison between these two techniques has already been performed (57). The findings of this retrospective cross-sectional case-control study must be further validated. The next step will be to analyze longitudinally the targeted-gene expression profile of rejections and to evaluate the dynamic changes before rejection and after its treatment.

I-B-5. Conclusion

We demonstrated that RT-MLPA is a suitable method to analyze FFPE EMB in the setting of a clinical transplant practice. A signature of 14 transcripts could distinguish between ACR, AMR, and absence of rejection. Integration of molecular assays to conventional histopathology represent the next step for the development of a reliable gold standard of rejection diagnosis.

Chapter I-C: Extension of intravascular activated mononuclear cells correlates with antibody-mediated rejection molecular activity, donor-specific antibodies and graft function.

I-C-1. Introduction

The 2013 International Society for Heart and Lung Transplantation working formulation for pathologic diagnosis of heart allograft AMR (pAMR classification) includes the presence of microvascular inflammation (MVI) as a major diagnostic criterion. Thus, the term “intravascular activated mononuclear cells” (IAMC) was chosen for cardiac MVI to take into account both swollen activated endothelial cells and intravascular inflammatory cells accumulation in microvascular lumens. In the pAMR classification, significant IAMC was defined as “more than occasional focal aggregates or scattered isolated foci of intravascular activated mononuclear cells” and thus no definitive threshold for IAMC has been proposed. Experiences from kidney transplantation have suggested that the assessment of the severity of MVI on kidney allograft biopsies (glomerulitis and peritubular capillaritis) is of interest not only to define a threshold for the pathologic diagnosis but also to evaluate the disease activity.

A similar evaluation of MVI could be a valuable tool in heart transplantation. As a prerequisite, we conducted the present study to evaluate the biological and clinical meanings of the extension of MVI in endomyocardial biopsies (EMB) using a semi-quantitative score. Our aim was to determine whether the MVI extension scale might be associated with AMR molecular activity, current rejection classifications, DSA and graft function at the time of EMB.

I-C-2. Patients and methods

I-C-2-a. Study design

An assessment of the extension of microvascular inflammation using a semi-quantitative scoring system was retrospectively performed in 398 endomyocardial biopsies from 268 heart transplant recipients. All EMB had molecular studies, either by microarray analysis (n=213) (32,45), or by targeted gene expression analysis on FFPE EMB (see chapter RT-MLPA). The extension of MVI was then compared i) to molecular rejection signatures, ii) to histopathology according to the last ISHLT classifications, iii) to the DSA status and iv) to the left ventricular ejection fraction (LVEF) at the time of the EMB.

I-C-2-b. Patients

Two-hundred and sixty-eight heart recipients from five referral French centers transplanted between January 2004 and June 2017 were included. All recipients underwent routine protocol EMB according to a predetermined schedule or for-cause EMB. Data were collected for donors' and recipients' gender and age, primary heart disease, cold ischemic time, and induction therapy. Donor specific antibody was evaluated by Luminex technology (Luminex LABScreen Single Antigen, One-Lambda Inc) at the time of the endomyocardial biopsy as previously described (45). An MFI value ≥ 500 were considered positive. We selected the highest rank of class I or II DSA MFI (immunodominant DSA). Left ventricular (LV) dysfunction at the time of EMB was defined as an unknown left ventricular ejection fraction (LVEF) $< 50\%$ (1) and/or a drop in LVEF $\geq 15\%$ compared to baseline LVEF.

I-C-2-c. Histology and immunohistochemistry

Endomyocardial biopsies were formalin-fixed, paraffin-embedded, and routinely stained with hematoxylin and eosin. Immunohistochemistry was performed in tissue sections with monoclonal rabbit anti-C4d (Cliniscience, Nanterre, France) and monoclonal anti-CD68 (DakoCytomation, Glostrup, Denmark) using the immunoperoxidase method, as previously described (45,63). All EMB were graded according to the ISHLT guidelines for acute cellular rejection (ACR) and AMR (29,59).

I-C-2-d. Evaluation of the extension of MVI: MVI score

All EMB (n=398) were assessed retrospectively for the extension of MVI by 3 pathologists, two senior heart transplant pathologists and a fellow transplant pathologist. The extension of MVI was evaluated semi-quantitatively using the percentage of the area with microvascular inflammation in capillaries and venules as described in Figure 14: score 0 representing 0% of MVI on the area (negative MVI); score 1 representing 0-10% of MVI on the area (minimal MVI); score 2 representing 11-50% of MVI on the area (focal MVI); score 3 representing >50% of MI on the area (diffuse MVI, Figure 14). Area of previous biopsy site, of replacement fibrosis and in close contact with foci of cellular rejection were excluded for the evaluation of MVI. All EMB were assessed independently by the 3 readers who were blinded for clinical data and for the initial histopathology diagnosis. In case of discrepancies between the readers, the slides were reviewed by the 3 readers altogether to achieve a final consensual score.

I-C-2-e. Molecular pathology

This study was based on the complementary approach of two types of molecular analysis. A pangenomic microarray technology was performed on 213 cases using an additional EMB (that is distinct from the biopsies used for histopathology, either snap frozen or stored in a nucleic acid preservation solution - RNAlater) and Affymetrix GeneChips (Affymetrix, Santa Clara, CA), as previously described (32,45). Microarray gene expression results for each EMB were summarized as Pathogenesis Based Transcript (PBT) scores as previously described and validated. AMR and TCMR classifiers were designed as previously described (66).

The second molecular approach was based on FFPE EMBs, allowing the evaluation of the extension of IAMC and the molecular assessment on the same paraffin block and biopsies. As described in the previous chapter, we applied the RT-MLPA technique to FFPE-EMB.

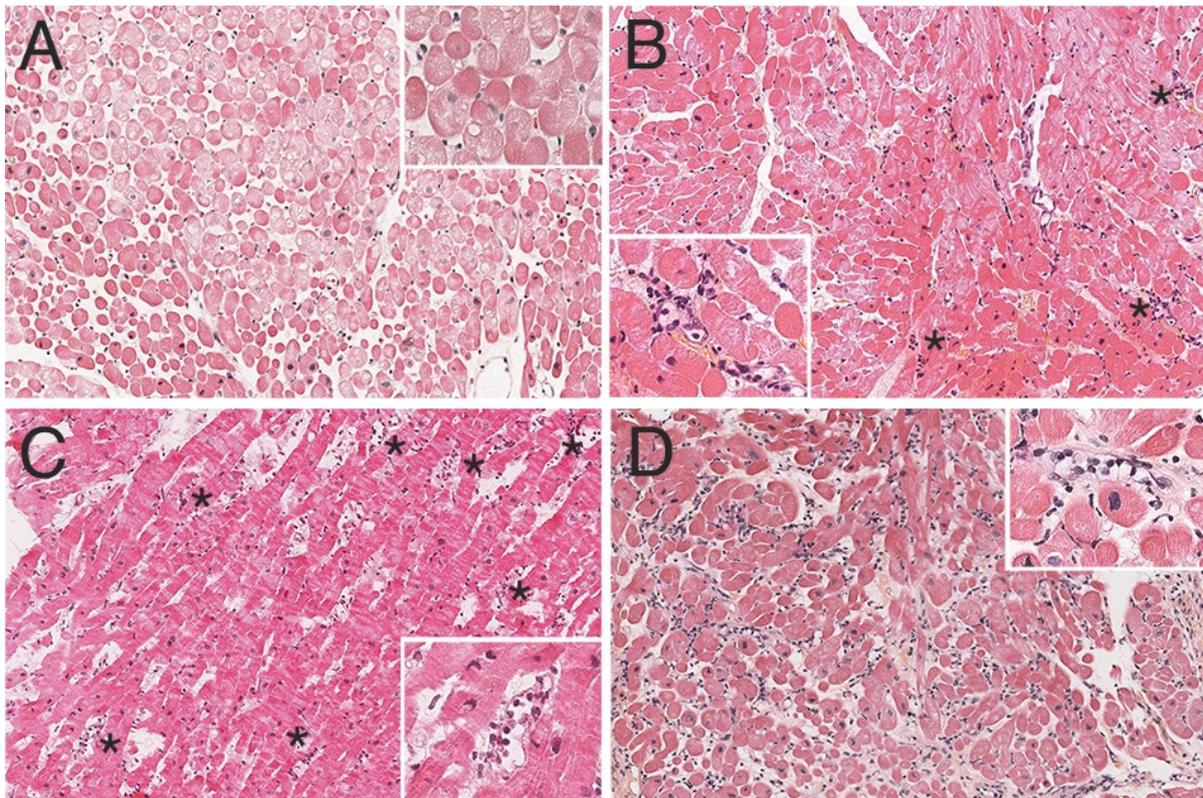


Figure 14: Semi-quantitative assessment of microvascular inflammation using the MVI score.

A: MVI score 0, absence of microvascular inflammation (insert show no inflammatory cell in capillaries); B: MVI score 1, minimal microvascular inflammation defined by presence of inflammatory cells in the capillaries and venules in 10 and less percent of the surface of the biopsy; C: MVI score 2, focal microvascular inflammation defined by presence of inflammatory cells in the capillaries and venules in more than 10 percent and less than 50 percent of the surface of the biopsy; D: MVI score 3, diffuse microvascular inflammation defined by presence of inflammatory cells in the capillaries and venules in 50 and more percent of the surface of the biopsy. Inserts in B-D show similar numbers of inflammatory cells in the most affected vessel. (H&E stain x50 and x200 in insert).

I-C-2-d. Statistical methods

We provide mean (SD) values for descriptive analyses of continuous variables. We compared means and proportions with Student's t test, ANOVA, or χ^2 test (Fisher's exact test when appropriate). We compared the expression of transcripts between groups by using geometric means, less sensitive to outliers. To analyze the association between microvascular inflammation and LV dysfunction, we performed a multivariable logistic regression model. We performed a principal components analysis with the `dudi.pca` module of the `ade4` package (version 1.5-1) of R (version 2.10.1). Statistical significance was set at $p \leq 0.05$. All tests were 2-sided. Statistical analyses were performed using STATA 15.0 (StataCorp LP, College Station, TX) and R software.

I-C-3. Results

I-C-3-a. Patients characteristics, histopathology and distribution of MVI grade

The clinical characteristics of the patients and the EMB are summarized in Table 5. EMB without rejection represented 31.2% of the cases (n=124). One hundred and eighty-six EMB were classified as AMR (56 pAMR1(I+), 32 pAMR1(H+), 96 pAMR2 and 2 pAMR3). One hundred and ninety-seven EMB were classified as ACR (1R: n=141, 2R: n=39, 3R: n=17). A small fraction of the EMB (n=8, 2%) had mixed rejection based of the association of AMR \geq pAMR2 and ACR \geq 2R. Overall rejection EMB were more prevalent in RT-MLPA case-control series that was enriched with rejecting cases. The extension of microcirculation inflammation was evaluated in the 398 EMB using the MVI score. Thirty-nine (9.8%), 56 (14.1%) and 46 (11.5%) EMB were classified in MVI grade 1, grade 2 and grade 3, respectively, while 257 EMB were classified as MVI grade 0.

I-C-3-b. Molecular profiles according to MVI score

Figure 15 gives the results of the molecular analysis according to the extension of MVI (MVI score) using microarrays (15-A/B) or RT-MLPA (15-C/D) analysis. We first tested whether the selected PBT, AMR and TCMR classifiers were correlated to the extension of MVI (MVI score). Regarding microarrays analysis, as shown in the Principal Component Analysis (Figure 15-A), most of the cases with MVI grade 0 and MVI grade 1 clusterized on the left side of the PCA while MVI grade 2 and MVI grade 3 clusterized on the right side of the PCA and were strongly driven by the PBT associated with AMR (ENDAT, DSAST, GRIT1, NK and QCMAT) and the AMR classifier (probABMR). Values of individual PBT and classifiers according to MVI score are given in Figure 15-B. ENDAT, DSAST, GRIT1, QCMAT, NK and TCB values strongly increased with the MVI score. MVI score was significantly associated with AMR classifier (probABMR) but not with ACR classifier (probTCMR).

	Overall	Microarray	RT-MLPA	p value
Patients' characteristics	n=268	n=128	n= 140	
Donor age	41.5 ±14	41.6 ±13.9	41.4 ±14.2	0.90
Male donor gender, n (%)	182 (68)	87 (68)	95 (68)	0.98
Recipient age	54.6 ±14.5	53.9 ±14.8	54.7 ±14.4	0.68
Male recipient gender, n (%)	195 (72.8)	96 (75)	99 (70.7)	0.43
Cold ischemic time min	189 ±58	199 ±55	180 ±61	0.01
Reason for transplant, n (%)				0.02
Dilated	126 (47)	57 (44.5)	69 (49.3)	
Ischemic	79 (29.5)	34 (26.6)	45 (32.1)	
Congenital	21 (7.8)	17 (13.3)	4 (2.9)	
Other	42 (15.7)	20 (15.6)	22 (15.7)	
Induction therapy, n (%)				0.93
ATG	202 (77.7)	95 (76.6)	107 (78.7)	
Basiliximab	50 (19.2)	24 (19.4)	26 (19.1)	
Other	8 (3.1)	5 (4)	2 (2.2)	
Anti-HLA DSA during follow-up	132 (51)	73 (59.3)	59 (43.4)	0.01
Endomyocardial biopsies' characteristics	n=398	n=213	n= 185	
EMB ≥ 1-year post-transplant, n (%)	201 (50.5)	100 (47)	101 (54.6)	0.13
“For cause” EMB, n (%)	52 (13.1)	15 (7)	37 (20)	<0.001
LVEF at EMB	62 ±12	65.1 ±10.6	59.7 ±12.1	0.01
ISHLT 2013 pAMR grade, n (%)				0.02
pAMR0	212 (53.3)	128 (60.1)	84 (45.4)	
pAMR1 (I+)	56 (14.1)	16 (7.5)	16 (8.7)	
pAMR1 (H+)	32 (8)	30 (14.1)	26 (14.1)	
pAMR2	96 (24.1)	38 (17.8)	58 (31.3)	
pAMR3	2 (0.5)	1 (0.5)	1 (0.5)	
ISHLT 2005 TCMR grade, n (%)				<0.001
0R	201 (50.5)	101 (47.4)	100 (54)	
1R	141 (35.4)	97 (45.6)	44 (24)	
2R	39 (9.8)	12 (5.6)	27 (15)	
3R	17 (4.3)	3 (1.4)	14 (8)	
MVI grade, n(%)				<0.001
0	257 (64.6)	148 (69.5)	109 (58.9)	
1	39 (9.8)	21 (9.9)	18 (9.7)	
2	56 (14.1)	33 (15.5)	23 (12.5)	
3	46 (11.5)	11 (5.1)	35 (18.9)	
Anti-HLA DSA at EMB, n (%) *	166 (57.8)	94 (50.5)	72 (71.3)	0.001

Table 5: Characteristics of patients and of endomyocardial biopsies.

The 213 The microarray EMB were included from one retrospective case-control enriched with rejecting cases and one prospective cohort that reflects the routine biopsy recruitment. The 185 RTMLPA biopsies were included from one retrospective case-control cohort enriched with rejecting cases. This explains the differences observed between the 2 groups, Affymetrix and RTMLPA, regarding the DSA status at the time of the biopsy, type of BEM (protocol/cause) and distribution of histological diagnoses on BEMs.

ATG means anti-thymocyte globulins, DSA: donor-specific antibodies, EMB: endomyocardial biopsy, LVEF: left ventricular ejection fraction, MVI= microvascular inflammation.

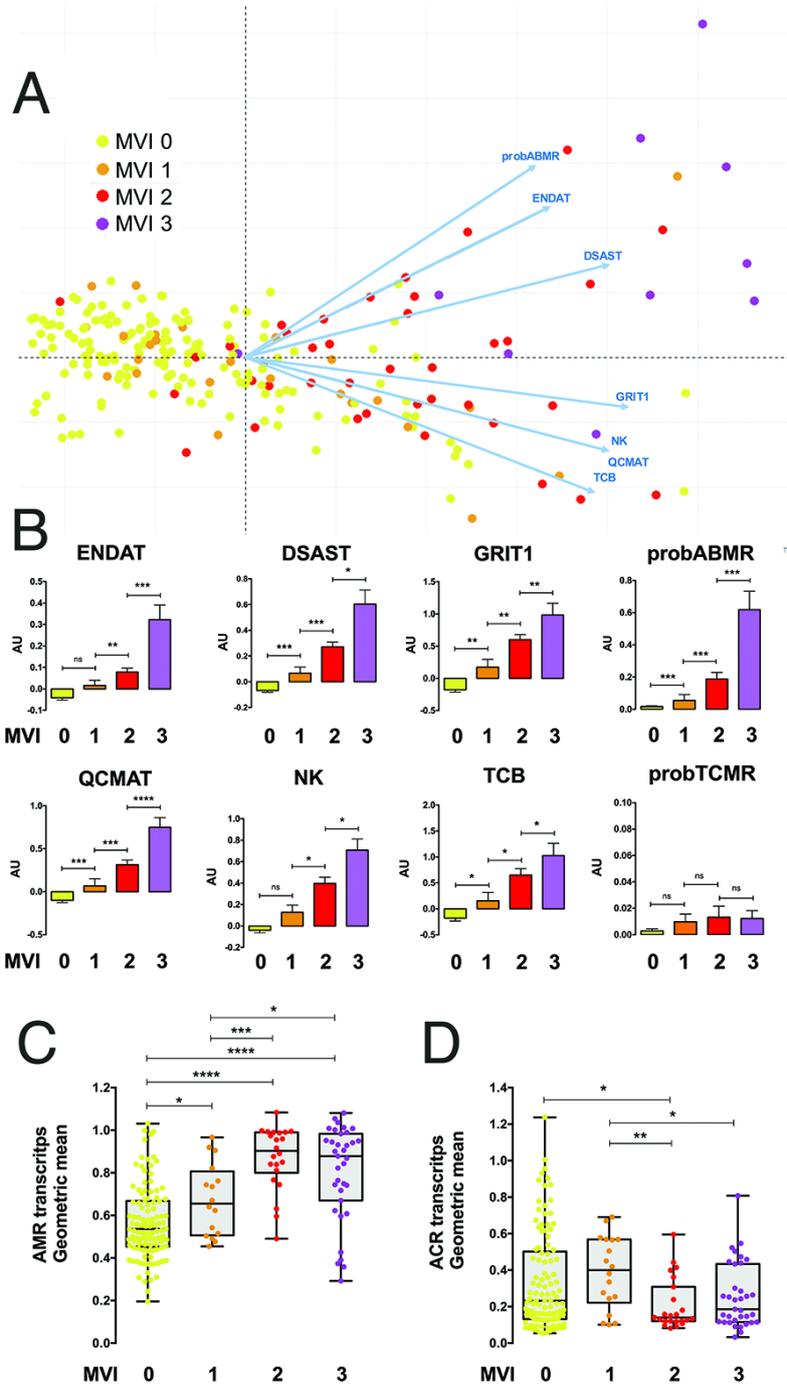


Figure 15: Molecular correlates of extension of microvascular inflammation MVI score.

A-B: Microarray analysis: **A: Unsupervised principal component (PC) analysis of MVI based on molecular profiles.** Each dot represents one of 231 EMB samples for the microarray analysis. They are colored by their MVI scores and distributed by their molecular features as determined using principal component analysis. The principal component analysis is based on six PBT (ENDAT, DSAST, GRIT1, NK, QCMAT and TCB) and one classifier score (probABMR). PC1, principal component 1 (accounts for x% of total molecular variance); PC2, principal component 2 (accounts for x% of total molecular variance); **B: Individual results for intragraft expression of pathogenesis based transcripts** (PBT: DSAST, ENDAT, GRIT1, QCMAT, TCB) and AMR and ACR classifiers (probABMR and probTCMR) according to MVI score (MVI 0: n=158; MVI 1: n=26; MVI 2: n=38; MVI 3: n=10; median and SEM); **C-D: RT-MLPA analysis:** **C: Median of geometric mean of AMR-associated transcripts according to MVI score;** **D: Median of geometric mean of ACR-associated transcripts according to MVI score** (MVI 0: n=109; MVI 1: n=18; MVI 2: n=23; MVI 3: n=35; median and min-max values). Statistical significances are *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001.

We then analyzed the molecular signals of selected rejection-associated transcripts in the same EMB that was used for the initial pathologic diagnosis and MVI scoring. Figure 15 C-D gives the results of RT-MLPA analysis with the geometric mean of transcripts associated with AMR (ROBO4, TM4SF1, PLA1, GNLY, CXCL11, CXCL9, CCL4 and FCGR3; Figure 15C) and ACR (CCL18, ADAMDEC1 and CD8A ; Figure 15D) according to MVI score.

Altogether, these results from independent series and techniques, show that the extension of MVI correlated with the intensity of the AMR-related molecular signals, but not with the ACR signals.

I-C-3-c. MVI score and ISHLT ACR and pAMR classifications

The detailed distribution of MVI score in the 398 EMB is given in Table 6 and illustrated in Figure 16-A. Each EMB was graded according to both ACR and pAMR classifications. MVI score was strongly associated with pAMR grade ($p < 0.001$). Thus, MVI 2 and MVI 3 were present in pAMR1(H+) and pAMR2/3 categories and was absent in ACR 2/3R in absence of concomitant AMR. As shown in Table 6, ACR grades did not influence MVI score in each subgroup of pAMR category.

Regarding immunohistochemistry, MVI score was significantly associated with C4d positivity with 11%, 20%, 37% and 57% of C4d positive cases in MVI score 0, MVI score 1, MVI score 2 and MVI score 3, respectively. MVI was also significantly associated with CD68 positivity with 25%, 56%, 66% and 87% of CD68 positive cases in MVI score 0, MVI score 1, MVI score 2 and MVI score 3, respectively.

I-C-3-d. MVI score and DSA

DSA status at the time of EMB was available for 281 biopsies (70.6% of the EMB). The percentage of positive-DSA cases increased with the extension of MVI from 42.9% to 94.3% (42.9%, 50%, 78.3%, and 94.3% of MVI score 0, 1, 2 and 3, respectively, $p < 0.001$). Mean MFI of the immunodominant DSA increased with the MVI score ($p < 0.001$). When the MFI was categorized (negative, 500-2,299, 3000-9,999 and $> 10,000$), the percentage of cases with the highest MFI (3000-9,999 and $> 10,000$ categories) significantly increased with the MVI score (Figure 16-B).

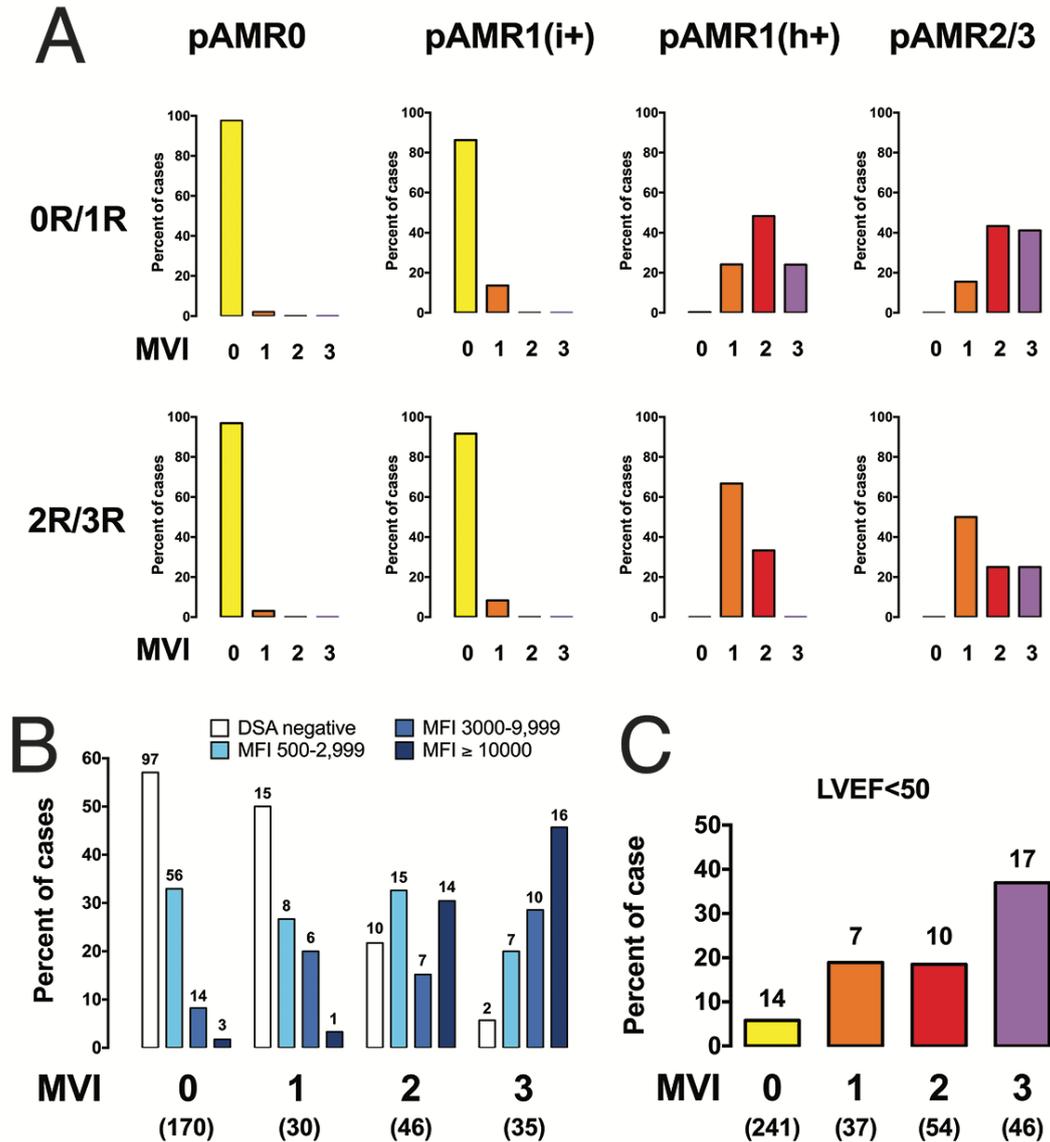


Figure 16: Relationship between Extension of microvascular inflammation (MVI score) and ISHLT classifications of rejection, MFI of immunodominant DSA and impaired graft function at the time of endomyocardial biopsy.

A: Distribution of MVI grades according to rejection categories based on ISHLT ACR and pAMR classifications in 398 EMB: MVI is strongly associated with pAMR1(h+) and pAMR2/3 categories and was absent in pure cellular rejection ACR 2R/3R. (Details (n in each categories and statistical significance) are presented in Table 5); **B: Relationship between MVI score and DSA:** Evaluation of DSA concomitant to the EMB was available in 281 biopsies. MFI of the Immunodominant DSA (MFI ID DSA) were grouped according to 4 categories (negative, 500-2,999, 3000-9,999 and >10,000). The percentage of each MFI ID DSA category is given for each MVI score. High MVI scores (MVI 2 and MVI 3) are enriched in cases with high MFI ID DSA (3000-9,999 and >10,000 categories), ($p < 0.001$, the number of cases is given on the top of each column). **C: Relationship between MVI score and graft function:** Assessment of cardiac function by measurement of Left ventricular ejection fraction (LVEF) at the time of EMB was available in 378 cases. The percentage of cases with cardiac dysfunction (LVEF below 50) is given in each MVI score ($p < 0.001$, the number of cases is given on the top of each column).

	n	Overall	MVI 0	MVI 1	MVI 2	MVI 3	p	
Clinical characteristics								
Time HTx-EMB > 1 year	398							
No		197 (49.5)	143 (55.6)	20 (51.3)	20 (35.7)	14 (30.4)	0.002	
Yes		201 (50.5)	114 (44.4)	19 (48.7)	36 (64.3)	32 (69.6)		
Type of EMB	398							
Protocolar		346 (86.9)	245 (95.3)	30 (76.9)	44 (78.6)	27 (58.7)	<0.001	
For cause		52 (13.1)	12 (4.7)	9 (23.1)	12 (21.4)	19 (41.30)		
LVEF at EMB (%)	378	63.1 (11.1)	64.9 (9)	61.5 (12.7)	62.8 (9.8)	55.6 (16.8)	<0.001	
LVEF ≤ 49% at EMB	378							
No		330 (87.3)	227 (94.2)	30 (81.1)	44 (81.5)	29 (63.0)	<0.001	
Yes		48 (12.7)	14 (5.8)	7 (18.9)	10 (18.5)	17 (37.0)		
Donor-specific antibodies at EMB								
MFI of ID DSA, Median (IQR)	281	677 (0-3000)	0 (0-1078)	315 (0-2651)	2164 (514-11081)	7343 (820-12495)	<0.001	
DSA status, n(%)	281							
Negative		124 (44.1)	97 (57.1)	15 (50)	10 (21.7)	2 (5.7)	<0.001	
MFI 500-2,999		86 (30.6)	56 (32.9)	8 (26.7)	15 (32.6)	7 (20)		
MFI 3,000-9,999		37 (13.2)	14 (8.2)	6 (20)	7 (15.3)	10 (28.6)		
MFI ≥ 10,000		34 (12.1)	3 (1.8)	1 (3.3)	14 (30.4)	16 (45.7)		
Pathological diagnosis								
pAMR grade		ACR grade						
0	212	0R	124 (58.5)	122 (98.4)	2 (1.6)	0 (0)	0 (0)	0.61*
		1R	55 (25.9)	53 (96.4)	2 (3.6)	0 (0)	0 (0)	
		2R	21 (9.9)	20 (95.2)	1 (4.8)	0 (0)	0 (0)	
		3R	12 (5.7)	12 (100)	0 (0)	0 (0)	0 (0)	
1(I+)	56	0R	21 (37.5)	19 (90.5)	2 (9.5)	0 (0)	0 (0)	0.91*
		1R	23 (41.1)	19 (82.6)	4 (17.4)	0 (0)	0 (0)	
		2R	9 (16.1)	8 (88.9)	1 (11.1)	0 (0)	0 (0)	
		3R	3 (5.3)	3 (100)	0 (0)	0 (0)	0 (0)	
1(H+)	32	0R	8 (25)	0 (0)	2 (25)	3 (37.5)	3 (37.5)	0.70*
		1R	21 (65.6)	1 (4.8)	5 (23.8)	11 (52.4)	4 (19.0)	
		2R	3 (9.4)	0 (0)	2 (66.7)	1 (33.3)	0 (0)	
		3R	0	0 (0)	0 (0)	0 (0)	0 (0)	
2/3	98	0R	48 (49.0)	0 (0)	7 (14.6)	21 (43.7)	20 (41.7)	0.34*
		1R	42 (42.9)	0 (0)	7 (16.7)	18 (42.8)	17 (40.5)	
		2R	6 (6.1)	0 (0)	3 (50)	1 (16.7)	2 (33.33)	
		3R	2 (2.0)	0 (0)	1 (50)	1 (50)	0 (0)	

Table 6: Clinical and pathologic characteristics according to MVI score.

ACR means acute cellular rejection, AMR: antibody-mediated rejection, EMB: endomyocardial biopsy, ID DSA: immunodominant donor-specific antibody, MVI: microvascular inflammation.

* Effect of ACR grade in each sub-group of AMR grade. ** Effect of AMR grade.

I-C-3-e. MVI score and graft function

Finally, we assessed the association between the MVI score and graft function at the time of the biopsy. Forty-eight patients (48/378, 12.7%) were diagnosed with left ventricular (LV) dysfunction. The percentage of cases with LV dysfunction increased with the extension of MVI (5.8%, 18.9%, 18.5%, and 37% for MVI score 0, 1, 2 and 3, respectively, $p < 0.001$, p for trend < 0.001 , Figure 16-C). Table 7 gives the main characteristics of EMB with concomitant LV dysfunction. Graft dysfunction was more frequent in “for cause” EMB and was significantly associated with MVI grade ($p < 0.001$), pAMR grade ($p < 0.001$), and the presence of DSA ($p < 0.001$), but not with ACR ($p = 0.28$)

and time post-transplant ($p=0.46$). After adjustment for the presence of DSA, ACR, the delay between transplantation and the type of EMB, the MVI grade remained an independent risk factor for LV dysfunction (taking MVI-0 as reference: MVI-1: OR=2.69, CI95%=0.83-8.73; MVI-2: OR=2.60, CI95%=0.93-7.24; MVI-3: MVI 1: OR=5.34, CI95%=1.93-14.74; $p=0.01$).

	n	Overall	LV dysfunction	No LV dysfunction	p value
EMB \geq 1-year post-transplant, n (%)	378	192 (50.8)	22 (45.8)	170 (51.5)	0.46
“For cause” EMB, n (%)	378	51 (13.5)	24 (50)	27 (8.2)	<0.001
MVI grade, n(%)	378				
0		241 (63.7)	14 (29.2)	227 (68.8)	<0.001
1		37 (9.8)	7 (14.6)	30 (9.1)	
2		54 (14.3)	10 (20.8)	44 (13.3)	
3		46 (12.2)	17 (35.4)	29 (8.8)	
ISHLT 2013 pAMR grade, n (%)	378				
pAMR0		198 (52.4)	11 (22.9)	187 (56.7)	<0.001
pAMR1 (I+)		54 (14.3)	5 (10.4)	50 (15.1)	
pAMR1 (H+)		31 (8.2)	4 (8.3)	26 (7.9)	
pAMR2		93 (24.6)	27 (56.3)	66 (20.0)	
pAMR3		2 (0.5)	1 (2.1)	1 (0.3)	
ISHLT 2005 ACR grade, n (%)	378				
0R		190 (50.3)	22 (47.8)	168 (50.9)	0.28
1R		132 (34.9)	21 (43.8)	111 (33.7)	
2R		39 (10.3)	2 (4.2)	37 (11.2)	
3R		17 (4.5)	3 (6.2)	14 (4.2)	
Anti-HLA DSA at EMB, n (%)	266	151 (56.8)	33 (86.8)	118 (51.8)	<0.001

Table 7: Clinical and pathologic characteristics according to the presence of a left ventricular dysfunction at the time of endomyocardial biopsy.

ACR means acute cellular rejection, AMR: antibody-mediated rejection, EMB: endomyocardial biopsy, DSA: donor-specific antibody, MVI: microvascular inflammation.

I-C-4. Discussion

The present study demonstrates that the severity of MVI, based on a semi-quantitative evaluation of its extension has biological and clinical meanings. MVI score was associated with AMR molecular activity, pAMR classification, DSA and graft function at the time of EMB. Using two complementary approaches, pangenomic (performed on an additional EMB) and targeted gene transcriptomics (done on the same EMB),

we confirm that MVI, recognized at the microscopic level reflects pathogenic processes resulting from DSA interaction with the graft endothelium. These processes include endothelial activation, gamma-interferon response, and leukocyte infiltration with high levels of macrophages, T-cells and NK-cells transcripts. Interestingly, the level of expression of these molecular markers are up-regulated as early as MVI 1 and raised with MVI scores 2 and further increased with MVI score 3, suggesting that histological and molecular activities evolve in parallel. This would substantiate the acceptance of the MVI score as a marker of activity of heart allograft AMR. This is reinforced by the association of the MVI score with C4d and CD68 staining and by the fact that EMB with high MVI scores had higher MFI of immunodominant DSA. Finally, MVI score was associated with graft dysfunction as assessed by a LVEF below 50% at the time of the index EMB.

Currently, the endomyocardial biopsy serves as a primary diagnostic tool for the diagnosis of AMR according to the 2013 ISHLT pAMR classification. This is a 4 grades composite classification: pAMR0 (no AMR), pAMR1(i+) (immunopathogenic AMR), pAMR1(h+) (histologic AMR), pAMR2 (proven AMR) and pAMR3 (severe AMR), considering IAMC and immunohistochemical criteria (C4d, CD68). Although the ISHLT Consensus has taken important steps to improve the pathologic diagnosis of antibody-mediated rejection, an important gap remains in terms of histologic diagnosis accuracy and clinical-pathologic correlations to determine the indications for a therapeutic intervention. Except for the nowadays rare pAMR3 grade that has been associated with survival, the pAMR classification has not been associate with graft outcome. In a previous study, we have not been able to differentiate the molecular signatures from pAMR1(H+) and pAMR2/3 biopsies, probably reflecting the heterogeneity in terms of MVI severity. In the present study we systematically evaluated the distribution of MVI in EMB graded for both pAMR and ACR ISHLT classifications. We found that MVI score was correlated to the pAMR classification but not to the ACR classification as it was associated with AMR (probABMR classifier) but not ACR (probTCMR classifier) molecular profiles. However, various MVI scores are found in both pAMR1(H+) and pAMR2/3 suggesting that MVI score and pAMR classification are not redundant but give complementary information. We believe that, like it has been shown in kidney transplantation, the severity of MVI would be of importance to accurately phenotype cardiac allograft AMR.

In the present study, we did not analyze the cellular composition of the inflammatory infiltrate, which might be important point for a precise histopathologic phenotyping. We have previously demonstrated that the type of inflammatory cells within the microvascular compartment may greatly vary in both heart and kidney AMR with various proportions of macrophages, T and B lymphocytes and NK cells. Such a heterogeneity in the cell infiltrate may reflect different subtypes of AMR and should be investigated in the future. Recent advances in histopathology, such as multiplex immunofluorescence analysis, that allows analysis of several labelled cell types on the same slide, cellular compartmentalization and computer assisted cell counting, may be of great interest to progress on this particular point. Correlation with molecular analysis performed on the same EMB will be warranted.

This study must be interpreted in the context of its limitations. Although multicentric for the selection of cases, MVI scoring was only performed by 2 senior readers from the same research team. Thus, the question of reproducibility of the MVI score should be further evaluated in prospective unselected studies, together with the determination of a cut-off of MVI for the diagnosis of AMR and the assessment of the evolution of MVI score after rejection therapies.

I-C-4. Conclusion

This study demonstrated that the extension of MVI evaluated according to a semi-quantitative score was associated with AMR molecular activity, DSA and graft dysfunction. The MVI score gave additional information to the current pAMR classification, although our study was not designed to answer the question of the prognostic impact on graft survival of the MVI score.

Chapter I-D: Synthesis of part 1 – molecular biology in heart transplantation.

Through the application of two distinct molecular biology approaches, we have been able to demonstrate the potential contribution of these techniques to the diagnosis of heart allograft rejection, particularly AMR. We have shown (i) that RT-MLPA, a technique for the analysis of expression profiles of preselected transcripts, was applicable to FFPE-EMB, that the concordance between unsupervised molecular and pathologic diagnoses was good and that the discordant cases could provide materials for discussion in the difficult diagnosis of allograft rejection; and (ii) that these molecular biology techniques could be of interest to refine the pathologic diagnosis of AMR by highlighting the link between the extension of microvascular inflammation in EMB and AMR molecular activity, thus validating a pathologic 4-scale classification of microvascular inflammation (Figure 17).

These studies illustrate our belief that molecular pathology and histopathology could be complementary to progress towards a histo-molecular phenotype of rejection that might be one of the keys to a better understanding and a homogenization of the diagnosis of AMR across centers.

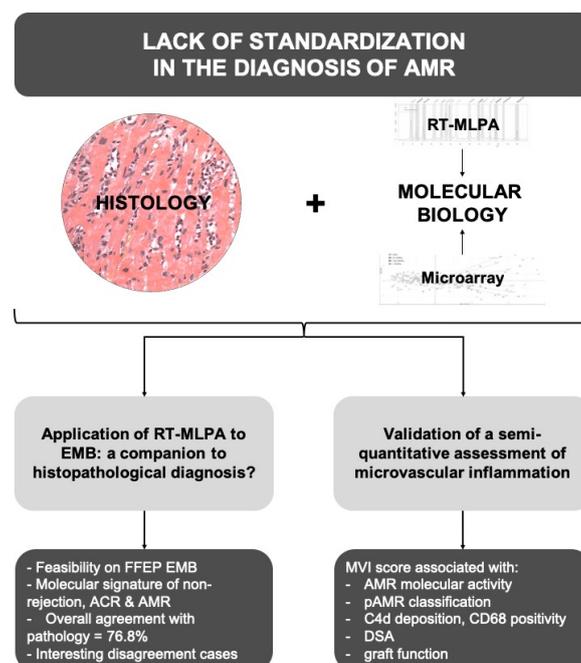


Figure 17: Summary of results of molecular biology studies.

Part 2.

Risk stratification of Donor-Specified Antibodies and Antibody-mediated rejection-related complications

In the quest for **personalized medicine**, heart transplantation must improve its standards for monitoring patients. Current approaches to screening for complications of allosensitization involve **iterative invasive protocol tests**, whether coronary angiography to detect CAV or endomyocardial biopsies to detect allograft rejection. Recent epidemiological data have shown that the incidence of these two complications has decreased over time (11,68). The current cost-effectiveness of these screening tests is low and should be compared with their potential complications.

Numerous risk factors/markers for the development of CAV or allograft rejection have been described in the literature, however, the **absence of an integrated tool** that enables an estimate of the risk of events for an individual makes the passage from a protocol medicine to a personalized medicine impossible.

In a first step towards the improvement of the stratification of the risk of events, we aim to accurately define the **epidemiology and natural history of CAV and allograft rejection** and to determine the **risk factors** independently associated with these complications. In order to do this, we will apply innovative methodologies to large cohorts of highly phenotyped heart transplant recipients.

We formulate the following hypotheses: i) that independent risk factors for allograft rejection are identifiable at the time of transplantation in a large population of heart transplants, which would make it possible to assess the risk of rejection early after transplant; and ii) that there are common evolutive profiles of CAV identifiable by latent class mixed models, constituting a first step towards individual risk stratification of CAV development (Figure 18).

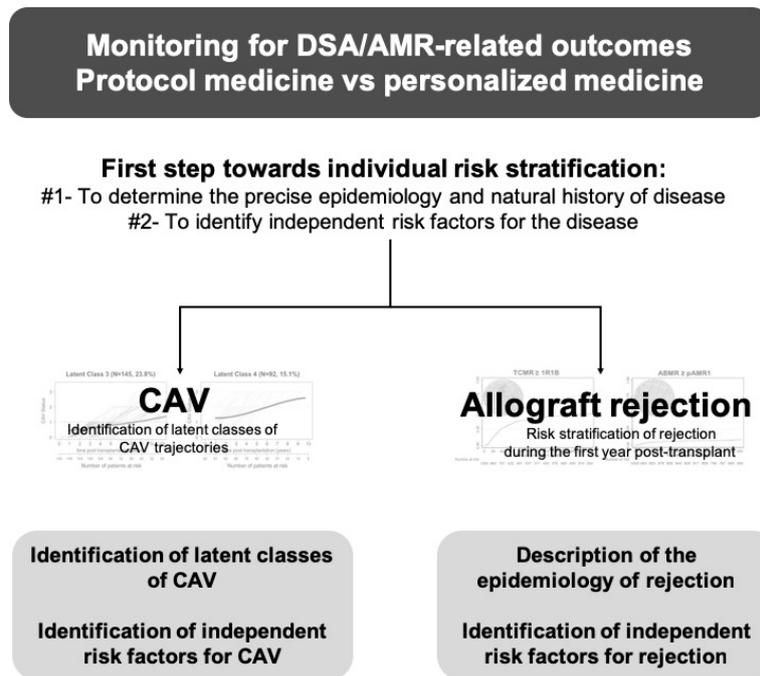


Figure 18: Objectives of the part 2: risk stratification.

Chapter II-A: Risk stratification

One of the primary objectives of biomedical research is the identification of risk factors for a disease or a clinical event that can stratify the risk of its occurrence, at least at the population level. Many statistical models exist and depend on the type of event or variables that we are trying to explain. Often in the biomedical field, the analyzed criterion is binary (disease / no disease), and the time of occurrence of the event is added to the analysis. In this typical case, the Cox model is the most relevant and frequently used model. This model makes it possible to determine independent risk factors for the occurrence of the disease that are assigned a β coefficient corresponding to the importance of their effect on the time of occurrence of the event analyzed. We will describe this model in detail in the introduction and will apply it in a first epidemiological study aiming to identifying independent risk factors for biopsy-proven rejection during the first year of transplantation.

However, this type of model suffers from several limitations. In particular, the Cox models are limited to the analysis of the occurrence of a first clinical event and cannot apprehend either the recurrence or the evolution of the severity of the disease after its diagnosis. Moreover, the Cox model is not able to fully understand the longitudinal character of iterative test results from the same patient. Statistical models such as generalized linear models can be a means of apprehending these issues by taking into account the correlation between results of repetitive tests performed on the same patient. However, they do not make it possible to take into account the variability of responses between individuals. We will then discuss the interest in mixed models applied to heart transplantation, which allow not only the analysis of longitudinal data, but also the introduction of random effects such as inter-cluster variability, which is the first step towards individual risk stratification. It is this type of model that we will apply to CAV in a second epidemiological study.

II-A-1. Identification of prognostic factors using Cox regression model

II-A-1-a. Cox semi-parametric model

This model was introduced in 1972 by David Cox and remains the most used model in right-censored survival analyzes and medical studies. This model takes into account several explanatory variables to explain the occurrence of an event (parametric part) without making assumptions on the survival distributions and the hazard function (non-parametric part). The model expresses the hazard function as the product of a basic hazard function $h_0(t)$ common to all individuals and considered as an unknown function, and a parametrically explicit regression function, $\exp(\beta'Z)$ where β' is a p-vector of unknown regression coefficient. The proportional risk model of Cox is written as:

$$h(t, Z) = h_0(t) \exp(\beta'Z)$$

This model underlies two hypotheses. Firstly, the relationship between the hazard function and covariates is **loglinear**. There are several methods to evaluate the log linearity deviation of continuous variables (statistics and / or graphs). When the variable does not satisfy the log linearity hypothesis, the common practice is to transform the continuous variable into a categorical variable. Secondly, the ratio of the instant hazards for two subjects i and j of characteristics Z_i and Z_j depends only on Z_i and Z_j and does not depend on time (**proportional hazard ratios** hypothesis). In other words, if an individual has a risk of death at some initial time point that is twice as high as that of another individual, then at all later times the risk of death remains twice as high. The proportional hazards ratios hypothesis can be verified by a graphical method or a statistical method. The statistical method is the most robust and is the preferred solution. It is based on the Schoenfeld residue analysis. The residues are calculated for each event date and correspond to the difference between the profile of the individual with the event and the profile of the individuals at risk on that date. If residues are distributed in the same way over time, the assumption of proportional hazards is verified. If this is not the case, then: i) the Cox model can be stratified on this variable (loss of the estimation of the impact of this variable on the event), ii) it is possible to introduce a time-dependent covariate (the covariate is then the same variable but with

an interaction with time), and iii) it is possible to create a model divided into two parts by considering the analysis separately over two periods.

II-A-1-b. Interpretation of the β coefficients

The interpretation of the exponentiated coefficients is the risk ratio for the increase in a unit of the specific covariant. The validity of this risk report is based on the assumption of proportional hazard ratios. If z_1 is a variable with two modalities, $z_1 = \{1/0\}$ among the p explanatory variables, then the risk ratio (RR) to this variable is written as:

$$RR_{z_1=1/z_1=0} = \frac{h_0(t_i) \exp(\beta_1) \exp(\sum_{j=2}^p \beta_j z_{i,j})}{h_0(t_i) \exp(\sum_{j=2}^p \beta_j z_{i,j})} = \exp(\beta_1) \forall t_i$$

Patients with the explanatory variable $\{z_1=1\}$ have an $\exp(\beta_1)$ -fold risk of failure compared to those with the explanatory variable $\{z_1=0\}$. The relative risk for variable z_1 is said to be adjusted for all other variables, i.e. with all other variables constant. It is constant over time since it only depends on the regression coefficient β_1 .

Hypothesis H_0 required testing of the equality of the hazard functions of each modality of the explanatory variable z_1 : $\{\beta_1=0 \Leftrightarrow RR_{z_1=1/z_1=0}\}$. The alternative hypothesis H_1 is most often bilateral: $\{\beta_1 \neq 0 \Leftrightarrow RR_{z_1=1/z_1=0}\}$. If H_0 is rejected, there is a difference in survival between the two populations defined by the variable z_1 .

II-A-1-c. Discrimination of the statistical model

When testing a new prognostic factor, it is essential to test its additional value. Indeed, if this factor is independently associated with the event, its contribution compared to existing models needs to be determined. The question is whether this new factor provides additional and complementary information and whether its application in clinical practice is relevant. If so, this factor is considered discriminant. The discriminative capacity of a model is defined by its ability to separate individuals who made the event from those who did not, and thus consists of separating individuals with different prognoses. One of the classical methods of evaluating discrimination is the analysis of an ROC curve. This is a graphical representation of the sensitivity of a factor for the event on the y-axis versus 1-specificity on the x-axis. This amounts to

evaluating the rate of true positives according to the rate of false positives. The associated index is the area under the ROC curve named AUC. This provides information on the probability that the result of the test for two people with and without the event can make the correct diagnosis. Thus, the test is perfectly discriminating if the AUC is 1. In this case, when evaluating two people the test will always distinguish the person having the disease from the one not having it. A test that is no better than chance will have an AUC of 0.5.

II-A-1-d. Construction of prognostic tools based on integrative models

After having identified the factors independently associated with the event and the additive value of new factors, the final step is the development of adequate prediction tools to enable risk stratification. A score is created according to the β coefficients attributed by the final multivariate model to each variable. These coefficients represent the relative and individual contribution of the variables to the survival distribution.

II-A-2-e. Statistical model performance

The analysis of the performance of the model involves the study of its calibration and discrimination, already discussed in the previous paragraphs.

To quantify the overall performance of a model, it is essential to take into account its calibration corresponding to the distance between the predicted events and the observed events. These distances between the events observed and predicted are related to the notion of the goodness of fit of a model, with the best models having smaller distances between the predicted results and the observed results. Calibration is essential since it refers to the ability of the model to produce accurate, unbiased predictions in similar patient groups. It is an indispensable preliminary step before the study of model discrimination since a discriminating, but poorly calibrated model has limited clinical value. The calibration can be measured by a graphical method using calibration curves or a statistical method, such as Hosmer-Lemeshow for survival models. The Hosmer-Lemeshow statistical model or its extension for survival data is derived from n balanced groups of patients defined by the quantiles of the probability predicted by the model. The measured bias is defined as the sum of the differences between the mean of the predicted probabilities and the mean of the probabilities observed in each group of similar patients. The calibration of the model is then

appreciated by the p-value of the test which is based on this sum. When this sum is less than 0.1, the calibration is not adequate.

Discrimination. As previously defined, discrimination is the ability of the model to discriminate individuals who made the event from those who did not, and thus is the model's ability to separate individuals with different prognoses (see above).

II-A-2. Introduction to the interest in mixed models in heart transplantation

The Cox model is limited to the analysis of a first clinical event and is not the most suitable model for the exploration and analysis of longitudinal data. Models specific to the analysis of longitudinal data, such as generalized linear models, can be a means of understanding the problems of the repetition of tests in the same individual. However, they do not take into account the variability in responses between individuals and, therefore, do not allow individual risk stratification. Hence, the goal of our research is to be able to generalize our results to all individuals in the population, not just those observed. We will, therefore, want to take into account the inter-cluster variance (inter-subject, inter-individual variability). The goal is to allow certain regression parameters to vary from cluster to cluster, via the introduction of random effects. Thus, various sources of the heterogeneity of the population can be taken into account. A mixed model is a statistical model in which we consider both fixed effects, which will intervene at the level of the model mean and random effects, which will intervene at the level of the variance of the model. A model is called mixed when there is at least one factor of each nature. In a second epidemiological study we will apply these models. We will use the particular case of latent classes mixed models, adapted to the analysis of categorical longitudinal data (grade of severity of the CAV according to the ISHLT). These models postulate the existence of latent variables, which are unobservable directly, but whose effects can be measured or observed. This approach allows the unsupervised categorization, that is, without a priori, of groups of patients. Each class includes a homogeneous group of individuals sharing a common evolutive profile.

II-A-3. Risk factors for allograft rejection after heart transplantation

Several clinical, laboratory, and immunological parameters might help to stratify the risk of allograft rejection after heart transplantation.

The time between transplantation and EMB is an important element of the risk of rejection. The risk of acute cellular rejection is the highest during the first weeks and decreases over time (69). The dynamic risk of AMR is less described but depends on the presence of pre-formed DSA (70). The effectiveness of protocol endomyocardial biopsies, has been well demonstrated during the first year, is less certain between 1 and 5 years and is lost for EMB after 5 years of transplantation, outside populations at risk (71).

While many studies have investigated the impact of both induction and basal immunosuppression on the risk of allograft rejection following cardiac transplantation (72–75), there are few data available on the intrinsic risk to the patient. Two important factors have, however, been described. The first is the ethnicity of the recipient. African-American patients have a higher risk of rejection than Caucasian patients, which justifies appropriate monitoring and immunosuppression protocols (71). A prior history of rejection is an important risk factor for the development of a new episode of rejection. This parameter should be taken into account in order to stratify the risk of subsequent rejection (34,76). In fact, late rejections occur most often in patients who have had one or more episodes of rejection early after transplantation (77). The risk of AMR depends on the presence or absence of pre-formed DSA (70), and on the development of DSA de novo after transplantation (78,79).

The stratification of the basal risk of rejection, i.e., at the time of transplantation, has not been studied in large cohorts of patients by analyzing a large number of potential risk factors for rejection. We will analyze the epidemiology and identify the risk factors for rejection in the first epidemiological study.

II-A-4. Risk factors for CAV

The situation of the coronary endothelium, at the border between the donor and the recipient, places it at the center of complex interactions between many immunological and non-immunological factors responsible for chronic aggressions leading to endothelial activation, chronic inflammatory responses, proliferation signals and finally chronic remodeling and diffuse narrowing of the coronary arteries (80,81).

Numerous risk factors for the development of CAV have been described. This literature has been enriched by the use of endocoronary imaging techniques, which are able to detect with a high sensitivity minimal changes in intimal thickness between two tests. Several syntheses of this abundant literature have been published (82). These CAV risk factors are traditionally categorized into (i) traditional cardiovascular risk factors of the donor responsible for transmitted atherosclerosis (83) (age, gender, diabetes, tobacco consumption etc. (84,85)); (ii) traditional cardiovascular risk factors of the recipient after transplantation (especially dyslipidemia (86)); (iii) non-immunological transplant-specific factors (ischemic time, cause of death of the donor, CMV infection (87,88)); and iv) immunological transplant-specific factors (acute cellular rejections, AMR, DSA (19,20,84,89)).

Some authors highlight that most of the studies analyzing CAV risk factors ultimately focused on the study of a limited number of potential factors, limiting the impact of their results (82). The epidemiological approach usually applied to the determination of risk factors for CAV is dichotomous (presence or absence of the disease, evolution or no change in intimal thickness), sometimes taking into account the delay in onset of the disease but neglecting the evolutionary profiles of the disease.

We therefore conducted a systematic review of the published literature for studies of trajectory-based assessment of CAV in heart transplant recipients. We searched PubMed for publications from January 1st, 2008 until June 30th, 2019 using the terms (((cardiac allograft vasculopathy) OR ((cardiac allograft vasculopathy) AND "Risk Factors"[Mesh])). To capture parameters relative to longitudinal changes in CAV, we also used the terms "repeated measure", "repeated measures", "repeated measurements", "trajectory", "trajectories", and "slope." AND (((("Natural History"[Mesh]) OR "Disease Progression"[Mesh]) OR "Latent Class Analysis"[Mesh])). To focus the search on Heart transplantation, we used the terms "heart transplantation", "heart transplant". Based on this approach, a comprehensive

search strategy was conducted using several databases (Medline, Embase, Cochrane, and Scopus).

The search yielded 249 articles. Ninety-eight did not address CAV, including studies on other solid organ transplants (n=46), other fields of heart transplantation (n=46) and animal models or in vitro studies (n=8). Sixty-six studies did not include longitudinal CAV data, including case-reports, reviews, editorials (n=36), and cross-sectional studies (n=30), leaving 85 studies that studied the progression of CAV. Among them, 60 studies considered CAV as a dichotomous endpoint (presence or absence), neglecting the severity and subsequent progression of CAV and 24 small retrospective studies compared the evolution of the mean of IVUS-derived parameters between groups at two points, neglecting the longitudinal characteristics of CAV data and subsequent CAV progression. Finally, no studies were derived from large patient cohorts with systematic monitoring and a specific design aimed at risk stratification and protocol repeated CAV assessment. Furthermore, none investigated CAV trajectories, none integrated a large spectrum of potential immune and non-immune risk factors and none were validated in large cohorts with different transplant allocation systems and management practice.

Our aim was to apply an innovative statistical model able to analyze the longitudinal data of coronary angiograms in a large cohort of patients allowing us to unmask the evolutive profiles of CAV over time.

Chapter II-B: Epidemiology and risk factors of biopsy-proven rejection during the first-year post-transplant.

This work has been presented as full oral communication at the 2019 ISHLT annual meeting (Orlando, April 2019) and at the 2019 ESOT meeting (Copenhagen, September 2019).

II-B-1. Introduction

Cardiac allograft rejection remains an important cause of graft loss and death (7). The diagnosis of rejection is based on the histological analysis of a myocardial fragment obtained by endomyocardial biopsy (EMB). The vast majority of the rejections are sub-clinical, and their diagnosis is based on a large number of iterative protocol EMB, as recommended by international societies (34). EMB is an invasive and non-risk-free procedure (90). Complications, which although rare at the level of an EMB (approximately 1% of cases, all complications combined), concern 20% of patients due to the repetition of EMB (76). The cost-effectiveness of this tests is low, since it is estimated that less than 10% of biopsies show signs of rejection, most of which are considered to be minimal signs, and that more severe forms of rejection are only present in 2% of the EMB (76). Strategies aimed at limiting the number of EMB during the first year seem feasible, however, the reported experiences have never adapted the screening protocol to the baseline risk of rejection (91). Tools, whether biological or epidemiological, to rationalize the number of protocol BEM are therefore an unmet medical need. Research on non-invasive biomarkers of rejection has been particularly active for more than 15 years. However, only very few of them have been applied in clinical practice. Only the ALLOMAP test is both recommended by the ISHLT guidelines and recognized by the US and European drug regulatory authorities (34,92). ALLOMAP is a score derived from the analysis of the expression of an 11-gene panel in peripheral blood mononuclear cells that enables the detection of acute cellular rejection $\geq 2R$ with good sensitivity and specificity (92,93). However, the limits of this test are numerous (a lack of sensitivity, screening only grade 2R or more cellular

rejections, cost). The search for donor-derived cell-free DNA in the blood of the recipient is a particularly promising method that is the subject of an abundant literature, but has not yet been validated on a large scale (94–96).

Our hypothesis is that an epidemiological approach allowing adaptation of the biopsy screening protocol to the risk of rejection could make it possible to rationalize the number of protocol biopsies. Although some risk factors for allograft rejection have been identified in the literature, such as the recipient's age, a prior history of rejection, and the presence of DSA, a risk stratification tool incorporating all of these factors has not been developed (70,71,76). As a first step towards personalized medicine in heart transplantation, our goal is to analyze the epidemiology of allograft rejection and to identify independent risk factors for rejection measurable at the time of the transplant.

II-B-2. Materials and methods

III-B-2-a. Patients and design

We performed a retrospective observational multi-center center study. We included all consecutive HTx recipients from two French heart transplantation reference centers (La Pitié-Salpêtrière Hospital, Paris, and Georges Pompidou European Hospital, Paris) transplanted between 2004 and 2016 and who had at least one EMB during the first-year post-transplant. During the study period, 1,250 patients were transplanted and 197 of these never had any EMB. Thus, 1,053 patients were included in our study. Patients were followed until January 2019. Our study complies with the Declaration of Helsinki, and the institutional review board approved the protocol. Informed consent was obtained at the time of inscription on the waiting list. Baseline donor and recipient characteristics were obtained from the prospective national registry CRISTAL database (French National Agency for Organ Procurement). We collected prospectively the results of EMB and clinical events during the first year (see the data collection section in the Supplementary Appendix). The design of our study is described in Figure 19.

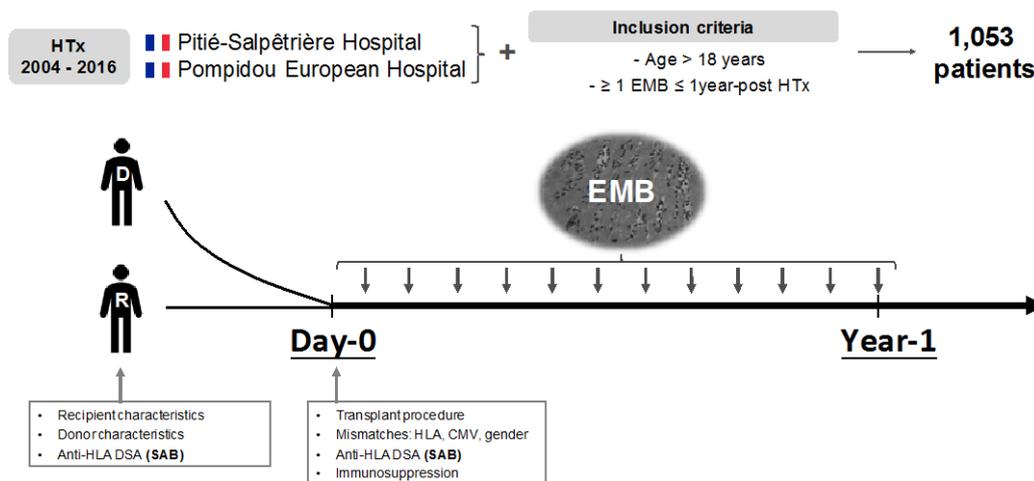


Figure 19: Design of the study.

Pre-transplant, day of transplant, and early post-transplant data were collected to identify the risk factors for biopsy-proven rejection during the first-year post-transplant.

II-B-2.b. Anti-HLA antibodies screening and endomyocardial biopsies

Circulating donor-specific antibodies against HLA-A, HLA-B, HLA-Cw, HLA-DR, HLA-DQ and HLA-DP were assessed using single-antigen flow bead assays. The pre-transplant and day of transplant sera of patients transplanted before 2011 were retrospectively reassessed using Luminex SAB technology in a reference laboratory using a pre-specified protocol (see the Methods section in the Supplementary Appendix). Beads showing MFI > 500 were considered as a positive result. Heart allograft pathology data were recorded according to the ISHLT classifications (see the Methods section in the Supplementary Appendix) (29,59). As recommended by ISHLT guidelines, only C4d grade 3 capillary staining was considered positive for pAMR1(I+). Due to the different practices between the two centers, CD68 and CD163 positivity were not considered as pAMR1(I+).

II-B-2.c. Evolution of practices over time

During the study period, the scheduled number of protocol biopsies during the first year was reduced from 15 to 13 in 2012. In 2009, as described in the third part of this manuscript, a specific prophylactic treatment for transplantation with pre-formed DSA was applied at La Pitié-Salpêtrière Hospital. Patients transplanted with MFI \geq 1,000 pre-formed DSA were prospectively treated with peri-operative plasmapheresis and IVIg. These elements led us to define three monitoring periods: period #1 from 2004 to 2008, period #2 from 2009 to 2011 and period #3 from 2012 to 2016.

II-B-2.d. Definition of outcomes

Significant rejection was defined as acute cellular rejection \geq 1R1B (i.e., 1R1B, 1R2, 2R, or 3R) and/or antibody-mediated rejection \geq pAMR1 (i.e., pAMR1(H+), pAMR(I+), pAMR2-3).

II-B-2.e. Statistical Analysis

Continuous variables were described using means and standard deviations (SDs) or the median and the interquartile range, as appropriate. We compared means and proportions between groups using the Student's t-test, analysis of variance (ANOVA) (Mann-Whitney test for DSA mean fluorescent intensity) or the chi-square test (or Fisher's exact test if appropriate). Survival functions were estimated according to the Kaplan-Meier method. Cox univariate regression and logistic univariate regression were used to evaluate the association between clinical and biological factors and outcomes. Candidate factors were selected when the univariate likelihood ratio test p-value was less than 0.10. Descending selection was used, and the final multivariate model considered significance at 0.05. The statistical analysis was performed using STATA (version 15).

II-B-3. Results

II-B-3-a. Characteristics of patients and endomyocardial biopsies

A total of 1,053 patients who were transplanted during the study period had at least one EMB during their first-year follow-up and were included in the study. One hundred and twenty-eight patients (12.2%) died during the first year. The median duration of follow-up was 354 days (quartile 1 = 315 days, quartile 3 = 363 days), representing a total follow-up of 868 patient-years. The main characteristics of donors, recipients, transplantation and immunosuppression are described in Table 8. The results of the pathologic analysis of the EMBs are summarized in Table 9. A total of 13,676 BEMs was performed, representing an average of 12.99 ± 5.5 BEM per patient. The distribution of EMBs over time during the first year is described in Figure 20 (overall and rejection EMBs). The number of BEMs per patient decreased from 14.6 ± 6.1 to 10.8 ± 3.7 after the introduction of a new screening protocol in 2012 (Figure 21).

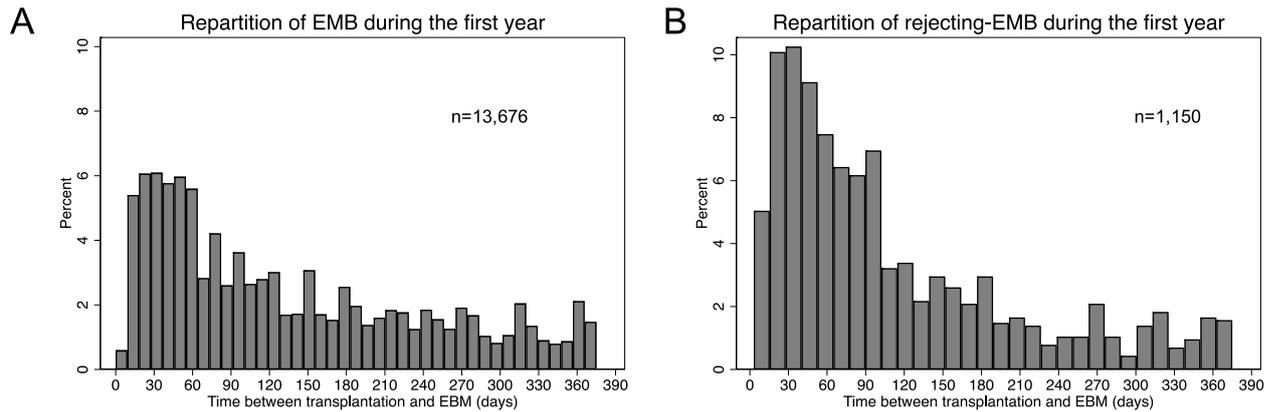


Figure 20: Repartition of (A) overall EMB and (B) rejecting-EBM according to the time post-transplant.

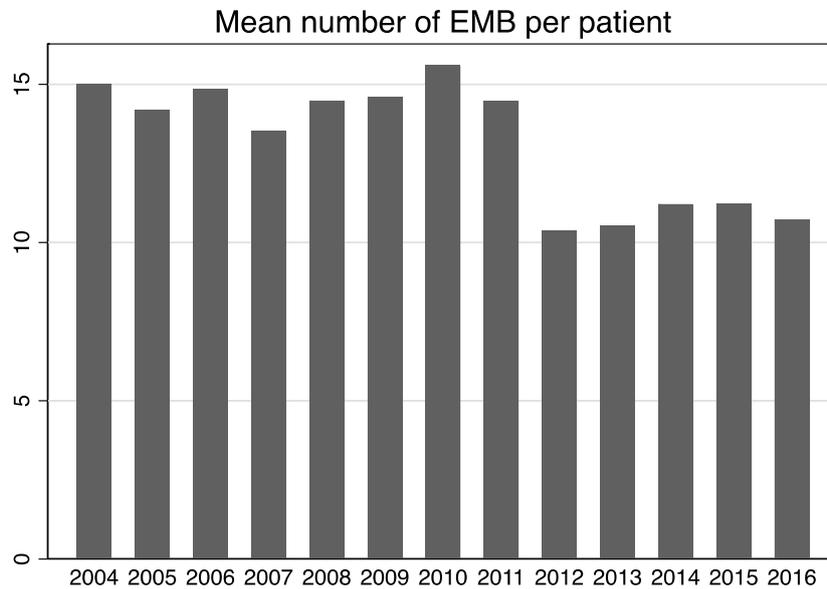


Figure 21: Evolution of the mean number of EMB per patient performed during the first-year post transplant.

The most commonly diagnosed type of rejection was 1R1A acute cellular rejection (n = 4,077, 29.8%). Significant acute cellular rejection signs \geq 1R1B were found in 896 BEMs (6.6%) and patterns of AMR \geq pAMR1 were found in 351 BEMs (2.6%). Overall, significant signs of cellular rejection and/or AMR were present in 1,150 BEM (8.4%). At the patient level, 457 (43.4%) had at least one episode of cellular rejection \geq 1R1B, 195 (15.5%) at least one AMR episode \geq pAMR1 and 520 (49.4%) at least one episode of significant rejection (\geq 1R1B and/or \geq pAMR1) in the first year. The comparison of patients' characteristics between the rejection and non-rejection groups is described in Table 8.

	All patients n=1,053		No rejection n=532		Rejection n=521		p
	N		N		N		
Donor characteristics							
Age (years), mean (SD)	1,053	45.3 (13.3)	532	46.0 (13.5)	521	44.62 (13.0)	0.09
Gender male, No. (%)	1,053	695 (66.0)	532	338 (63.53)	521	357 (68.52)	0.09
Cause of death, No (%)	1,053		532		521		
Cerebrovascular		517 (49.10)		262 (49.25)		255 (48.94)	
Traumatic		349 (33.14)		177 (33.27)		172 (33.01)	0.86
Anoxia		146 (13.87)		75 (14.10)		71 (13.63)	
Other		41 (3.89)		18 (3.38)		23 (4.41)	
BMI (kg/m ²), mean (SD)	1,053	25.3 (4.7)	532	25.3 (4.6)	521	25.3 (4.8)	0.97
Recipient characteristics							
Age (years), mean (SD)	1,053	48.4 (13.15)	532	49.6 (13.1)	521	47.2 (13.1)	0.004
Male gender, No. (%)	1,053	825 (78.35)	532	412 (77.44)	521	413 (79.27)	0.47
Caucasian ethnicity, No. (%)	1,053	761 (72.27)	532	395 (74.25)	521	366 (70.25)	0.15
Cardiomyopathy, No. (%)	1,053		532		521		
Dilated		473 (44.92)		252 (47.37)		221 (42.42)	
Ischemic		330 (31.34)		159 (29.89)		171 (32.82)	
Congenital		50 (4.75)		22 (4.14)		28 (5.37)	0.52
Retransplantation		26 (2.47)		15 (2.26)		14 (2.69)	
Other		174 (16.52)		87 (16.35)		87 (16.70)	
Long term MCS, No. (%)	1,053	139 (13.20)	532	61 (11.47)	521	78 (14.97)	0.09
History of pregnancy, No. (%)	986	110 (11.16)	498	60 (12.05)	488	50 (10.25)	0.37
Prior History of solid organ transplantation, No. (%)	1,053	27 (2.56)	532	12 (2.26)	521	15 (2.88)	0.52
eGFR (MDRD, mL/min), mean (SD)	761	77.18 (29.7)	376	74.0 (30.2)	385	80.3(28.8)	0.004
Mechanical ventilation, No. (%)	1,053	32 (3.04)	532	12 (2.26)	521	20 (3.84)	0.14
ECMO at transplant, No. (%)	1,053	266 (25.26)	532	132 (24.81)	521	134 (25.72)	0.74
Diabetes mellitus at transplant, No. (%)	1,050	180 (17.14)	532	92 (17.29)	518	88 (16.99)	0.90
BMI (kg/m ²), mean (SD)	1,053	24.4 (4.4)	532	24.3 (4.8)	521	24.4 (4.0)	0.53
Transplant baseline characteristics							
Combined transplantation, No. (%)	1,053	53 (5.03)	532	43 (8.08)	521	10 (1.92)	<0.001
Type of transplantation, No. (%)	1,053		532		521		
Heart		1,000 (94.97)		489 (91.92)		511 (98.08)	
Heart + Kidney		30 (2.85)		24 (4.51)		6 (1.15)	<0.001
Heart + Liver		23 (2.18)		19 (3.57)		4 (0.77)	
Cold ischemic time (min), mean (SD)	1,052	185.2 (57.2)	532	186.4 (56.4)	520	184.1 (57.2)	0.51
Cold ischemic time > 4h, No. (%)	1,052	191 (18.16)	532	97 (18.23)	520	94 (18.08)	0.95
CMV mismatch (D+/R-), No. (%)	1,002	187 (18.66)	504	107 (21.23)	498	80 (16.06)	0.04
Immunosuppressive therapies							
Induction therapy, No (%)	1,053		532		521		
ATG		964 (91.55)		492 (92.48)		472 (90.60)	0.27
IL-2R inhibitors		89 (8.45)		40 (7.52)		49 (9.40)	
Type of calcineurin inhibitors (post-transplant), No. (%)	1,053		532		521		
Cyclosporine		971 (92.21)		485 (91.17)		486 (93.28)	0.20
Tacrolimus		82 (7.79)		47 (8.83)		35 (6.72)	
Immunology							
Anti-HLA DSA, pre-formed (MFI ≥ 500), No (%)	1,038	324 (31.21)	522	142 (27.20)	516	182 (35.27)	0.005
HLA mismatches (A-B-DR), No (%)	1,005	5.01 (0.93)	505	4.90 (0.98)	500	5.12 (0.87)	<0.001

Table 8: Baseline characteristics of included patients

ATG means anti-thymocyte globulins, BMI = body mass index, DSA = donor-specific antibodies, ECMO = extra corporeal membrane oxygenation, eGFR = estimated glomerular filtration rate, MCS = mechanical circulatory support.

Overall population	
N = 1,052 patients	
Endomyocardial biopsies	
Overall number	13,676
Number per patient, mean (SD)	12.99 (5.5)
Acute cellular rejection	
ACR 0 , No. of EMB (% of EMB)	8,703 (63.64)
ACR 1R1A , No. of EMB (% of EMB)	4,077 (29.81)
ACR 1R1B , No. of EMB (% of EMB)	715 (5.23)
ACR 1R2 , No. of EMB (% of EMB)	9 (0.07)
ACR 2R3A , No. of EMB (% of EMB)	147 (1.07)
ACR 3R , No. of EMB (% of EMB)	25 (0.18)
Antibody-mediated rejection*	
pAMR0 , No. of EMB (% of EMB)	13,325 (97.43)
pAMR1(I+) , No. of EMB (% of EMB)	74 (0.54)
pAMR1(H+) , No. of EMB (% of EMB)	268 (1.96)
pAMR2/3 , No. of EMB (% of EMB)	9 (0.07)

Table 9: Diagnosis of EMB

II-B-3-b. Epidemiology of rejection in the first year

The incidence of the first significant rejection during the first year is presented in Figure 22-A/C. Beyond the third month of transplantation, very few new patients presented a first episode of rejection without having presented in the first three months. The probability of identifying a rejection with a protocol EMB was highest in the early BEMs and then gradually decreased over time (Figure 22-D). Two hundred and eighty-seven patients (27.3%) had at least one relapse of rejection in the first year. Rejection recurrences were distributed as follows: 134 patients (12.7%) had 2 rejections, 64 patients (6.1%) 3 rejections, 40 patients (3.8%) 4 rejections and 49 (4.6%) patients \geq 5 rejections.

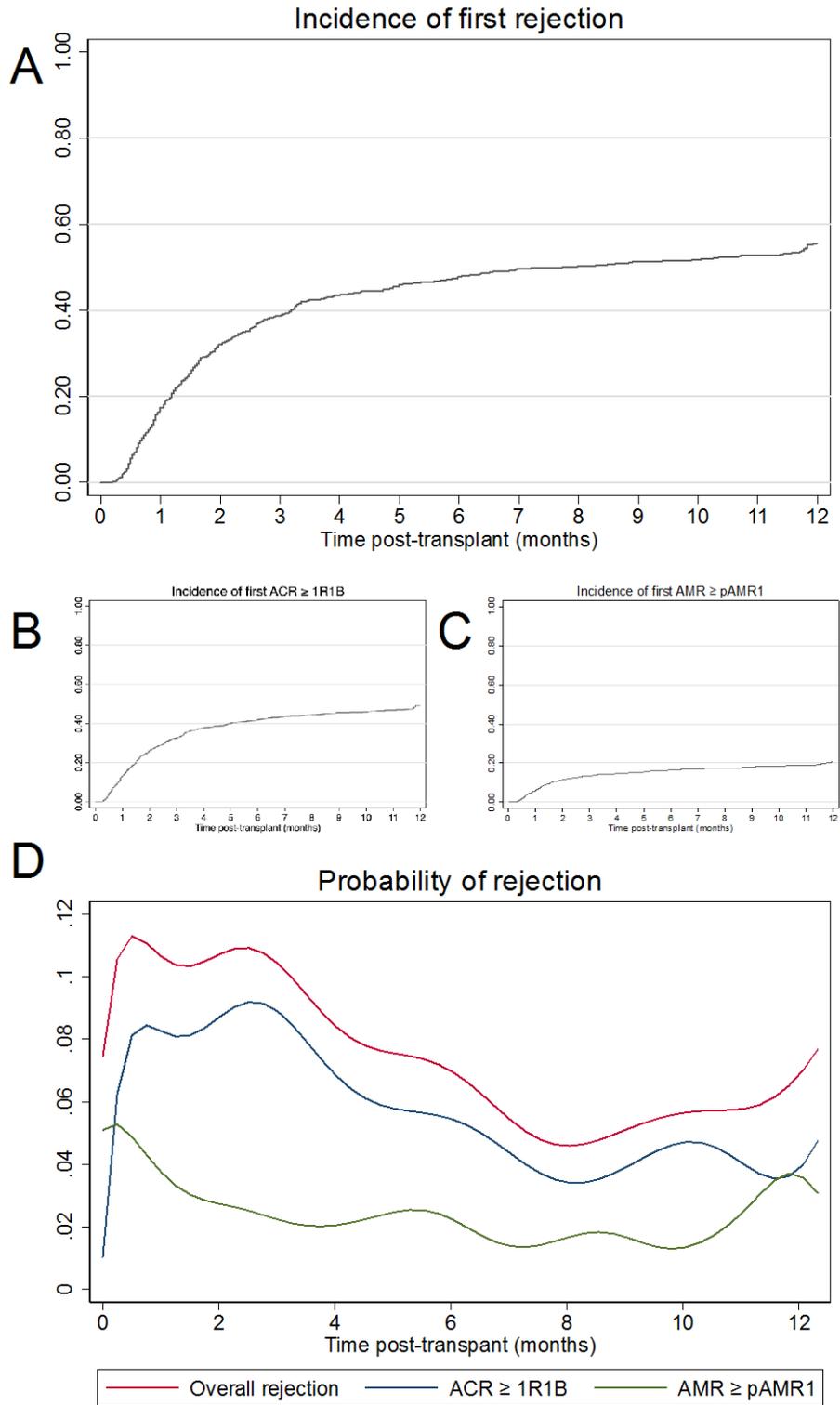


Figure 22: Epidemiology of allograft rejection during the first year after heart transplantation

A- Incidence of first significant-rejection (ACR \geq 1R1B and / or AMR \geq pAMR1).

B- Incidence of the first acute cellular rejection \geq 1R1B.

C- Incidence of first antibody-mediated rejection \geq pAMR1.

D- Dynamic prevalence of rejection during the first year according to the time post-transplant. Interpretation: for example, the probability of finding significant signs of rejection on an EMB performed 3 months after the transplantation is about 10%, at 8 months post-transplant, about 5%.

II-B-3-c. Risk Factors for Allograft Rejection during the first-year post-transplant

In univariable analyses, we identified 9 variables associated with the risk of developing a rejection during the first year: the recipient's age (per 10-year increment: HR = 0.89, 95% CI = 0.84-0.95, $p < 0.001$), the presence of long-term mechanical circulatory support at the time of transplant (HR = 1.39, 95% CI = 1.09-1.78, $p = 0.01$), the donor's age (per 10-year increment: HR = 0.94, 95% CI = 0.88-1.01, $p = 0.07$), a sex mismatch between the donor and the recipient (HR = 0.84, 95% CI = 0.69-1.03, $p = 0.08$), the combined character of transplantation (compared to isolated heart transplantation: HR = 0.29, 95% CI = 0.15-1.54, $p < 0.001$), the presence of a CMV mismatch (donor positive / recipient negative: HR = 0.76, 95% CI = 0.60-0.97, $p = 0.02$), the type of induction (induction by IL-2R inhibitors compared to induction at thymoglobulin: HR = 1.36, 95% CI = 1.01-1.83, $p = 0.05$), the number of mismatches HLA A-B-DR (for each increase of one HLA mismatch: HR = 1.20, 95% CI = 0.09-1.32, $p < 0.001$) and the presence of pre-formed DSA at the time of transplantation (MFI ≥ 500 , HR = 1.43, 95% CI = 1.19-1.71, $p < 0.001$). The results are summarized in Table 10.

In multivariable analyses, five variables were independently associated with the risk of rejection during the first year: the recipient's age (per 10-year increment: HR = 0.90, 95% CI = 0.85-0.95, $p = 0.003$), the type of transplantation (combined vs isolated: HR = 0.25, 95% CI = 0.13-0.47, $p < 0.001$), the type of induction (induction by IL-2R inhibitors compared to induction with thymoglobulin: HR = 1.58, 95% CI = 1.17-2.16, $p = 0.003$), the number of HLA AB-DR mismatches (for each mismatch increase: HR = 1.17, 95% CI = 1.06-1.29, $p = 0.001$) and the presence of pre-formed DSA (MFI ≥ 500 , HR = 1.46, 95% CI = 1.21-1.75, $p < 0.001$). The results are detailed in Table 11.

The calibration of the model was good ($p = 0.91$). On the other hand, the discrimination of the model evaluated by a ROC curve proved to be bad (Figure 23-A). The area under the curve of the ROC curve was calculated at 0.64.

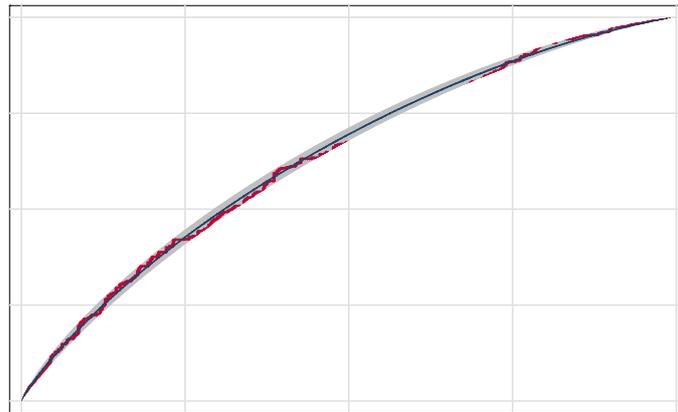


Figure 23: $ACR \geq 1R1B$ and/or $AMR \geq pAMR1$: discrimination of the Cox model.

II-B-3-d. Risk Factors for acute cellular and antibody-mediated rejections during the first-year post-transplant

We then analyzed separately the risk factors for $ACR \geq 1R1B$ and for $AMR \geq pAMR1$. After multivariable analyses, we identified five independent risk factors for ACR during the first year: the recipient's age (per 10-year increment: HR = 0.91, 95% CI = 0.84-0.97, $p = 0.005$), the type of transplantation (combined vs isolated: HR = 0.20, 95% CI = 0.09-0.43, $p < 0.001$), the type of induction (induction by IL-2R inhibitors compared to induction with thymoglobulin: HR = 1.86, 95% CI = 1.36-2.55, $p < 0.001$), the number of HLA AB-DR mismatches (for each mismatch increase: HR = 1.13, 95% CI = 1.02-1.25, $p = 0.02$) and the presence of pre-formed DSA (MFI ≥ 500 , HR = 1.25, 95% CI = 1.03-1.53, $p = 0.027$). The discrimination of the model was poor (AUC = 0.62).

After multivariable analyses, we identified three independent risk factors for AMR during the first year: the recipient's age (per 10-year increment: HR = 0.78, 95% CI = 0.66-0.91, $p = 0.002$), the presence of pre-formed DSA (MFI ≥ 500 , HR = 2.01, 95% CI = 1.30-3.12, $p = 0.002$) and the ischemic time (per 1-hour increment: HR = 1.33, 95% CI = 1.04-1.68, $p = 0.021$). The discrimination of the model was poor (AUC = 0.66).

Characteristics	Variable	Label	Number of patients	Number of patients with ≥ 1 rejection	HR	95% CI	p
Recipient characteristics	Age	(per 10-yr increment)	1,053	521	0.89	[0.84-0.95]	<0.001
	Gender	Male	825	413	1	-	
		Female	228	108	0.98	[0.79-1.21]	0.83
	Caucasian ethnicity	Yes	761	366	1	-	
		No	292	155	0.94	[0.78-1.13]	0.52
	Cardiomyopathy	Dilated	473	221	1	-	
		Ischemic	330	171	0.72	[0.49-1.10]	
		Congenital	50	28	0.82	[0.55-1.22]	0.46
		Retransplantation	26	14	0.78	[0.51-1.20]	
		Others	174	87	0.91	[0.48-1.73]	
	Long term MCS	No	914	443	1	-	
		Yes	139	78	1.39	[1.09-1.78]	0.01
	History of pregnancy	No	876	438	1	-	
		Yes	110	50	0.95	[0.71-1.28]	0.75
	Prior history of solid organ transplantation	No	1,026	506	1	-	
Yes		27	15	1.21	[0.72-2.02]	0.48	
Pre-HTx ECMO	No	787	387	1	-		
	Yes	266	134	1.13	[0.93 - 1.38]	0.22	
Diabetes mellitus at transplant	No	870	430	1	-		
	Yes	180	88	1.04	[0.82 - 1.31]	0.75	
Donor characteristics	Age	(per 10-yr increment)	1,053	521	0.94	[0.88- 1.01]	0.07
	Gender	Male	695	357	1	-	
		Female	358	164	0.87	[0.73-1.05]	0.15
	Cause of death	Cerebrovascular	88	23	1	-	
		Anoxia	237	51	0.71	[0.43-1.16]	0.32
Other		80	23	1.05	[0.59-1.87]		
		10	3	1.09	[0.33-3.64]		
Transplant characteristics	Gender mismatch	No	731	385	1	-	
		Yes	322	136	0.84	[0.69-1.03]	0.08
	Combined transplantation	No	1,000	511	1	-	
		Yes	53	10	0.29	[0.15-1.54]	<0.001
	Type of transplantation	Heart	1,000	511	1	-	
		Heart + Kidney	23	4	0.32	[0.14-0.71]	<0.001
		Heart + Liver	28	6	0.25	[0.09-0.67]	
	Cold ischemia	(per 1-min increment)	1,052	521	1.00	[0.998-1.001]	0.93
Cold ischemia	≤ 4 hours	861	426	-	-		
	> 4 hours	191	94	1.03	[0.82-1.28]	0.83	
CMV mismatch (D+/R-)	No	815	418	1	-		
	Yes	287	80	0.76	[0.60-0.97]	0.02	
Immunosuppressive therapies	Induction	ATG	964	472	1	-	
		IL2-R inhibitors	89	49	1.36	[1.01-1.83]	0.05
	Type of calcineurin inhibitors	Cyclosporine	971	486	1	-	
	Tacrolimus	82	35	0.86	[0.61-1.22]	0.39	
Immunology	Number of HLA mismatches A-B-DR	(per 1-MM increment)	1,005	500	1.20	[0.09-1.32]	<0.001
	Pre-formed DSA	No	714	334	1	-	
	MFI ≥ 500	Yes	324	182	1.43	[1.19-1.71]	<0.001

Table 10: Risk factors for biopsy-proven rejection during the first year: univariable Cox model analysis.

Variables	Label	Number of patients	Number of patients with ≥ 1 rejection	HR	95% CI	LRT p-value
Recipient Age	per 10-yr increment	995	499	0.90	[0.85-0.95]	0.003
Type of transplantation	Isolated Heart	947	489	1	-	
	Combined	51	10	0.25	[0.13-0.47]	<0.001
Type of induction therapy	ATG	914	453	1	-	
	IL2-R inhibitors	84	46	1.58	[1.17-2.16]	0.003
Number of HLA mismatches (A-B-DR)	per 1-mismatch increment	995	499	1.17	[1.06-1.29]	0.001
Pre-formed DSA (MFI ≥ 500)	No	679	319	1	-	
	Yes	319	180	1.46	[1.21-1.75]	<0.001

Table 11: Risk factors for biopsy-proven rejection during the first year: multivariable Cox model analysis.

II-B-3-e. Sensitivity analyses

Different sensitivity analyses were conducted. First, a stratification over the period (2004 to 2008, 2009 to 2011 and 2012 to 2016) did not change the final model, including the effect of pre-formed DSA on the risk of rejection: recipient's age (per 10-year increment: HR = 0.92, 95% CI = 0.85-0.97, $p = 0.005$), type of transplant (combined compared to isolated heart transplants: HR = 0.25, 95% CI = 0.14-0.48, $p < 0.001$), type of induction (IL-2R inhibitors compared to ATG: HR = 1.58, 95% CI = 1.16-2.14, $p = 0.004$), number of HLA A-B-DR mismatches (per 1 mismatch increment: HR = 1.17, 95% CI = 1.06-1.29, $p = 0.001$) and presence of pre-formed DSA (HR = 1.60, 95% CI = 1.32-1.95, $p < 0.001$). Similarly, the stratification on the center did not modify the final multivariate model, either in terms of statistical significance or the significance of the effect (HR and OR).

II-B-4. Discussion

In a large cohort of heart transplant recipients, we were able to accurately determine the epidemiology of allograft rejection during the first year and to identify five independent risk factors for rejection during this period, which were identifiable at the time of transplantation: the young age of the recipient, an induction therapy based on IL-2R inhibitors compared to anti-thymocyte globulins infusions, an isolated heart transplant compared to a combined transplantation (heart-kidney or heart-liver), the number of A-B-DR HLA mismatches and the presence of pre-formed DSA (MFI ≥ 500). Cox models, although well calibrated, have been disappointing in terms of discrimination.

The main strengths of our study concern the sample size, the fact there was a retrospective analysis of EBM by two expert pathologists, the high level of patients' phenotyping (anti-HLA antibodies screening based on the reference method even for transplantations performed before 2009), and the analysis of many potential risk factors for rejection.

The risk of rejection evolves during the first year. These rejection dynamics have already been described for acute cellular rejection but never for AMR (76). While significant patterns of rejection are found in approximately 10% of EMB achieved in the first 3 months, this probability falls to less than 5% between 6- and 11-months post-transplant. The increased probability of rejection we observed at the end of the first year can have several explanations, such as the development of de-novo DSA and an increase in the time interval between two biopsies thus increasing the risk of more severe rejection due to it being identified later.

The discrimination of a statistical model is its ability to identify patients who will present the disease from others who will not, in our case, the patients who will experience rejection in the first year from those who will not. The discrimination of our models, evaluated by the area under ROC curve, proved to be poor and was lower than 0.65. Such discrimination does not allow the practical application of this statistical model to a patient's scale. Its diagnostic performance is too limited and the confidence in this model too low to adapt the biopsy screening. Therefore, we did not derive a scoring system from these models. Several explanations for these poor statistical performances can be evoked. First, the diagnosis of rejection might be open to discussion. It is difficult to establish a dichotomization of an endomyocardial biopsy result. The threshold used to define acute cellular rejection $\geq 1R1B$ is not a consensus. ISHLT guidelines do not recommend the treatment of subclinical $1R1B$ rejections (34,59). This definition has the effect of overrepresenting cellular rejections among all rejections in our cohort. A definition threshold $\geq 2R$ could have been chosen. However, our local experience led us to treat $1R1B$ rejections, even when sub-clinical. Thus, the epidemiology of grade $2R$ rejection is different in our cohort compared to North American practices. Moreover, more and more molecular data suggest that $1R1B$ rejections are molecularly closer to $\geq 2R$ rejections than to non-rejection biopsies (50). Similarly, the threshold for the definition of AMR is questionable. In addition to the diagnostic difficulties of the $pAMR1(H+)$ rejection already described in the first part of

the thesis, molecular data suggest that pAMR1(I +) rejections would behave more like biopsies without rejection than pAMR1(H +) or pAMR2-3 rejection biopsies (33,45). Secondly, although we have carried out extensive data collection, our base could benefit from being supplemented by the addition of evolutive data over the first year, such as the modifications in immunosuppressive therapy, the analysis of anticalcineurin dosages, de novo DSA development or the evolution of the MFI of pre-formed DSA after transplantation. Finally, the statistical models used were not optimal for the analysis of longitudinal data. The Cox model is a model for analyzing the occurrence of a first clinical event and is therefore not the best model for longitudinal data analysis. Mixed models represent the next stage of statistical development to be applied to our cohort. Not only can these models take into account the recurrence of the disease and the correlation between the results of multiple EMB carried out on the same patient, but they also can incorporate random effects related to the inter-cluster variation.

Although large and well phenotyped, our cohort has management specificities that could limit the application of our results to foreign cohorts, particularly those from North America. The almost systematic induction by anti-thymocyte globulins and the transplantation with pre-formed DSA are peculiarities of our centers. Internationally, more than half of patients do not receive any induction therapy. The most commonly used induction is IL-2R inhibitors followed by polyclonal induction, accounting for only 22% of patients (compared to 91.6% in our cohort) (14). Similarly, the most prescribed calcineurin inhibitor at the international level is tacrolimus, prescribed for more than 85% of patients worldwide, compared to for only 7.8% in our cohort (14). The small number of tacrolimus patients probably explains why this factor does not appear to be protective in our cohort (HR = 0.86 with a wide confidence interval), despite tacrolimus patients having been shown to have fewer rejections than patients given cyclosporin (75).

II-B-4. Conclusion

The risk of allograft rejection evolves during the first year. We identified five independent risk factors for rejection in the first year following heart transplantation: recipient's age, type of induction, type of transplantation, number of HLA mismatches and the presence of pre-formed DSA.

Chapter II-C: Identification and characterization of Trajectories of Cardiac Allograft Vasculopathy After Heart Transplantation: A Population Based Study.

Article submitted to Circulation and presented as full oral communication at the 2019th ISHLT annual meeting.

II-C-1. Introduction

Whereas the short term survival after HTx has improved considerably in the last decades, the survival beyond 1 year post-transplant has remained unchanged over years despite important progresses in immunosuppression and patients' care (7,8,97). In this setting, cardiac allograft vasculopathy (aka CAV), a diffuse intimal thickening leading to progressive narrowing of the coronary arteries, has a prevalence of 50% at ten years after transplantation, making this injury the third cause of late mortality and the leading cause of late allograft dysfunction (7,25,98).

The current gold standard for CAV monitoring after HTx relies on protocol angiograms, which guides clinical management of patients including prevention strategies, therapeutic changes, and also potential endpoint for clinical trials (99,100).

Current approaches for investigation CAV have been limited by registry data including convenience samples with limited number of measures for a single patient. Importantly no study to date has been primarily designed towards a prospective protocol based and standardized assessments of CAV together with thoroughgoing patient characterization including immunological and biological profile, histological phenotypes and information on treatments. To avoid making any prior assumptions on the number and shapes of CAV trajectories, we used a contemporary unsupervised trajectory-based approach known as latent class mixed modeling (101). This model has already demonstrated its clinical relevance in different medical settings such as depression, atherosclerosis, or disability by revealing unrecognized profiles of patients and by contributing to a deeper understanding of the evolution in time of chronic diseases (102–104).

Identifying relevant CAV trajectories and their respective determinants is an unmet need and requires additional level of understanding and characterization over the current paradigm of CAV staging. Given the long-term course of CAV development post-transplant, Such information might be useful to provide a more nuanced picture of disease progression, which may ultimately contribute to guiding the care of heart TX patients.

To achieve this goal, we performed a longitudinal prospective observational based cohort study including consecutive heart transplant recipients from different cohorts from Europe and US. We used informations gathered by protocol-driven repeated CAV asesements performed together with clinical, biological, histological and immunological phenotyping. We determined whether a trajectory-based approach could identify universal prototypes of CAV course and could stratify patients into distinct, mutually-exclusive CAV trajectories and identify the respective contribution of immune and non-immune determinants of these trajectories over 10 years.

II-C-2. Patients and methods

II-C-2.a. Study design and participants

Derivation cohort

The derivation cohort included 815 consecutive European patients over 18 years of age who were prospectively enrolled at the time of heart transplantation in two French referral HTx centers (La Pitié-Salpêtrière Hospital, Paris, n = 510 and Georges Pompidou European Hospital, Paris, n = 99) and one Belgium center (Leuven University Hospital, n = 206), transplanted between January 1, 2004 and December 31, 2015. and who were alive at 1-year post transplant and having two coronary angiograms during follow-up (from one-year to ten-year post-transplant). All data were anonymized and entered at the time of transplantation, at 3 months, 6 months, 1-year post-transplant and at each transplant anniversary using a standardized protocol to ensure harmonization across study centers. Day of transplant and 1-year post-transplant visits included an extensive clinical, biological and functional evaluation (See the methods section and the study protocol in the **Supplementary Appendix** for detailed data collection procedures). Data were retrieved from the database on June 2019. All patients provided written informed consent at the time of transplantation.

Validation cohort

External validation was conducted in 486 heart transplant recipients over 18 years of age from one northern American center (Cedars-Sinai Heart Institute, Los Angeles, transplantations between 2008 and 2016). Data sets from the validation center were collected as part of routine clinical practice and entered in the center’s databases in compliance with local and national regulatory requirements and sent anonymized to the Paris Transplant Group. The design of the study is described in Figure 24.

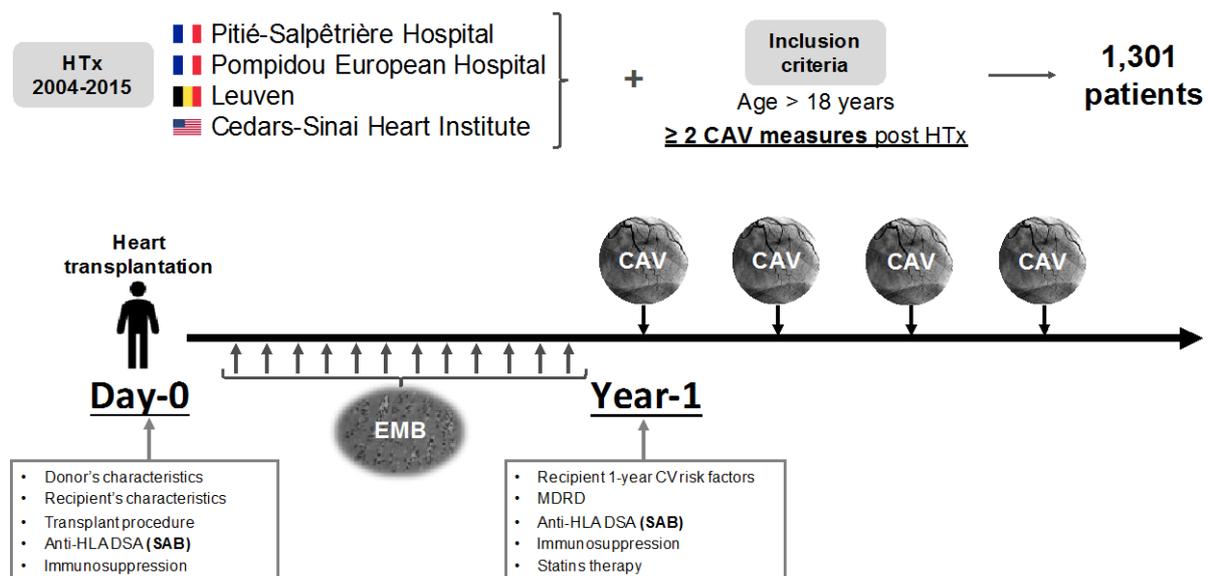


Figure 24. Design of the study.

II-C-2.b. Procedures and clinical protocols

All patients were followed from 1-year post-transplant (the index date of the analyses) until retransplantation, death, or date of final data extraction.

We defined the baseline period at 1-year after transplantation where the recipients underwent concomitant evaluation of angiogram, allograft pathology, usual blood tests (including serum creatinine, liver function, LDL-c, fasting glucose) and circulating anti-HLA antibody assessment according to a prespecified protocol. All patients in the study had at least 2 CAV measures after 1-year posttransplant (See Supplementary Appendix for the list of parameters assessed for the derivation cohort).

CAV angiograms were recorded per center protocol for all patients after transplantation. CAV was graded according to the current ISHLT classification as CAV 0 (not significant), 1 (mild), 2 (moderate) and 3 (severe) (35). European centers

protocols (derivation cohort) for follow-up data included CAV measurement at one year after transplantation and every two years thereafter as well as at the time of any clinically indication. The American center protocol included CAV measurement at 6-month one-year after transplantation and every year thereafter as well as at the time of any clinically indication.

In the European derivation cohort, a total of 2,742 reports of coronary angiograms were retrospectively and independently reviewed by three senior cardiologists. In case of discrepancies between observers or inconclusive report, the coronary angiograms were reviewed to reach an agreement (n = 454, 16.6%, mostly ISHLT CAV1). In the American validation cohort, a total of 1,968 coronary angiograms were analyzed. Two senior cardiologists graded all coronary angiograms according to the summarized report collected prospectively during patient follow-up.

Circulating donor-specific antibodies against HLA-A, HLA-B, HLA-Cw, HLA-DR, HLA-DQ and HLA-DP were assessed using single-antigen flow bead assays (see the Methods section in the Supplementary Appendix). Heart allograft pathology data were recorded according to the ISHLT classifications in the derivation and validation cohorts (see the Methods section in the Supplementary Appendix) (29,59). During the first-year post-transplant, a total of 12,803 EMB were performed in the derivation cohort.

II-C-2.c. Outcome measures

The primary outcome was CAV trajectories after transplantation. The secondary outcome was progression to all-cause mortality or retransplantation. The outcomes were prospectively assessed in the derivation and validation cohorts at each transplant anniversary, up to June 30, 2019.

II-C-2.d. Statistical Analysis

Continuous variables were described using means and standard deviations (SDs) or median and the interquartile range, as appropriate. We compared means and proportions between groups using Student's t-test, analysis of variance (ANOVA) (Mann-Whitney test for DSA mean fluorescent intensity) or the chi-square test (or Fisher's exact test if appropriate).

Derivation of post-transplantation CAV trajectories

CAV trajectories were identified over 10 years after transplantation using latent class mixed models (101). Latent class mixed models characterize trajectories in repeated measurements, with the assumption that several underlying subpopulations (i.e, the latent classes) can be detected (104). This approach requires neither the same number of measures per patient nor the same timepoints of measurement. CAV grading at baseline (1-year after transplantation) and CAV slopes were specific to each subpopulation identified. We compared the linear trajectory models with non-linear models, including quadratic (t^2 effect) and splines, and confirmed the suitability of linear models used for trajectory identification. Furthermore, we tested different link functions to identify the best fitting model. At model convergence, each patient was assigned posterior likelihoods of belonging to each CAV trajectory. Patients were assigned the class to which they had the highest likelihood of belonging.

Definition of the optimal number of CAV trajectories

The number of CAV trajectories was defined according to *i)* the Bayesian Information Criterion (BIC) and the Akaike Information Criterion (AIC), *ii)* the discrimination (i.e, the ability of the model to specifically classify patients in the CAV trajectories), *iii)* the entropy (i.e, the ability of the model to identify distinct CAV trajectories) and *iv)* the interpretability of the model, as previously published (see further details in Supplementary appendix) (102,104,105).

Determinants of CAV trajectories

In the derivation cohort, the associations between CAV trajectories and clinical, histological, functional, and immunological parameters at the time of transplantation, during the first year and at one year post-transplant were assessed using multinomial logistic regression. Parameters associated with trajectories in the univariate analysis with p -value < 0.10 were thereafter included in the multivariable model. Stepwise backward elimination was performed to obtain the final multivariable.

We used R (version 3.2.1, R Foundation for Statistical Computing) and STATA (version 14, Data Analysis and Statistical Software) for the descriptive and survival analyses. Values of $p < 0.05$ were considered statistically significant, and all tests were 2-tailed.

II-C-3. Results

II-C-3-a. Characteristics of the derivation and validation cohorts

The European derivation cohort (n=815) and the US validation cohort (n=486) comprised a total of 1,301 included patients from 4 transplant centers between January 1st, 2004, and December 31st, 2016 corresponding to 9,298 patient-years. The median follow-up post-transplantation was 6.59 years (IQR 4.72). The summarized characteristics of the derivation and validation cohorts (overall, European derivation and US validation cohorts) are detailed in Table 12. Briefly, older donors were used in the European cohort as compared with the US cohort (43.6 ± 12.2 vs 34.8 ± 12.9 , $p < 0.001$). Recipients were younger (48.1 ± 12.8 vs 56.4 ± 12.7 , < 0.001) with less ischemic cardiomyopathy and cardiovascular risk factors at the time of transplant (diabetes mellitus, hypertension and obesity) in the European cohort compared with the American cohort.

The total number of CAV measures analyzed was 4,710 (3.6 ± 1.6 measures per patient), including 2,742 coronary angiograms in the derivation cohort (3.4 ± 1.3 per patient) and 1,968 in the validation cohort (4.0 ± 1.8 per patient). The ISHLT CAV grades were distributed as follows: 3,354 CAV grade 0 (71.21%), 847 CAV grade 1 (17.98%), 358 CAV grade 2 (7.60%) and 151 CAV grade 3 (3.21%) from year 1 to year 10. The CAV grades were higher in the European derivation cohort compared to the American validation cohort (Table 13). One hundred and sixty-six out of 1,301 (12.76%) were diagnosed with CAV grade > 0 in the first year post-Tx. The detailed results of coronary angiograms and their evolution across years post-transplant are described in Table 13. Two hundred and eighteen patients (27.3%) and 89 patients (20.1%) had detectable anti-HLA DSA, either pre-formed or de-novo with 69.81% of DSA being class II, in the derivation and validation cohorts, respectively. Overall, 108 patients (13.3%) and 76 patients (12.5%) from the derivation cohort presented with acute cellular rejection $\geq 2R$ and AMR $\geq pAMR1$, respectively. In the validation cohort, where only the information “treated rejections” was available, 32 (6.6%) and 28 (5.8%) patients presented with treated acute cellular rejection $\geq 2R$ and treated AMR $\geq pAMR1$, respectively. The detailed results of EMB are provided in Table 14.

	All patients (4 centers, n=1,301)		European derivation cohort (3 centers, n=815)		American validation cohort (1 center, n=486)		p
	N		N		N		
Donor characteristics							
Age (years), mean (SD)	1,299	40.29 (13.74)	815	43.56 (12.17)	484	34.77 (12.89)	<0.001
Gender male, No. (%)	1,202	833 (69.3)	815	556 (68.22)	385	340 (70.10)	0.48
Tobacco, No (%)	1,220	390 (31.97)	783	348 (44.44)	437	42 (9.61)	<0.001
BMI ($\geq 25\text{kg/m}^2$), No (%)	1,257	608 (48.37)	815	347 (42.58)	442	261 (59.05)	<0.001
Recipient characteristics							
Age (years), mean (SD)	1,301	51.23 (13.36)	815	48.14 (12.77)	486	56.43 (12.72)	<0.001
Male gender, No. (%)	1,300	975 (75)	815	632 (77.55)	485	343 (70.72)	0.006
Caucasian ethnicity, No. (%)	1,300	933 (71.77)	815	619 (75.95)	485	314 (64.74)	<0.001
Ischemic cardiomyopathy, No. (%)	1,298	453 (34.90)	815	265 (32.52)	483	188 (38.92)	0.019
Long term MCS, No. (%)	1,300	268 (20.61)	815	150 (18.40)	485	118 (24.33)	0.009
Prior history of hypertension, No. (%)	1,211	430 (35.51)	797	219 (27.48)	486	211 (50.97)	<0.001
Diabetes mellitus, No. (%)	1,281	261 (20.37)	813	109 (13.41)	468	152 (32.48)	<0.001
BMI $\geq 25\text{ kg/m}^2$, No. (%)	1,296	564 (43.52)	815	327 (40.12)	481	237 (49.27)	0.001
Transplant baseline characteristics							
Combined transplantation, No. (%)	1,300	83 (6.38)	815	33 (4.05)	485	50 (10.31)	<0.001
Cold ischemic time (min), mean (SD)	1,282	177.5 (58.6)	815	183.0 (55.3)	467	167.8 (62.8)	<0.001
CMV mismatch (D+/R-), No. (%)	1,266	247 (19.51)	815	164 (20.27)	486	83 (18.16)	0.36
Immunosuppressive therapies							
ATG induction therapy, No (%)	1,282	991 (77.30)	815	773 (94.85)	467	218 (46.68)	<0.001
1-year immunosuppressive regimen							
Cyclosporine	1,297	505 (38.86)	815	465 (76.35)	482	27 (5.60)	<0.001
Tacrolimus	1,297	792 (61.06)	815	144 (23.65)	482	455 (94.40)	<0.001
MMF	1,297	1,113 (85.81)	815	732 (89.82)	482	381 (79.05)	<0.001
mTOR-inhibitors	1,297	212 (16.35)	815	132 (16.20)	482	80 (16.60)	0.85
1-year post-transplant cardiovascular risk profile							
Treated hypertension, No (%)	813	592 (72.82)	813	592 (72.82)	-	-	-
Diabetes mellitus, No (%)	813	177 (21.77)	813	177 (21.77)	-	-	-
Statins, No (%)	1,275	1,178 (92.39)	789	692 (87.71)	486	470 (96.70)	<0.001
LDL-c $\geq 1\text{g/L}$, No (%)	1,126	536 (45.19)	794	386 (48.61)	392	150 (38.27)	0.001
Tobacco, No (%)	813	47 (5.78)	813	47 (5.78)	-	-	-
Immunology and histology							
Anti-HLA DSA, pre-formed & 1-year (MFI ≥ 500), No (%)	1,242	307 (24.72)	799	218 (27.28)	443	89 (20.09)	0.005
MFI of immunodominant DSA, pre-formed & 1-year							
None		935 (75.34)		581 (72.81)		354 (79.91)	
MFI: 500-2999		151 (12.17)		135 (16.92)		16 (3.61)	<0.001
MFI: ≥ 3000		155 (12.49)		82 (10.28)		73 (16.48)	
Class II anti-HLA DSA, pre-formed & 1-year, No (%)	308	215 (69.81)	217	150 (69.12)	91	65 (71.43)	0.69
Acute cellular rejection $\geq 2\text{R}$, No (%)	1,301	140 (10.76)	815	108 (13.25)	486	32* (6.58)	<0.001
Antibody-mediated rejection $\geq \text{pAMR1}$, No (%)	1,094	104 (9.51)	608**	76 (12.48)	486	28* (5.76)	<0.001
Follow-up							
Follow-up (years), median (IQR)	1,301	6.56 (4.72)	815	7.73 (5.14)	486	4.84 (3.23)	<0.001

Table 12: Baseline characteristics of patients (European derivation and American validation cohorts).

* Only the information “treated rejection” was available in the US validation cohort. ** The retrospective analysis of the histologic criteria of antibody-mediated rejection pAMR1(H+) was only performed in the two French centers. ATG means anti-thymocyte globulins, AMR: antibody-mediated rejection, BMI: body mass index, CMV: cytomegalovirus, DSA: donor-specific antibodies, IQR: interquartile range, MCS: mechanical circulatory support, MMF: mycophenolate mofetil.

	All patients (4 centers, n=1,301)	French cohort (2 centers, n=609)	Belgium cohort (1 center, n=206)	North American validation cohort (1 center, n=486)
Number of coronary angiograms	4,710	2,085	657	1,968
Number of CA per patient, mean (SD)	3.62 (1.58)	3.42 (1.47)	3.19 (1.23)	4.05 (1.75)
CAV grade (ISHLT), No (%)				
CAV 0	3,354 (71.21)	1,232 (59.09)	480 (73.06)	1,642 (83.43)
CAV 1	847 (17.98)	495 (23.74)	111 (16.89)	241 (12.25)
CAV 2	358 (7.60)	249 (11.94)	39 (5.94)	70 (3.56)
CAV 3	151 (3.21)	109 (5.23)	27 (4.11)	15 (0.76)

Table 13: Characteristics of coronary angiograms

The coronary angiograms were graded according to the ISHLT guidelines (35). Significantly higher grades of cardiac allograft vasculopathy were diagnosed in the European derivation cohort ($p < 0.001$). CA means coronary angiograms, CAV: cardiac allograft vasculopathy.

	Overall derivation cohort N = 815 patients	French centers N = 609	Belgium center N = 206
Endomyocardial biopsies			
Overall number	12,803	9,396	3,407
Number per patient, mean (SD)	15.73 (3.70)	15.45 (4.13)	16.53 (1.68)
Acute cellular rejection			
ACR 0, No. of EMB (% of EMB)	7,841 (61.25)	5,624 (59.86)	2,217 (65.07)
ACR 1R1A, No. of EMB (% of EMB)	4,058 (31.69)	3,152 (33.55)	906 (26.59)
ACR 1R1B / 1R2, No. of EMB (% of EMB)	765 (5.97)	497 (5.29)	268 (7.87)
ACR 2R3A, No. of EMB (% of EMB)	116 (0.91)	101 (1.07)	15 (0.44)
ACR 3R, No. of EMB (% of EMB)	23 (0.18)	22 (0.23)	1 (0.03)
Antibody-mediated rejection*			
pAMR0, No. of EMB (% of EMB)	-	9,191 (97.81)	-
pAMR1(I+), No. of EMB (% of EMB)	76 (0.59)	46 (0.49)	30 (0.88)
pAMR1(H+), No. of EMB (% of EMB)	-	90 (0.96)	-
pAMR2/3, No. of EMB (% of EMB)	-	69 (0.74)	-

Table 14. Detailed classification of endomyocardial biopsies in the European derivation cohort

Endomyocardial biopsies were graded according to the ISHLT guidelines (29,59).

* The retrospective analysis of the histologic criteria of antibody-mediated rejection pAMR1(H+) was only performed in the two French centers (Pitié-Salpêtrière & Georges Pompidou European Hospital, n=609).

ACR means acute cellular rejection, AMR: antibody-mediated rejection.

II-C-3-b. Identification and characterization of CAV trajectories in the derivation and external validation cohorts

Derivation cohort. We identified 4 mutually exclusive CAV trajectories over 10 years after transplantation (Figure 25); the model showed a good discrimination of 0.92 (0.96, 0.83, 0.93 and 0.88 for trajectory#1,#2,#3 and #4, respectively) and entropy of 0.82, meaning that the model adequately separated the trajectories.

External validation cohort. Latent class mixed models were applied in the independent external validation cohort from US. The best fitting model identified 4 profiles of CAV trajectories and confirmed the consistency of the 4 profiles previously demonstrated in the derivation cohort (Figure 26). In this geographically distinct cohort, the model showed an excellent discrimination of 0.97 (0.93, 0.97, 0.88 and 0.92 for trajectory#1,#2,#3 and #4, respectively).

CAV trajectory#1 was composed of patients with no CAV at baseline that remained stable over time (n=823, 63.26%, CAV slope per year = 0.00 ± 0.01). CAV trajectory#2 was composed of patients with no CAV at baseline, with late onset increase of CAV overtime starting at around four years post-transplant (n=79, 6.07%, overall CAV slope per year = 0.11 ± 0.12). CAV trajectory#3 was composed of patients with intermediate CAV grade at 1 year who experienced moderate increase during follow-up (n=261, 20.06%, CAV slope per year = 0.18 ± 0.16) Patients from CAV trajectory#4 presented with a pattern of intermediate baseline CAV and acceleration over time (n=138, 10.61%, CAV slope per year = 0.40 ± 0.50).

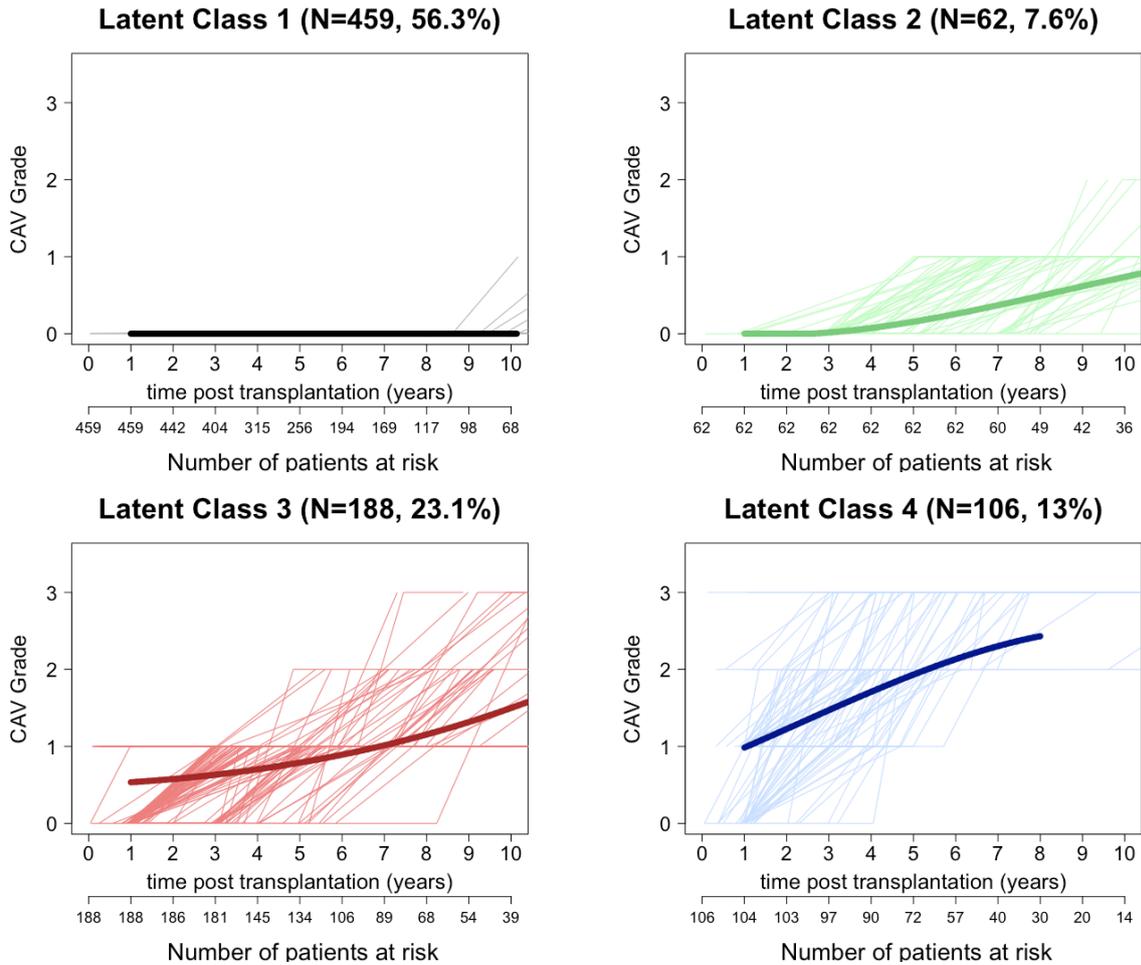


Figure 25. Distinct profiles of CAV trajectories identified post-transplantation in the European derivation cohort (n=815).

This figure represents the main profiles CAV grades identified with latent class mixed models. Each patient, represented by an individual CAV trajectory, is assigned to the class for which the membership probability is the highest. Discrimination = 0.92. CAV trajectory#1 was composed of patients with no CAV at baseline that remained stable over time; CAV trajectory#2 was composed of patients with no CAV at baseline, with slight increase of CAV overtime starting around four years post-transplant; CAV trajectory#3 was composed of patients with intermediate CAV grade at 1 year who experienced moderate increase during follow-up; Patients from CAV trajectory#4 presented with a pattern of intermediate baseline CAV and acceleration over time. Thick lines represent latent class trajectory; thin lines represent CAV individual patient trajectory.

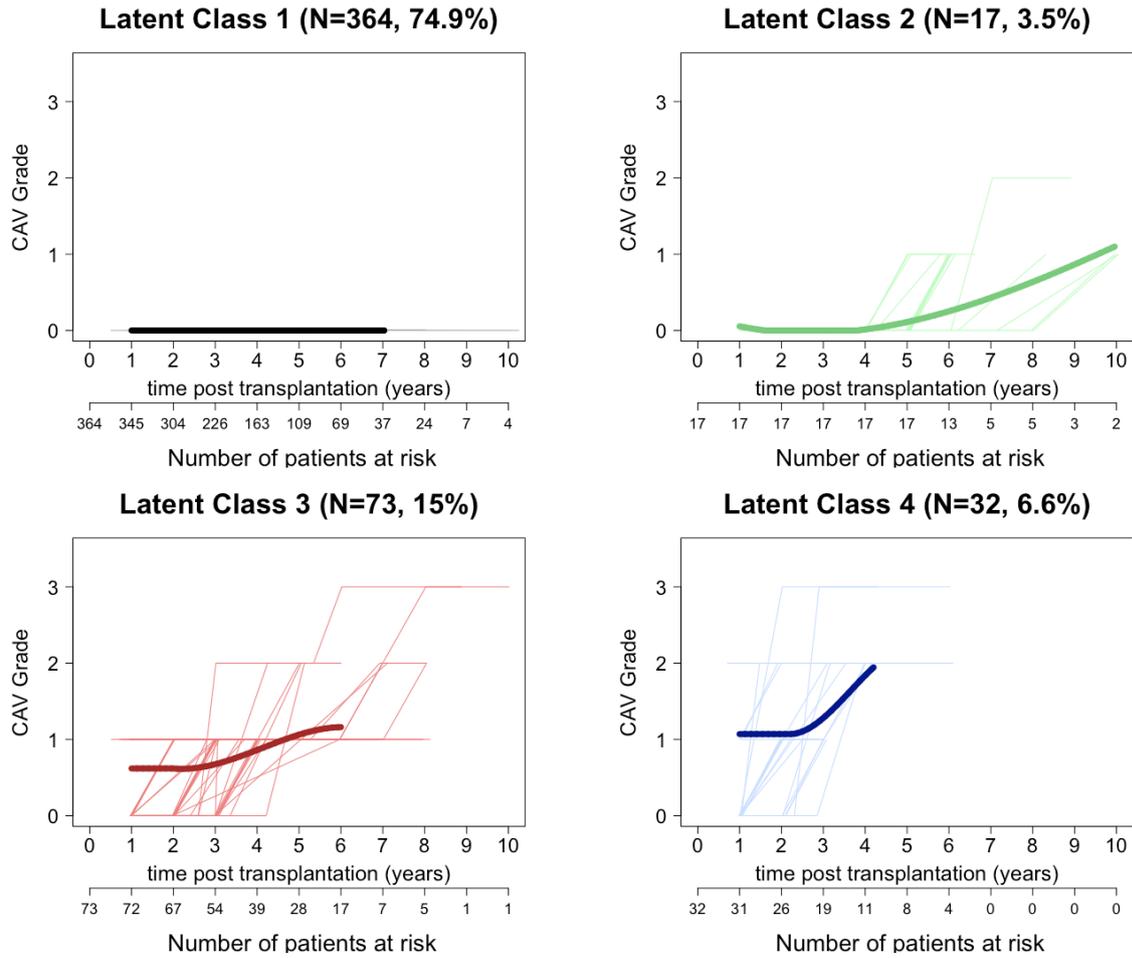


Figure 26: Cardiac allograft vasculopathy trajectories: external validation in the American cohort.

Latent class mixed models were applied to the American validation cohorts. CAV profiles identified in these independent analyses were similar to those identified in the derivation cohort with an excellent discrimination of 0.97. Thick lines represent latent class trajectory; thin lines represent CAV individual patient trajectory.

II-C-3-c. Clinical, functional, structural and immunological determinants of CAV trajectories

A total of 60 clinical, functional, structural and immunological factors of CAV trajectories were investigated. The main results of the univariate analysis conducted in the derivation cohort are reported in Table 15. We identified 17 significant variables associated with the trajectories: 8 donor related risk factors (age: $p < 0.001$; gender: $p = 0.002$; hypertension: $p = 0.035$; diabetes mellitus: $p = 0.003$; tobacco consumption: $p = 0.004$; body mass index $\geq 25 \text{ kg/m}^2$: $p = 0.002$; creatinine clearance $\leq 60 \text{ mL/min}$: $p < 0.001$; vascular cause of death: $p = 0.013$), 4 pre-transplant recipient variables (gender: $p = 0.081$; ischemic cardiomyopathy: $p = 0.022$; hypertension: $p = 0.081$; body mass index $\geq 25 \text{ kg/m}^2$: $p = 0.031$), one post-transplant maintenance immunosuppressive therapy variable (type of calcineurin inhibitor: $p < 0.001$), 4 recipient cardiovascular profile variables assessed at 1-year post transplant (diabetes mellitus: $p = 0.07$; statins therapy: $p = 0.09$; LDLc $\geq 1 \text{ g/L}$: $p = 0.002$; creatinine clearance: $p = 0.02$), 4 immunological parameters (presence of preexisting or de novo anti-HLA DSA: $p = 0.007$; class II immunodominant DSA: $p = 0.025$; cellular rejection grade $\geq 2\text{R}$ occurring during the first year post transplant: $p = 0.042$; episode of AMR occurring during the first year: $p = 0.049$).

After multivariable analysis, the following independent determinants of GFR trajectories were identified: i) donor age ($p < 0.001$), ii) donor gender ($p < 0.001$), iii) donor tobacco consumption ($p = 0.001$), iv) recipient LDL-c $\geq 1 \text{ g/dL}$ one year after transplantation ($p < 0.001$), v) recipient immunological profile as defined by the presence of preexisting or de novo circulating class 2 anti-HLA donor-specific antibodies ($p = 0.019$) and vi) allograft injury defined by acute cellular rejection $\geq 2\text{R}$ occurring in the first year post transplant ($p = 0.028$, Table 16).

	N		Trajectory 1 (n=475)	Trajectory 2 (n=52)	Trajectory 3 (N=179)	Trajectory 4 (N=109)	P
Donor parameters							
Age (10-year increment)	815	-		1.23 (1.00-1.52)	1.80 (1.54-2.10)	1.91 (1.57-2.33)	<0.001
Gender (ref=female)	815	-		1.50 (0.83-2.70)	1.30 (0.90-1.88)	2.59 (1.52-1.88)	0.002
Hypertension	788	-		1.42 (0.70-2.88)	1.56 (0.99-2.49)	2.08 (1.23-3.53)	0.035
Diabetes mellitus	790	-		1.02 (0.12-8.42)	2.87 (1.03-8.05)	3.11 (0.97-10.00)	0.003
Tobacco consumption	783	-		1.32 (0.77-2.26)	1.55 (1.09-2.20)	2.00 (1.30-3.09)	0.004
BMI >25 kg/m ²	815	-		1.61 (0.94-2.74)	1.64 (1.17-2.32)	1.92 (1.26-2.94)	0.002
Creatinine Clearance ≤60 (mL/min/1,73m ²)	815	-		1.57 (0.75-3.29)	1.08 (0.64-1.85)	0.95 (0.48-1.89)	<0.001
Vascular cause of death	815	-		1.11 (0.65-1.88)	1.56 (1.11-2.19)	1.75 (1.14-2.68)	0.013
Recipient parameters							
Age (10-year increment)	815	-		0.98 (0.96-1.00)	1.01 (0.99-1.02)	1.01 (0.99-1.03)	0.153
Gender (ref=female)	815	-		1.17 (0.62-2.21)	1.50 (0.98-2.28)	1.79 (1.02-3.13)	0.081
Ischemic cardiomyopathy	815	-		0.57 (0.30-1.08)	1.00 (0.70-1.45)	1.64 (1.07-2.53)	0.022
Prior history of hypertension	797	-		0.77 (0.40-1.47)	1.19 (0.81-1.75)	1.71 (1.08-2.70)	0.081
BMI (>25 kg/m ²)	815	-		1.11 (0.64-1.92)	1.43 (1.01-2.01)	1.77 (1.15-2.70)	0.031
Transplant baseline parameters							
Cold ischemic time (per 1-hour increment)	815	-		1.08 (0.81-1.45)	0.99 (0.82-1.18)	0.87 (0.69-1.09)	0.565
Combined transplantation	815	-		0.66 (0.15-2.89)	0.77 (0.32-1.83)	0.38 (0.09-1.65)	0.500
CMV mismatch (D+/R-)	809	-		1.15 (0.61-2.18)	1.07 (0.71-1.63)	0.82 (0.47-1.42)	0.800
Immunosuppressive therapies							
IL-2R inhibitor induction therapy (ref = ATG)	815	-		1.18 (0.34-1.10)	1.72 (0.83-1.56)	1.64 (0.67-4.00)	0.467
Tacrolimus CNI at 1 year (ref= Cyclosporine)	815	-		0.51 (0.26-1.01)	0.47 (0.30-0.72)	0.36 (0.20-0.66)	<0.001
Everolimus therapy at 1 year	815	-		1.54 (0.81-2.94)	1.00 (0.63-1.60)	0.87 (0.48-1.59)	0.557
1-year post-transplant cardiovascular risk factor							

Treated hypertension	813	-	0.78 (0.44-1.36)	1.10 (0.75-1.61)	1.71 (1.01-2.90)	0.123
Diabetes mellitus	813	-	1.04 (0.53-2.03)	1.52 (1.02-2.27)	1.70 (1.05-2.76)	0.070
Statins	789	-	2.86 (0.87-9.43)	0.79 (0.49-1.30)	1.38 (0.68-2.81)	0.090
LDL-c ≥ 1g/L	815	-	1.39 (0.82-2.37)	1.76 (1.25-2.49)	1.84 (1.20-2.82)	0.002
Tobacco	813	-	2.02 (0.79-5.18)	1.06 (0.49-2.27)	1.54 (0.67-3.55)	0.462
Creatinine clearance > 60 ml/min/1.73m²	813	-	1.30 (0.76-2.21)	1.10 (0.78-1.54)	0.55 (0.35-0.86)	0.020
Immunology and histology						
Anti-HLA DSA Pre-formed+1-year post HTx	799	-	0.45 (0.21-0.97)	1.49 (1.03-2.16)	1.31 (0.82-2.10)	0.007
Anti-HLA DSA Pre-formed+1-year post HTx	798					0.025
Class I		-	0.51 (0.15-1.71)	1.08 (0.57-2.02)	1.37 (0.67-2.80)	
Class II		-	0.42 (0.16-1.10)	1.72 (1.13-2.61)	1.30 (0.75-2.25)	
≥ 1 episode of acute cellular rejection ≥ 2R	815	-	2.39 (1.23-4.63)	1.25 (0.75-2.10)	1.79 (1.00-3.18)	0.042
Antibody-mediated rejection ≥ pAMR1	608	-	1.78 (0.82-3.84)	1.21 (0.69-2.14)	0.53 (0.22-1.31)	0.049

Table 15: Determinants of CAV trajectories in the derivation cohort

This table shows the association of clinical, immunological, functional and structural parameters associated with CAV trajectories in univariate multinomial regression analysis. The trajectory of reference was trajectory#1, including patients with no CAV at baseline and stable over time.

ATG means anti-thymocyte globulins, AMR: antibody-mediated rejection, BMI: body mass index, CMV: cytomegalovirus, DSA: donor-specific antibodies, MCS: mechanical circulatory support.

	Trajectory 1 (n=475)	Trajectory 2 (n=52)	Trajectory 3 (N=179)	Trajectory 4 (N=109)	P
Donor parameters					
Age (per 10-year increment)	-	1.27 (1.03-1.58)	1.91 (1.62-2.25)	2.14 (1.73-2.65)	<0.001
Gender (ref=female)	-	1.64 (0.89-3.00)	1.82 (1.23-2.71)	3.69 (2.11-6.46)	<0.001
Tobacco	-	1.40 (0.82-2.39)	1.73 (1.20-2.50)	2.22 (1.40-3.51)	0.001
1-year post-transplant cardiovascular risk factor					
LDL-c \geq 1g/L	-	1.87 (0.80-2.35)	1.72 (1.19-2.47)	1.78 (1.13-2.80)	0.009
Immunology and histology					
Anti-HLA DSA pre-formed+ 1-year post HTx (ref=None)					0.019
Class I	-	0.47 (0.14-1.59)	0.87 (0.44-1.70)	1.03 (0.48-2.23)	
Class II	-	0.45 (0.17-1.17)	1.93 (1.23-3.02)	1.49 (0.83-2.68)	
Acute T-cell mediated rejection \geq 2R during the 1st year	-	2.39 (1.22-4.68)	1.29 (0.75-2.21)	1.98 (1.07-3.65)	0.028

Table 16: Factors associated CAV trajectories in multivariate analyses.

This table shows the association of clinical, immunological, functional and structural parameters associated with CAV trajectories in multivariate multinomial regression analysis. The trajectory of reference was trajectory#1, including patients with no CAV at baseline and stable CAV grade over time.

II-C-3-d. Projection of patient individual 10 years CAV trajectories based on determinants assessed at the time of transplant and in the first year after transplant

Based on the determinants identified in previous analyzes, we built an online interfacing to provide clinicians with a ready-to-use tool that projects the CAV trajectories (developed with the Shiny package on R: https://transplantpredictionsystem.shinyapps.io/personalized_CAV_trajectories/).

Clinicians can enter individual parameters of one patient and 4 likelihoods of belonging for each trajectory are provided, corresponding to the personalized likely future CAV progression of the patient, as well as the corresponding allograft survival and the mean slope of CAV per year of the predominant CAV trajectory.

II-C-3-e. Association between CAV trajectories and overall cause mortality

We found that the trajectories were also associated with patient death. During the follow-up, a total of 198 patients died overall. We found that CAV trajectories 3 and 4 were associated with higher mortality (10-years patient survival of 73.43% (95%CI=65.18-80.02), 51.89% (95%CI=38.76-63.51), respectively) as compared with trajectories 1, and 2 that were characterized by 10-years patient survival of 80.01 (95%CI=76.38-84.82) and 83.49% (95%CI=71.34-90.80), respectively (log-rank test: $p < 0.001$, Figure 27).

II-C-3-f. Sensitivity analyzes

Various sensitivity analyses were performed to test the robustness and generalizability of the CAV trajectories and determinants in different subpopulations and clinical scenarios.

i) CAV trajectories and center effect: to take into account a possible bias in the assessment of the determinants of the CAV trajectories, the center was also entered in the final multivariable model and did not modify the set of independent parameters associated with trajectories. When the multivariable model was performed adding to the derivation cohort the external validation cohort, the 6 independent determinants of CAV trajectories identified in primary analyzes remained unchanged. *ii) Immune determinants of CAV trajectory*: Replacing the variable “DSA at transplant or in the first-year” by “DSA at transplant” did not have any impact on the final model. When histological antibody mediated rejection was forced in the final multivariable model, it showed a trend for association ($p=0.07$) with CAV trajectory, but was outperformed by the circulating anti HLA DSA status. *iii) Consistency of eGFR trajectories according to timing of baseline CAV assessment*: We tested and confirmed the robustness of the profiles of CAV trajectories when the CAV evaluation started before 1-year post-transplant (the starting point of our study), with a discrimination of 0.89. We also confirmed the consistency of the trajectories when the baseline CAV was assessed at 2 years after transplantation (discrimination of 0.90).

Last, the similar four-trajectory pattern was found when performing LCCM analyses stratified by countries of the derivation cohort.

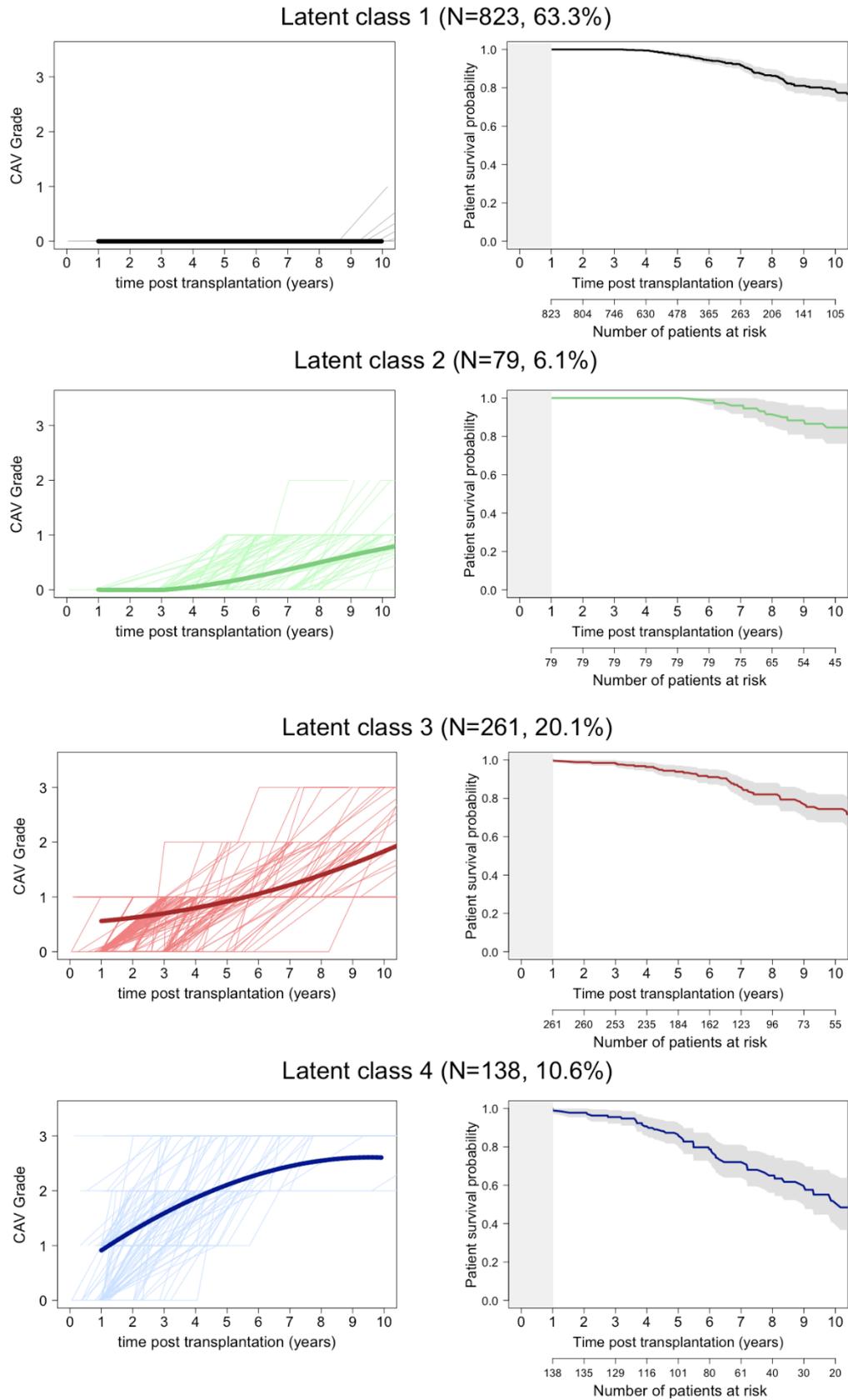


Figure 27: Description of overall mortality according to the CAV trajectory in the overall cohort (n=1,301).

Overall survival was significantly lower in trajectories #3 and #4 compared to trajectories #1 and #2 (p<0.001).

II-C-4. Discussion

In this international study of 1,301 carefully-phenotyped heart transplant recipients with protocol-based repeated assessment of CAV over 10 years, (4,710 coronary angiographies), combined with histology (12,803 biopsies) and immune profiling, we identified for the first time using unsupervised approach 4 distinct trajectories of long-term CAV progression. We demonstrated that the 4 trajectories were consistent in geographically distinct cohorts recruited in Europe and the US, which is remarkable given their distinct allocation systems, patient characteristics and management practices. We found that these trajectories were quite varied for several different conditions related to donor and recipient characteristics, ongoing diseases processes and immunological profile that can be determined at an early stage after transplantation.

II-C-4-a. Study strengths and novelties

Despite the importance of angiogram evaluation as standard of care after heart transplantation, no study has characterized long-term trajectories using large unselected transplant cohorts with a prespecified longitudinal CAV monitoring together with a patient deep level phenotyping including donor and recipient characteristics, histology, immunology and treatment. Beyond the novel study design, the original advantage of our trajectory approach over traditional analysis is its ability to map the CAV course and classify individuals into distinct, mutually exclusive groups. Hence this approach not only helps to conceptualize the long term trajectories of CAV but also allows us to probe the population heterogeneity and the susceptibility of change in CAV over the life course. Furthermore, we believe that identifying the profiles of evolution in time of a chronic disease with an unsupervised approach and investigating whether these profiles manifest consistently across centers and countries may provide important guidance on the potential universality of the disease, their determinants, which has great implications for future research in particular for counseling patients, and also for choosing therapies.

II-C-4-b. Clinical significance of the CAV trajectories

The four 4 distinct trajectories identified represent different profiles of CAV evolution over time. CAV trajectory^{#1} is characterized by the absence of CAV overtime, trajectory^{#2} by a mild and late onset of CAV, trajectory^{#3} by an early onset with progressive evolution of CAV and trajectory^{#4} by an early onset with rapid evolution of CAV. From a clinical perspective, those findings might help to refine CAV management. On the one hand, the coronary angiogram monitoring protocol could be adapted to the evolving risk of CAV and therefore to the likelihood of belonging to each trajectory. Based on the determinants identified and the results of baseline coronary angiograms, we build an online interfacing to provide clinicians with a ready-to-use tool that projects the CAV trajectories. Patients with a high likelihood of belonging to trajectory^{#1} might be monitored less intensively. On the opposite, patients with a high likelihood of belonging to trajectories^{#3} and ^{#4}, at risk of CAV progression, might benefit from a closer follow-up. On the other hand, these trajectories might improve prognosis stratification after heart transplantation since trajectories^{#3} and ^{#4} are associated with a significant higher risk of overall mortality.

II-C-4-c. Significance of immune determinants of CAV

This study also emphasizes the interplay between cardiac transplant donor and recipient characteristics, the allograft immune profile, and existing allograft injury and the long-term CAV trajectories. The associations between these characteristics and the trajectories CAV suggest mechanistic explanations. Class II DSA appears to be an important trigger of CAV in our cohort. Both experimental and clinical studies support these immune determinants of CAV. The passive transfer of DSA to immunodeficient mice promote the development of an early and severe CAV, even in complement KO mice (17,18). Heart transplant recipients with preformed class II DSA were at increased risk of accelerated CAV as detected by consecutive volumetric three-dimensional intravascular ultrasound (19). The expression of class II HLA antigens by the endothelial cells requires their activation. Traditional cardiovascular risk factors associated with Trajectory^{#3}, both donor-derived and from the recipient, might contribute to the endothelial inflammation and activation, thus increasing the number of potential class-II DSA targets. More and more data suggest that the Natural-Killer cell (NK) and ADCC plays a critical role in the development of immunological

atherosclerosis. Mice treated with a depletive NK cells agent or genetically deficient in mature NK cells had a significantly reduced DSA-induced CAV (18). FCGR3A-VV polymorphic variant, which encodes the highly responsive NK cells CD16 Fc receptor, was identified as an independent baseline predictor of cardiac allograft vasculopathy (21).

We found that acute cellular rejection $\geq 2R$ component is an independent risk factor for CAV progression. On the one hand, severe cellular rejection might acutely accelerate CAV by promoting local inflammation and the recruitment of other immunological cells. On the other hand, cellular rejection has been recognized as a risk factor for the development of de-novo DSA (106). In the French derivation cohort, patients with ≥ 1 episode of acute cellular rejection $\geq 2R$ were twice as likely to develop de-novo DSA as patients without acute cellular rejection (17/60 (25.3%) compared to 55/315 (14.9%), $p = 0.01$). Finally, we found marginal associations between antibody mediated rejection and CAV trajectories in our cohort, contrasting with robust association with the presence of preexisting or de novo anti-HLA DSA. DSA is probably a much more sensitive markers of allo-sensitization than AMR. Although acute and severe antibody mediated rejection has been associated with fulminant forms of CAV, this entity is rare and chronic allograft inflammation induced by allo-sensitization is probably a much more trigger of CAV at a population level. Chronic antibody-mediated rejection has not been properly identified in heart allografts in contrary to kidney transplantation. This sub-clinical disease process might not be captured by 1-year histology.

II-C-4-d. Universality of the 4 trajectories profiles and prospects for patient risk stratification and monitoring

Remarkably, despite the identification without any preconceptions of 4 CAV trajectories in the derivation cohort, very similar trajectories were detected in North-American independently. This validation of the same set of trajectories for patients in different healthcare systems promotes the idea that the same universal factors drive the evolution of CAV over the world which we further supported by identifying the determinants of CAV trajectories in the derivation cohort, and in the overall pooled derivation and validation cohorts. Furthermore, although the proportions of patients in each trajectory differed somewhat between the cohorts, reflecting intrinsic variability in the demographics and heart transplant practices across nations, our results

nonetheless suggest promise for using these profiles to first categorize heart transplant recipients and then tailor the plan for future allograft monitoring and therapy based on the category. Consequently, to make trajectory-based monitoring of patients feasible in contemporary practice, we developed an easy-to-use online interface that allows clinicians to predict the personalized likely future of CAV trajectory of any given patient. This approach to determining patient trajectories brings wider dimension to the traditional approaches that are either derived from one single angiogram result and selected but limited parameters, or are instead based on few measurements in non protocol based cohorts. Taking into account the evolution of CAV in the long-term could therefore takes advantage of more information about subtle changes and therefore could be an important complement to current medical practices. In addition, given the advancement of technology, especially the growing use of electronic medical records, trajectory assessment will conceivably have improved accuracy and may not be an overwhelming task for clinical practitioners to perform in the future.

II-C-4-e. Clinical trials

Although not validated by American and European drug regulatory authorities, CAV is a major clinical endpoints for past and ongoing clinical trials in heart transplantation since this pathology represents one the leading cause of death or retransplantation late after transplant (JHLT 2018 annual report). Endocoronary imaging modalities, either intravascular ultrasound or optical coherence tomography, have the ability to detect early coronary lesions while the coronary angiogram remains normal. The change in maximal intimal thickness from baseline to one year or from one to five year post-transplant has been associated with mortality, nonfatal major adverse cardiac events and development of angiographic CAV (15,16). All not-withdrawn CAV-focused studys registered on clinicaltrials.com (n=18) have an IVUS/OCT endpoint. However, endocoronary imaging procedures have specific complications and are not available in all centers.(107) Moreover, coronary angiography coupled with assessment of cardiac alograft function maintains the highest level of evidence for CAV monitoring according to ISHLT guidelines.(108) We propose the possibility that the latent class trajectory approach also holds promise as an outcome measure. Indeed, for most of trials, the duration of follow-up is too brief to expect a large change in CAV.

II-C-4-f. Study limitations

Several limitations of the study should be noted. First, CAV grading is subject to measurement error. However, given our prospective design, any error would have probably attenuated identification of 4 distinct trajectories, whereas we were able to consistently identify the trajectories across different cohorts. Besides, in the derivation cohort, all CAV were reassessed by 2 independent observers. vSecondly, a limited number of trajectories were derived that may not accurately reflect every possible CAV profiles. However, the good discrimination of our trajectory building model indicate that these trajectories can parsimoniously summarize the predominant features of patient allografts in our population without a significant loss of information. Third, some classes – especially trajectory^{#4} – have relatively high inter-patient variability. Yet, since our goal was to give the overall picture of the CAV in large and diverse cohorts, we considered such variability acceptable. Last, there are unmeasured characteristics of the recipients and allografts such as donor genetic profiles, non HLA antibodies or inflammatory circulatory biomarkers that may well affect CAV trajectories and could be explored in future studies.

II-C-5. Conclusion

In conclusion, this analysis takes an original approach to analyzing and characterizing for the first-time trajectories of long-term CAV after heart transplantation associated with overall mortality. We also found that the identified CAV trajectories and their determinants could be generalizable and transportable across centers in different continents. Our results provide an important new tool for improving the approach to monitoring and risk stratifying heart transplant recipients, potentially paving the way towards therapeutic interventions.

Chapter II-D: Synthesis of epidemiological studies.

By using a population-based approach and applying two different epidemiological approaches, we were able to describe the epidemiology and natural history of allograft rejection and CAV, two important complications of allosensitization after heart transplantation, and to determine their risk factors.

We were able to show that allograft rejection had an evolutionary epidemiology during the first year, with a maximum risk of rejection during the first 3 months followed by a gradual decrease in risk and to identify 5 independent risk factors for rejection: recipient's age, type of induction, type of transplantation (isolated vs combined), number of HLA mismatch and presence of pre-formed DSA.

Using latent class mixed models, we identified four mutually exclusive CAV trajectories derived in a European cohort and validated on a US cohort. We identified five independent risk factors associated with these trajectories: donor age, donor gender, donor smoking, post-transplant dyslipidemia and the presence of class II DSA. (Figure 28).

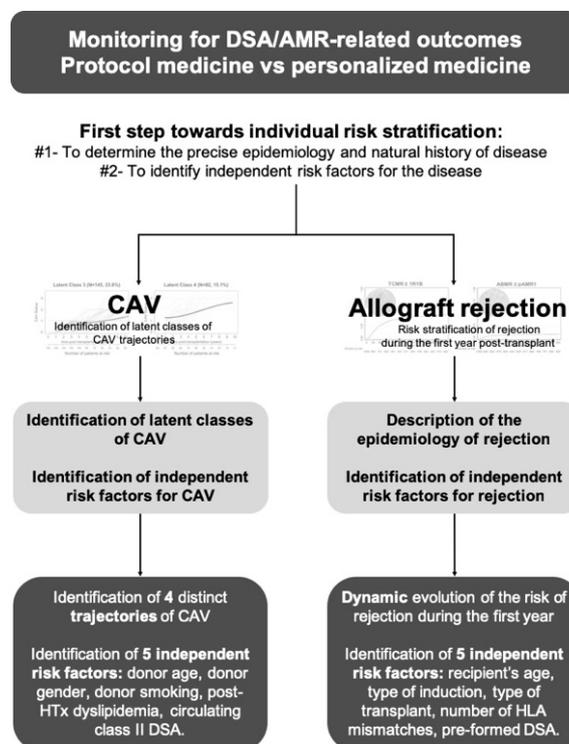


Figure 28: Synthesis of the results of the epidemiological studies.

Part 3.

Clinical studies to better characterize the outcomes of at-risk populations

As previously discussed in the introduction section, the therapeutic management of allosensitization and AMR is **heterogeneous** between centers and there is no consensus due to the lack of clinical data and therapeutic trials. Two populations at high immunological risk are particularly concerned by this lack of data: the patients transplanted with **preformed DSA** and the patients presenting the most severe forms of **AMR**, who are refractory to usual therapies and characterized by a severely depressed graft function responsible for a **cardiogenic shock**.

Our objective in this clinical part is to report the experience of our center with these two types of patients in order to better describe and understand their clinical events (Figure 29).

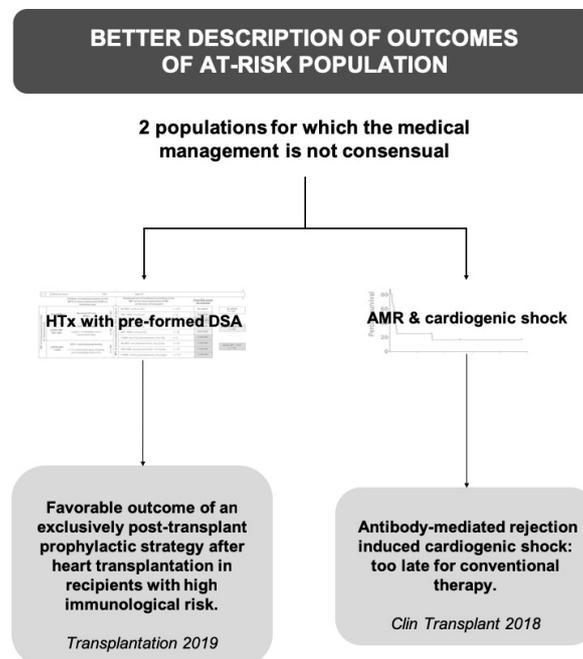


Figure 29: Better understanding of outcomes of at-risk populations.

Chapter III-A: Favorable outcome of an exclusively post-transplant prophylactic strategy after heart transplantation in recipients with high immunological risk.

Article published in *Transplantation*, 2019 July.

III-A-1. Heart transplantation with pre-formed anti-HLA donor-specific antibodies

The number of sensitised patients on the heart transplant waiting list has increased over time, and now presents a recurrent issue (109). Pre-transplant desensitisation therapies are the most widely used strategy for these patients. Several protocols have been evaluated, but a consensus has not been reached. While most of these protocols achieve acceptable post-transplant outcomes (27,110), they suffer from several limitations: longer waiting time and worse outcomes for sensitised patients (111), potential post-transplant infectious complications, and a higher cost of therapy. An exclusively post-transplant strategy has not yet been evaluated.

In France, a higher priority status is granted to transplant candidates on Extra-Corporeal Membrane Oxygenation (ECMO) or inotropes than those on long-term mechanical circulatory support. Emergency access to heart transplantation (HTx) is limited to 4 days. Due to this particular allocation policy, approximately half of patients undergo “high-emergency” transplantation, and therefore, cannot be included in a desensitisation program. Our local strategy is to perform HTx regardless of the presence of preformed donor-specific anti-HLA antibodies (pfDSA). We believe that granting rapid access to HTx for sensitised patients is crucial, particularly for high-risk patients. Therefore, we established an exclusively post-transplant prophylactic strategy consisting of perioperative management of pfDSA (polyvalent immunoglobulins [IVIg] +/- plasmapheresis sessions, according to the level of pfDSA, combined with induction therapy) and systematic treatment of subsequent antibody-mediated rejection. Here, we report a retrospective analysis of this prospective

protocol. Our aim was to describe the outcomes of HTx recipients with pfDSA at our center.

III-A-2. Patients and methods

III-A-2-a. Patients

We performed a retrospective analysis of a prospective protocol. We included all consecutive first non-combined HTx recipients at our institution between January 1, 2009 and December 31, 2015. Patients were followed until January 2017. Our study complies with the Declaration of Helsinki, and the institutional review board approved the protocol. Informed consent was obtained at the time of enrolment into the study. Baseline donor and recipient characteristics were obtained from the prospective national registry CRISTAL database (French National Agency for Organ Procurement).

III-A-2-b. Study outcomes

The primary endpoint was all-cause mortality. Secondary endpoints included: (i) biopsy-proven rejection-free survival (cellular rejection $\geq 2R$ and/or ABMR, pAMR 1–3 according to the International Society for Heart and Lung Transplantation [ISHLT] guidelines (112,113)), ii) primary graft dysfunction (PGD; the requirement for ECMO support after HTx, corresponding to the severe PGD-LV scale of ISHLT (114), in patients without pre-HTx ECMO, as those patients are maintained under ECMO for at least 24 to 48 h after HTx at our center), and iii) cardiac allograft vasculopathy (CAV)-free survival (CAV grade 1 or higher according to ISHLT guidelines (35)).

The safety endpoints included (i) bleeding complications (assessed by bleeding requiring surgical management during the first week after HTx and the number of packed red blood cells [PRBC] and platelet concentrates transfused during the first week), and (ii) infectious complications (assessed as the incidence of bloodstream infections during the first 2 weeks after HTx).

III-A-2-c. Immunosuppressive protocol

Immunosuppression after HTx was based on an induction therapy with rabbit anti-thymocyte globulin (rATG; Thymoglobuline; Genzyme, Lyon, France; 1.5 mg/kg/day for 5 days, treatment time: after plasmapheresis, before IVIg) for transplant patients

with pfDSA, and rATG or basiliximab (Simulect, Novartis, Basel, Switzerland) for other patients. Prophylactic immunosuppressive therapy included calcineurin inhibitors, mycophenolate mofetil and corticosteroids with posology recommended by ISHLT guidelines (115).

The decision to accept a graft was made regardless of the presence of pfDSA. A prophylactic protocol including perioperative management of pfDSA and systematic treatment of subsequent ABMR was applied to patients transplanted with pfDSA. Perioperative management was adapted to the results of the virtual cross-match and to the level of pfDSA, as evaluated by the mean fluorescence intensity (MFI) of the immunodominant DSA (i.e., DSA with the highest MFI): (1) patients with MFI 500–1000 were treated with IVIg (0.5 g/kg over 4 consecutive days, total dose of 2 g/kg; Privigen; CSL Behring AG, Bern, Switzerland), (2) patients with MFI >1000 were treated with perioperative plasmapheresis sessions (one immediately before HTx, then four sessions over 4 consecutive days; 2/3 fresh frozen plasma and 1/3 albumin, fibrinogen substitution if serum fibrinogen < 2g/L) and IVIg. Initiation of the protocol was based on detection of anti-HLA antibodies in historical sera. The treatment was readjusted 2 to 3 days after HTx according to the MFI results at the time of HTx (Figure 30). Subsequent biopsy-proven ABMR, even subclinical ABMR, were treated with corticosteroid pulses, plasmapheresis (five sessions) and IVIg (2 g/kg).

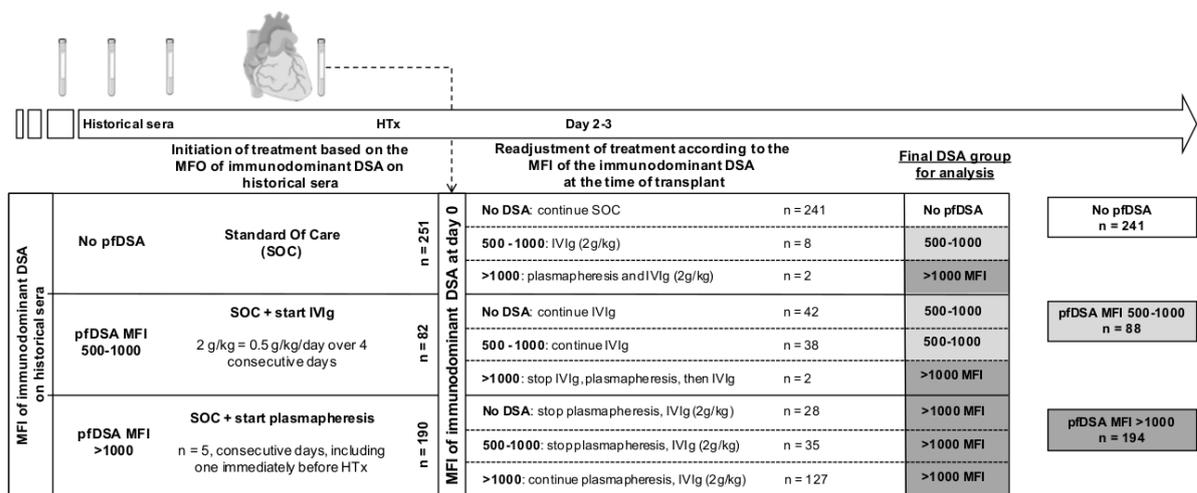


Figure 30: Study protocol.

Study protocol and evolution of repartition in the group with preformed donor-specific anti-HLA antibodies (pfDSA) between historical sera and day 0 serum. The initiation of treatment was based on the mean fluorescence intensity (MFI) values of immunodominant DSA in historical sera and readjusted according to the results for day 0 serum. SOC means Standard of Care.

III-A-2-d. Immunology

The HLA-typing of heart recipients as low-resolution class I HLA-A and HLA-B and class II HLA-DR and HLA-DQ was performed by PCR-SSO (LABType, One Lambda). Donor HLA A, B, DR and DQ typing was performed by CDC methods using tissue-typing trays (One Lambda) and controlled by molecular biology. The detection of anti-HLA antibodies pre- and post-HTx was based on Luminex single antigen bead technology (One Lambda, Canoga Park, CA, USA). The fluorescence of each bead is detected by a reader and recorded as the normalized MFI. Beads showing MFI values > 500 were considered positive. The pre-transplant screening for anti-HLA antibodies was performed every 3 months while on the waiting list. Retrospective crossmatches were performed by complement-dependent cytotoxicity. A pre-transplant sensitizing event was defined as pregnancy, transfusion and/or solid organ transplantation (other than heart transplantation) prior to heart transplantation.

III-A-2-e. Endomyocardial biopsies

Endomyocardial biopsies (EMBs) were performed, processed and examined according to current standards (115). Our routine EMB protocol includes 13 EMBs during the first year (the first is performed 15 days after HTx), followed by every 4 months for 2 years, then every 6 months for the subsequent 2 years. Additional biopsies were performed in case of clinical indication. Serial sections were cut from formalin-fixed paraffin-embedded EMB specimens and stained with hematoxylin, eosin and saffron for diagnosis. Immunofluorescence for C4d was performed (frozen section; C4d monoclonal Quidel Corporation 1/100; Polyclonal rabbit anti mouse FITC Dako). Only capillary staining for C4d was assessed. The EMBs were classified as cellular (0 to 3R) or ABMR (pAMR 0 to 3) according to the ISHLT guidelines (112,113). An expert pathologist retrospectively reviewed the following biopsies: for-cause EMB, all rejection EMB (ABMR and ACR 1R1B or more), and protocol EMBs performed at 1, 3 and 12 months after transplantation.

III-A-2-f. Cardiac allograft vasculopathy

Routine coronary angiograms were performed 1 year after HTx and then every 2 years afterwards, or in the case of unexplained left ventricular dysfunction. Staging of cardiac

allograft vasculopathy (CAV) was performed using the recommended ISHLT nomenclature (35).

III-A-2-d. Statistical analyses

Qualitative variables are presented as frequencies, and quantitative variables are presented as the mean and standard deviation (SD) or median and inter-quartile range (IQR) according to their distribution. To compare quantitative and qualitative variables, Student's t-test and the chi-square test were used, respectively. Survival functions were estimated with the Kaplan-Meier method, and survival rates and 95% confidence intervals (CI) were provided using Greenwood variance. Cox univariate regression was used to evaluate the association between clinical and biological factors and outcomes. Risk factors for PGD were analyzed by univariate logistic regression. Cumulative incidence function of CAV was defined as the time elapsed between HTx and the first CAV of grade ≥ 1 . Patients without at least one coronary angiogram were excluded from this analysis ($n = 139$). Multivariate models were then established. Candidate factors were selected when the univariate likelihood ratio test p-value was less than 0.10. Descending selection was used, and the final multivariate model considered significance at 0.05. Goodness-of-fit of multivariate logistic regression models were evaluated using Hosmer-Lemeshow test. Statistical analysis was performed with R software (version 3.4.1) using the 'survival' and 'cmprsk' packages.

III-A-3. Results

III-A-3-a. Characteristics of included patients

During the study period, 523 patients received their first non-combined HTx (nine re-transplantations were excluded). The recipient, donor and transplant characteristics are presented in Table 17. Half of patients underwent transplantation in an emergency context, including 111 patients (21.2%) who were under ECMO support.

Of the 523 patients, 241 had no DSA, 88 patients had pfDSA with MFI between 500 and 1000, and 194 patients had pfDSA with MFI >1000 in historical or day 0 sera. The proportion of sensitized patients increased over the study period. The evolution in group repartition between historical sera and day 0 sera is presented in Figure 30. None of our patients received pre-transplant desensitisation therapies.

	All patients N=523	No pfDSA N=241	MFI 500-1000 N=88	MFI > 1000 N=194	p-value
Recipient characteristics					
Age (years)	49.55±12.6	51.1±12.2	49.1±12	47.8±13	0.02
Gender (female) — no. (%)	119 (22.7%)	48 (19.9%)	15 (17%)	56 (28.9%)	0.03
Weight (kg)	72.1±14.1	71.4±13.7	74.7±14.6	71.8±14.5	0.11
Body mass index (BMI, kg/m ²)					0.78
< 18.5	26 (5%)	14 (5.8%)	3 (3.4%)	9 (4.6%)	
18.5 - 25	300 (57.3%)	133 (55.2%)	50 (56.8%)	117 (60.3%)	
25-30	152 (29.1%)	75 (31.1%)	28 (31.8%)	49 (25.3%)	
> 30	45 (8.6%)	19 (7.9%)	7 (8%)	19 (9.8%)	
Ischemic cardiomyopathy — no. (%)	176 (33.6%)	80 (33.2%)	26 (29.5%)	70 (36%)	0.55
Long term MCS (LVAD or TAH) — no. (%)	70 (13.4%)	17 (7%)	13 (14.8%)	40 (20.6%)	< 0.001
ECMO support at transplantation — no. (%)	111 (20%)	56 (23.2%)	12 (13.6%)	43 (22.1%)	0.15
Emergency HTx — no. (%)	303 (57.9%)	140 (58.1%)	46 (52.3%)	117 (60.3%)	0.44
Past history of cardiac surgery — no. (%)	102 (29.5%)	41 (17%)	18 (20.5%)	43 (22.1)	0.38
Pre-transplant sensitizing event	159 (30.4%)	67 (27.8%)	15 (17.1%)	77 (39.7%)	< 0.001
Mechanical ventilation — no. (%)	21 (3%)	12 (5%)	1 (1.1%)	8 (4.1%)	0.29
Dialysis — no. (%)	4 (0.8%)	2 (0.8%)	1 (1.1%)	1 (0.8%)	0.82
Creatinine clearance - day 0 (ml/min/1.73 m ²)					0.096
≥ 90	193 (36.9%)	90 (37.3%)	22 (25%)	81 (41.7%)	
≥ 60 and < 90	166 (31.8%)	79 (32.8%)	37 (42%)	50 (25.8%)	
≥ 30 and < 60	144 (27.5%)	63 (26.2%)	25 (28.4%)	56 (28.9%)	
< 30 or dialysis	20 (3.8%)	9 (3.7%)	4 (4.6%)	7 (3.6%)	
Total bilirubin - day 0 (mmol/l)	22.8±23.1	23.7±26	24.1±19	21.1±20.5	0.13
Donor characteristics					
Age (years)	46.1±13.7	46.3±13.4	44.5±14.2	46.6±13.9	0.52
Gender (female) — no. (%)	193 (36.9%)	84 (34.9%)	32 (36.4%)	77 (39.7)	0.59
Weight (kg)	75.4±15	75.5±16	74.8±16	75.6±14	0.68
Cause of death — no. (%)					0.43
Traumatic	87 (16.6%)	47 (19.5%)	16 (18.2%)	24 (12.4%)	
Cerebrovascular	326 (62.3%)	140 (58.1%)	56 (63.6%)	130 (67%)	
Anoxia	94 (18%)	46 (19.1%)	13 (14.8%)	35 (18%)	
Other	16 (3.1%)	8 (3.3%)	3 (3.4%)	5 (2.6%)	
Prior cardiac arrest — no. (%)	131 (25%)	66 (27.4%)	19 (21.6%)	46 (23.7%)	0.50
Norepinephrine > 3 mg/h — no. (%)	77 (14.7%)	31 (12.9%)	16 (18.2%)	30 (15.5%)	0.44
Transplant characteristics					
Sex mismatch (female D, male R) — no. (%)	119 (22.8%)	57 (23.6%)	20 (22.7%)	42 (21.6%)	0.89
Weight mismatch — no. (%)	43 (8.2%)	17 (7%)	9 (10.2%)	17 (8.8%)	0.57
Cold ischemic time (min)	188±57	187±59	182±60	191±53	0.53
HLA A/B/DR/DQ mismatch (n)	5.9±1.5	5.7±1.4	5.9±1.7	6±1.6	0.12
MFI of immunodominant DSA	1095±2663	0	340±389	2798±3800	< 0.001
Cumulative MFI of DSA	1810±6139	0	448±599	4677±9411	< 0.001
Induction therapy (ATG) — no. (%)	509 (97.5%)	232 (96.7%)	86 (97.7%)	191 (98.4%)	0.28

Table 17. Characteristics of included patients.

LVAD means left ventricular assist device; MCS, mechanical circulatory support, TAH, total artificial heart.

III-A-3-b. Donor-specific antibodies

Patients with pfDSA MFI >1000 were younger and were more likely to be female, under mechanical circulatory support and to have had a pre-transplant sensitizing event (Table 17). The median MFI value for immunodominant DSA at day 0 was 1536 (776–2816) in this group. One-quarter of patients had pfDSA MFI >3000. The repartition of immunodominant DSA MFI and cumulative MFI is presented in Figure 31. Seventeen patients had a retrospective positive CDC-LCT crossmatch (8.7%).

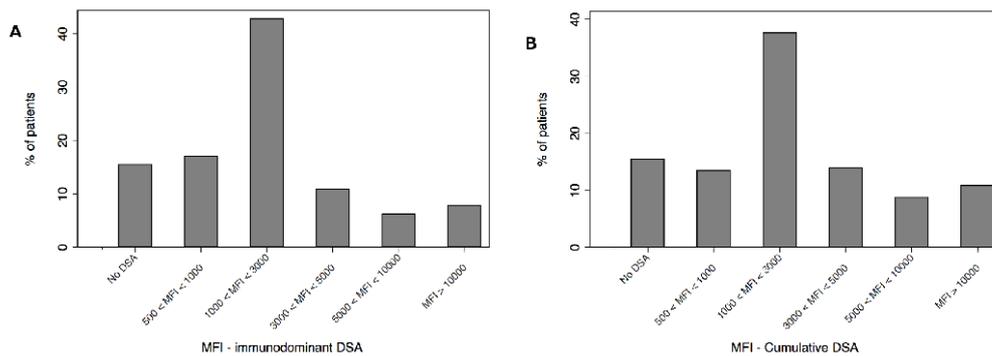


Figure 31: Mean Fluorescence Intensity of donor-specific antibodies.

Distribution of patients according to preformed donor-specific anti-HLA antibody (pfDSA) mean fluorescence intensity (MFI) on the day of transplant in patients with pfDSA MFI of >1000 in historical or day of transplant sera (n = 194). (A) Immunodominant DSA. (B) Cumulative MFI of all DSA.

We observed a significant decrease in the amount of DSA, as assessed by the MFI of the immunodominant DSA and cumulative MFI, between day 0 serum and post-plasmapheresis serum and between day 0 serum and 1-month serum ($p < 0.001$ for all comparisons, Figure 32).

In patients transplanted without pfDSA (n = 241), 187 (77.6%) had at least one screening for *de novo* DSA (dnDSA) during the first year. Among them, a dnDSA was detected in 54 patients (28.9%). Anti-HLA antibodies not directed against the donor HLA were present on historical and/or day 0 sera in 108 patients (57.8%) and was a significant risk factor for dnDSA development (incidence of dnDSA at one-year: 40.8 and 12.7% in patients with and without pre-formed anti-HLA antibodies not directed against the donor, respectively, $p < 0.001$).

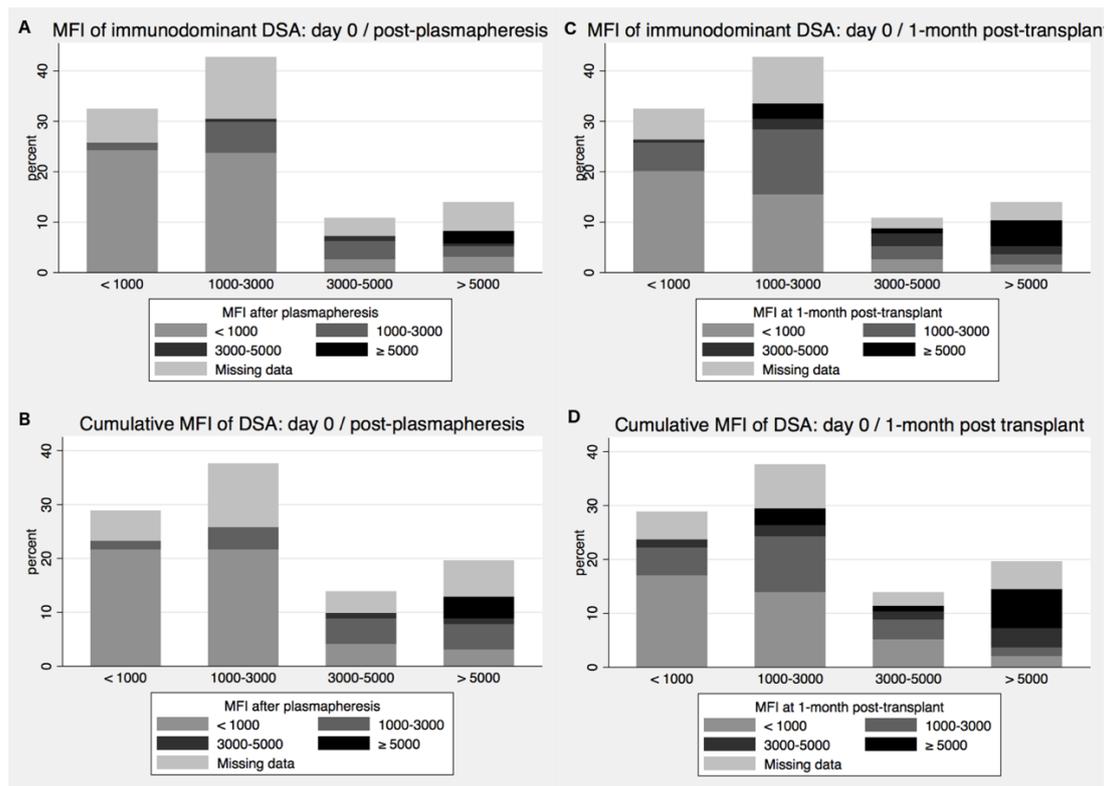


Figure 32. Evolution of MFI after peri-operative management in patients with preformed donor-specific anti-HLA antibody (pfDSA) mean fluorescence intensity (MFI) levels >1000.

Evolution of MFI after peri-operative management in patients with preformed donor-specific anti-HLA antibody (pfDSA) mean fluorescence intensity (MFI) levels >1000. Evolution of MFI of DSA between day 0 and post-plasmapheresis sera (A, B, n = 139, missing values: n = 55) and between day 0 and one-month post-transplant sera (C, D, n = 153 patients, missing values: n = 41). (A, C: immunodominant DSA; B, D: cumulative MFI of DSA). Categories are determined according to the MFI at the day of transplant (immunodominant or cumulative). The evolution of MFI after plasmapheresis and at one-month is described in each subgroup.

III-A-3-c. Primary endpoint: survival after transplantation

The median follow-up was 4.06 years (IQR 1–7.98). Survival rates at 1 and 3 years after transplantation were 81.1% (95% CI [78, 84]) and 73.6% (95% CI [70, 78]), respectively, and were not significantly different between groups (Figure 33 and Table 18). In the univariate analyses, donor age, weight and gender, recipient age and creatinine clearance, weight mismatch and ischemic time were associated with the risk of death, regardless of DSA group or DSA MFI (Table 19). In the multivariate analyses, donor age, recipient age, weight mismatch and cold ischemic time remained associated with mortality (Table 20).

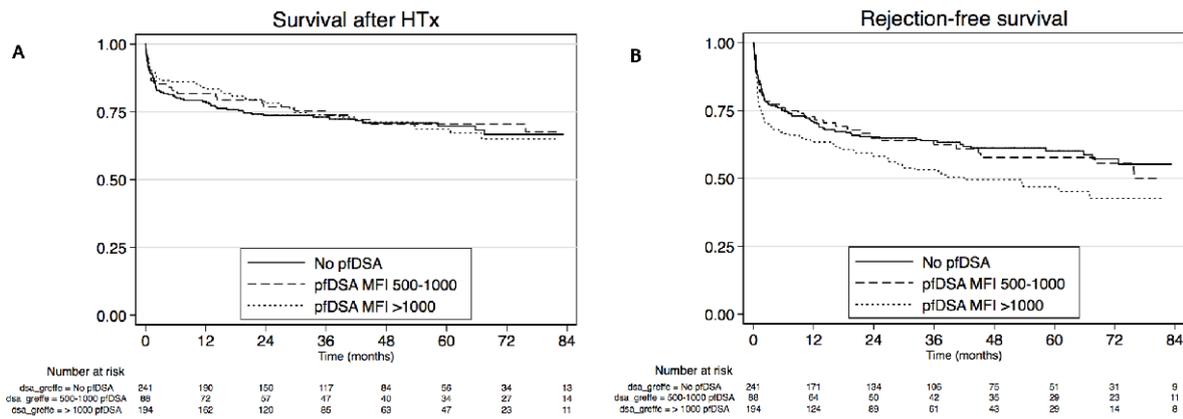


Figure 33. Primary and secondary outcomes.

(A) Survival after heart transplantation (n = 523). (B) Biopsy-proven rejection-free survival (cellular rejection $\geq 2R$ and antibody-mediated rejections; n = 523). Survival was not significantly different between groups ($p=0.92$). Rejection-free survival was worse in patients with pfDSA MFI >1000 ($p=0.05$).

III-A-3-d. Secondary endpoints

Rejection-free survival was significantly worse in patients with pfDSA MFI >1000 (Figure 33-B and Table 18). This difference was driven by a four-fold increase in the risk of biopsy-proven ABMR at 1 year (Figure 34). A total number of 116 biopsy-proven ABMR, mostly subclinical (93%), were diagnosed in 73 patients during the entire follow-up. In the multivariate analyses, only DSA status at transplantation and cold ischemic time remained independent risk factors for death or rejection. Rejection-free survival was no longer significantly different between groups when defining rejection as ACR $\geq 2R$ and/or pAMR ≥ 2 (Table 18).

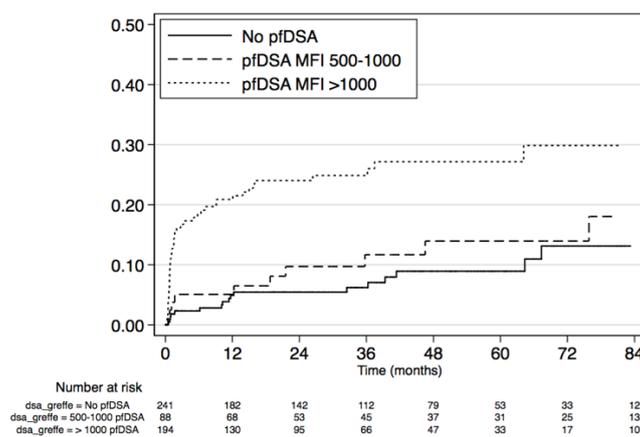


Figure 34. Incidence of biopsy-proven antibody-mediated rejection (ABMR) (deaths not shown).

Patients with preformed donor-specific anti-HLA antibody (pfDSA) mean fluorescence intensity (MFI) levels >1000 had a four-fold increase in the risk of biopsy-proven antibody-mediated rejection 1 year following heart transplantation ($p<0.001$).

	All patients N=523	No pfDSA N=241	MFI 500-1000 N=88	MFI > 1000 N=194	p-value
Primary outcome					
Survival (%)					
1-year (%)	81.1%	78.8%	81.8%	83.5%	Log rank 0.92
3-year (%)	73.6%	73%	73.8%	73.9%	
5-year (%)	69.6%	69.8%	70.5%	68.6%	
Secondary outcomes					
Biopsy-proven rejection-free survival (%)					Log rank 0.05
1-year (%)	68.6%	71%	72.7%	63.9%	
3-year (%)	59.7%	63.8%	62.5%	53.1%	
5-year (%)	55%	60%	57.8%	46.9%	
Biopsy-proven rejection-free survival (%)					Log rank 0.68
1-year (%)	72.4%	71.8%	73.9%	72.5%	
3-year (%)	63.9%	65.7%	64.6%	61%	
5-year (%)	58.7%	61.8%	58.2%	54.6%	
Biopsy proven ACR ≥ 2R (death censored)					Log rank 0.21
1-year (%)	8.6%	8.4%	10.8%	8%	
3-year (%)	11.7%	10.3%	15.6%	11.9%	
5-year (%)	13.6%	10.3%	19.9%	14.7%	
Biopsy proven AMR (pAMR ≥ 1) (death censored)					Log rank < 0.001
1-year (%)	11%	4.9%	5%	21%	
3-year (%)	14.2%	6.2%	11.7%	24.9%	
5-year (%)	16.7%	8.9%	13.9%	27.1%	
Biopsy proven AMR (pAMR ≥ 2) (death censored)					Log rank < 0.001
1-year (%)	3.4%	1.4%	0%	7.3%	
3-year (%)	3.9%	1.4%	0%	8.6%	
5-year (%)	3.9%	1.4%	0%	8.6%	
Primary Graft Dysfunction					0.14
Number at risk (without pre-HTx ECMO) no. (%)	412 139 (33.7%)	185 55 (29.7%)	76 24 (31.6%)	151 60 (39.7%)	
CAV grade 1 or more — no. (%)					0.98
Number at risk (patients with ≥ 1 CA)	384	173	65	146	
1-year (%)	16.7%	15%	18.5%	17.8%	
3-year (%)	27.6%	27.5%	24.6%	29%	
5-year (%)	30.7%	31%	28.6%	30.8%	
Safety outcomes					
Transfusion of PRBC (Yes or no) — no. (%)	369 (70.5%)	150 (62.2%)	49 (55.7%)	170 (87.6%)	< 0.001
Transfusion of PRBC (n)	6.5±7.2	5.4±7.1	4.9±6.2	8.5±7.2	< 0.001
Transfusion of platelets (Yes or no) — no. (%)	311 (59.5%)	124 (51.4%)	45 (51.1%)	142 (73.2%)	< 0.001
Transfusion of platelets (n)	1.4±1.9	1.2±1.9	1.1±1.7	1.7±1.9	0.006
Bleeding requiring surgical management	94 (18%)	40 (16.6%)	16 (18.2%)	38 (19.6%)	0.72
Blood stream infections — no. (%)	45 (8.6%)	23 (9.5%)	6 (6.8%)	16 (8.2%)	0.72

Table 18. Primary, secondary and safety endpoints.

The risk factors for PGD were analyzed in the subgroup of 412 patients without pre-HTx ECMO for the reasons discussed above. The rate of PGD was similar between groups, although we observed a non-significant increase in the prevalence among patients with pfDSA MFI >1000 (Table 18). However, after the multivariate analyses, only the cold ischemic time, donor gender and donor norepinephrine use >3 mg/h were associated with PGD (Hosmer-Lemeshow test: p=0.81). Results remained unchanged when including also in the analysis pre-HTx ECMO-patients. There was no difference in CAV incidence between the groups (Table 18). After multivariate analyses, only donor age was found to be associated with CAV (<40 years old: reference; >40 years old: HR=4.66; 95%CI=2.6-8.35, p<0.001).

		Number of patients	Number of deaths	HR	95% CI	p	
Recipient characteristics	Age (per 10-yr increment)	523	149	1.215	[1.054-1.401]	0.005	
	Gender	Male	404	113	1	-	0.53
		Female	119	36	1.13	[0.77-1.64]	
	Weight (per 5-kg increment)	523	149	0.990	[0.94-1.05]	0.74	
	BMI	< 18.5 kg/m ²	26	10	1.65	[0.85-3.18]	0.39
		≥ 18.5 and < 25 kg/m ²	297	76	1	-	
		≥ 25 and < 30 kg/m ²	155	49	1.26	[0.88-1.82]	
		≥ 30 kg/m ²	45	14	1.15	[0.65-2.04]	
	Ischemic cardiomyopathy	No	347	96	1	-	0.74
		Yes	176	53	1.06	[0.75-1.48]	
	Long term MCS	No	453	132	1	-	0.44
		Yes	70	17	0.82	[0.49-1.36]	
	Pre-HTx ECMO	No	412	121	1	-	0.77
		Yes	111	28	0.94	[0.62 - 1.42]	
	Emergency HTx	No	220	70	1	-	0.32
		Yes	303	79	0.85	[0.61-1.17]	
	Past history of cardiac surgery	No	421	116	1	-	0.21
Yes		102	33	1.29	[0.87-1.89]		
Mechanical ventilation	No	502	145	1	-	0.26	
	Yes	21	4	0.59	[0.22-1.60]		
Creatinine clearance (ml/min/1.73 m ²)	≥ 90	193	43	1	-	0.08	
	≥ 60 and < 90	166	48	1.27	[0.84-1.92]		
	≥ 30 and < 60	144	49	1.55	[1.02-2.33]		
	< 30 or dialysis	20	9	2.18	[1.06-4.48]		
Total bilirubin (per 10mmol/l increment)		432	121	1.03	[0.97-1.11]	0.36	
Donor characteristics	Age (per 10-yr increment)	523	149	1.163	[1.024-1.32]	0.017	
	Sex	Male	330	87	1	-	0.10
		Female	193	62	1.31	[0.94-1.82]	
	Weight (per 10-kg increment)	523	149	0.94	[0.89-0.99]	0.024	
	Cause of death	Traumatic	87	27	1	-	0.55
		Cerebrovascular	326	89	0.84	[0.55-1.29]	
		Anoxia	94	26	0.91	[0.53-1.56]	
		Other	16	7	1.46	[0.63-3.34]	
	Prior cardiac arrest	No	392	115	1	-	0.74
		Yes	131	34	0.94	[0.64-1.37]	
Norepinephrine > 3mg/h	No	446	125	1	-	0.52	
	Yes	77	24	1.15	[0.75-1.79]		
Transplant characteristics	Sex mismatch	No	404	111	1	-	0.29
		Yes	119	38	1.22	[0.84-1.77]	
	Weight mismatch	No	480	132	1	-	0.078
		Yes	43	17	1.62	[0.98-2.69]	
	Cold ischemic time (per 1-hour increment)		523	149	1.19	[1.006-1.41]	0.04
	HLA mismatch (per 1 MM increment)		523	149	0.94	[0.85-1.04]	0.27
	Preformed DSA status	No pfDSA	241	70	1	-	0.92
		pfDSA MFI 500-1000	88	25	0.92	[0.58-1.45]	
		pfDSA MFI >1000	194	54	0.94	[0.66-1.35]	
	Day 0: MFI of ID DSA (per 1000 increment)		523	119	1	[0.999-1.001]	0.79
	Day 0: Cumulative MFI of DSA		523	119	1.02	[0.999-1.004]	0.23
	Day 0: ID DSA MFI > 3000	No	475	134	1	-	0.73
Yes		48	15	1.10	[0.64-1.87]		

Table 19. Univariate analysis (Cox regression): all-cause mortality.

BMI means Body Mass Index, ECMO: extra-corporeal membrane oxygenation, HTx: heart transplantation, ID DSA: Immunodominant donor-specific antibodies, MCS: mechanical circulatory support, MFI: mean fluorescence intensity.

		Number of patients	Number of deaths	HR	95% CI	LRT p-value
Recipient age (per 10-yr increment)		523	149	1.19	[1.03-1.38]	0.016
Donor age (per 10-yr increment)		523	149	1.16	[1.02-1.32]	0.022
Weight mismatch	No	480	132	1.84	[1.09 -3.09]	0.03
	Yes	43	17			
Cold ischemic time (per 1-hour increment)		523	149	1.19	[1.09-1.39]	0.042

Table 20. Multivariate analysis (Cox regression): all-cause mortality.

III-A-3-e. Safety endpoints

Transplanted patients with pfDSA MFI >1000 (i.e., plasmapheresis group) received significantly more PRBC and platelet concentrates during the first week following HTx compared to other patients (5.2 ± 6.8 compared to 8.5 ± 7.2 units of PRBC in patients with plasmapheresis, $p < 0.001$; Table 18). After multivariate analyses, undergoing perioperative plasmapheresis sessions remained an independent risk factor for postoperative PRBC transfusion (OR = 3.97, 95% CI [2.3, 6.85], $p < 0.001$; other significant risk factors: pre-transplant and post-transplant ECMO support, prior history of cardiac surgery, long-term mechanical circulatory support at transplant; Hosmer-Lemeshow test: $p=0.83$). However, this did not translate into a significant increase in surgical revisions for bleeding. The risk of infectious complications, as assessed by the incidence of blood stream infections within the first 15 days after HTx, was not significantly different between groups (overall 8.6%; Table 18).

III-A-3-f. Exploratory analyses in patients with preformed donor-specific anti-HLA antibodies with a mean fluorescence intensity greater than 1000

In this group, we evaluated the impact of the MFI level on the day of transplant, representative of immunodominant DSA, on outcomes. While survival did not significantly differ between subgroups ($p = 0.37$; figure 35-A), patients with MFI >5000 experienced worse rejection-free survival than other patients ($p = 0.01$; Figure 35-B) due to a higher rate of early ABMR. This association remained significant once

adjusted for cold ischemic time. Although not statistically significant, we observed a trend toward an increase in the risk of PGD in patients with MFI>5000 (rate of PGD = 39.2%, 37.1%, 35.2% and 47.6% for MFI<1000, 1000-3000, 3000-5000 and > 5000, respectively). The incidence of CAV did not significantly differ between groups.

In patients with pfDSA MFI >1000, survival and rejection-free survival did not significantly differ between patients with or without pre-transplant sensitizing event (p = 0.99 and 0.25, respectively).

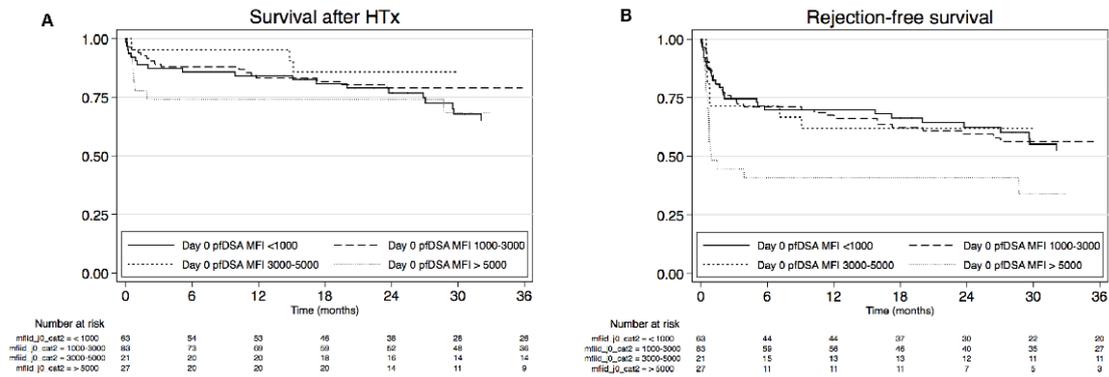


Figure 35. Exploratory analysis in the group of patients with preformed donor-specific anti-HLA antibody (pfDSA) mean fluorescence intensity (MFI) levels >1000.

(A) Overall survival after heart transplantation. (B) Biopsy-proven rejection-free survival (cellular rejection ≥2R and antibody-mediated rejection) according to day 0 MFI (n = 194). While survival did not significantly differ between subgroups (p = 0.37), patients with MFI >5000 experienced worse rejection-free survival than other patients (p = 0.01).

III-A-4. Discussion

We report a retrospective analysis of a prospective, single-center experience in the management of sensitised heart transplant recipients. We applied a post-transplant protocol to patients with pfDSA consisting of IVIg +/- perioperative plasmapheresis sessions combined with induction therapy, with systematic treatment of subsequent ABMR. None of the patients had received pre-transplant desensitisation therapies. We observed a significant decrease in MFI (both immunodominant and cumulative) after the peri-operative management. We found that overall survival, CAV-free survival and the incidence of PGD were not significantly different between groups. However, rejection-free survival was significantly worse in patients with pfDSA MFI greater than 1000, evidenced by a four-fold increased risk of early ABMR. Perioperative plasmapheresis increased the risk of requiring a transfusion during the first week after HTx but had no impact on the incidence of bloodstream infections.

Management of the increasing number of sensitised heart transplant candidates is challenging. Pre-transplant desensitisation therapy is the most widely used strategy, and data suggest that lowering PRA before HTx improves prognosis after transplantation (116). While different protocols have been evaluated, robust clinical trials are still lacking. Several issues and concerns might limit the widespread application of such pre-transplant therapies (27,110). Firstly, as they require a HLA-compatible donor, sensitised patients have longer waiting times, and subsequently, worse outcomes (111). Secondly, potential over-immunosuppression induced by these therapies might increase the risk of infectious complications. Thirdly, this strategy is costly because desensitisation protocols are based on expensive medications, and sensitised patients might require a bridge-to-transplant assist-device strategy due to the expected long waiting time. Finally, the initiation of these anti-HLA antibody therapies is based on the PRA, which evaluates the potential risk of being transplanted with DSA, but is not based on the real DSA, potentially leading to overtreatment. Rather than using pre-transplant desensitisation therapies, we established a post-transplant prophylactic strategy consisting of perioperative management of pfDSA and systematic treatment of ABMR. Our decision to perform HTx was made regardless of the presence of pfDSA. We believe that this protocol might improve access to transplantation of sensitised patients.

A recent ISHLT consensus stated that desensitization likely confers a benefit to the patients (28). The conventional goal of desensitization therapy is to achieve a negative crossmatch before proceeding with a transplant. As emphasized by ISHLT experts, there is, however, an alternative strategy whereby transplantation proceeds despite a positive virtual crossmatch and therapies are initiated to mitigate the impact of DSAs (28). We found that an exclusively post-transplant management of pfDSA resulted in a neutralization of the risk of DSA on post-HTx survival and might be a therapeutic option for sensitized patients.

The largest experience of solid organ transplantation with pfDSA comes from kidney transplantation. Lefaucheur et al. found that 8-year graft survival was significantly worse among patients with pfDSA compared with non-sensitised patients (117). While *de novo* DSA has been consistently associated with worse outcomes after HTx, the impact of pfDSA is less clear (118). Retrospective, single-center studies without a post-transplant prophylactic protocol suggest that they may be associated with worse

outcomes, particularly CAV and ABMR; however, their impact on survival or graft survival is highly debated in the field of heart transplantation (19,37,119). In our cohort, we observed similar survival but a four-fold higher risk of biopsy-proven ABMR at 1 year in transplant patients with pfDSA MFI greater than 1000.

Chronic rejection is widely recognized as a progressive process that poses significant risks of allograft loss and mortality after organ transplant (25). The main feature of chronic AMR across solid organ transplants is chronic vascular lesions. In cardiac allografts, these vascular injuries manifest as CAV. Furthermore, AMR has been associated with CAV and cardiovascular mortality (120). However, while late and symptomatic ABMR are associated with a higher risk of CAV progression, the ABMR in our study were early and mostly subclinical (20). We did not observe a higher incidence of CAV in sensitised patients, although a longer follow-up period is necessary to detect such potential chronic complications of ABMR and DSA (median follow-up in this study of 4.06 years).

As pointed out in a recent review, the choice of a clear cut-off value of MFI to predict post-transplant outcomes is highly controversial (121). When our protocol was set up in 2009, there was very little data on MFI cut-off values in the field of HTx. The presence of weak antibodies is likely to reflect the presence of memory antibody-producing cells, which are dormant pre-transplant but may be re-activated after transplant (121). Based on this, our local cut-off point was intentionally low in order to minimize immunological risks. Moreover, Svobodova et al. demonstrated that pfDSA MFI greater than 1000 is an independent risk factor for early ABMR after HTx (39). International experts reported that the most frequently cited MFI cut-off for identifying the antigens to avoid is 5000. We found a similar cut-off value in our exploratory analysis of the subgroup of sensitised patients: worse rejection-free survival, trend toward an increase in the risk of PGD. These findings raise caution concerning the use of this protocol in patients with MFI >5000.

More in depth characterization of pfDSA may have allowed for better prognosis stratification of post-transplant outcomes. There is growing evidence that suggests that simply using MFI values as the sole gauge for antibody strength is not sufficient. The ability of DSA to bind to C1q and the IgG isotype provides additional prognosis value (122,123). Kobashigawa et al. reported that using four antibody detection assays might optimize the selection of unacceptable antigens (124).

Although limited by the small sample size, our data suggest that patients with high MFI at transplant that remain high despite therapies are at increased risk of early events. This result emphasizes the need for DSA monitoring after immunosuppressive therapies. Reinforcement of therapies should be considered in poor or non-responders.

The results of the current study led to several changes to our practice. Firstly, due to the four-fold increase in the risk of ABMR, we reserve this protocol for patients requiring emergency access to HTx. Secondly, we treat patients transplanted with pfDSA MFI of >1000 with tacrolimus as a calcineurin inhibitor therapy since 2017 as it significantly decreases rejection compared to cyclosporine (75). Finally, despite the uncertainties regarding the ability of rituximab to prevent and/or treat ABMR, we are currently considering using this medication in patients with pfDSA MFI >5000 on top of the current therapy.

Compared to international cohorts, our patients have several particularities. Firstly, only 13.4% of our patients were under long-term mechanical circulatory support at the time of transplantation compared to the 42.9% of patients reported in the most recent ISHLT registry (10). Our data are consistent with French national data. Previous and current allocation policies in France give high-priority HTx access to patients under ECMO and/or inotropes, which might explain this important difference. Twenty percent of our patients were under ECMO support at the time of transplantation compared to 1% in the 2017 ISHLT registry (10). Second, alloimmune sensitization was almost twice more prevalent in our cohort in comparison to international registries, probably due to differences in definitions (pfDSA MFI >1000 in our cohort compared to PRA of >10% in registries).

It is important to highlight that re-transplantations were excluded from our study. Most of re-transplantations were combined heart-kidney transplantations and were treated with pre-transplant desensitization therapies according to our kidney-transplant team protocol. Since more than half of re-transplantation patients received pre-transplant desensitization therapies before transplantation, including re-transplanted patients in the analysis might have induced selection bias.

The present study should be interpreted within the context of its limitations. Firstly, our study does not include a control group for comparison, thus the independent impact of our prophylactic strategy on outcomes cannot be analyzed. However, a randomized

clinical trial could not have been performed due to ethical issues, as data published before the initiation of this protocol suggested that pfDSA is correlated with graft loss after HTx (38). Secondly, as this is a single center experience, external validation is required. Moreover, as discussed above, the MFI threshold was made as low as 1000, which could explain, in part, the good prognosis we observed in our sensitised patients. The number of patients with very high MFI (e.g., >10,000) was too low in our cohort to determine a value above which the transplantation should not be performed. Similarly, our exploratory analyses of the subgroup of patients with MFI >5000 are undeniably underpowered due to the small number of patients. Thirdly, the lack of C1q assay did not allow us to provide an in-depth characterization of pfDSA. Fourthly, we limited treatment of DSA to the peri-transplant period and to the AMR episodes whereas it has been widely demonstrated that IVIg-based treatment protocol shows a higher clearance efficacy if IVIg infusions are repeated over time (125–127). Repeated infusion of IVIG with a lower dosage may reduce subsequent incidence of ABMR. However, IVIg shortage and costs are two important limits for a wider use of IVIg. Fifthly, we acknowledge that post-transplant confounding factors that were not tested in the model might have influenced the risk of transfusions during the first week post-transplant. Sixthly, as discussed above, longer follow-up is necessary to monitor CAV progression potentially associated with pfDSA and ABMR. Finally, PRA is not routinely performed at our center, thus our strategy is not based on this result.

III-A-5. Conclusion

A post-transplant prophylactic strategy including perioperative management of DSA and systematic treatment of subsequent ABMR achieved favorable outcomes after HTx in patients transplanted with pfDSA.

Chapter III-B: Antibody-mediated rejection induced cardiogenic shock: too late for conventional therapy.

Article published in Clinical Transplantation 2018 May;32(5):e13253 (13)

III-B-1. Introduction

The clinical spectrum of AMR ranges from asymptomatic subclinical AMR to acute cardiogenic shock. Subclinical AMR has been associated with increased cardiovascular mortality (128). Allograft dysfunction at the time of AMR is an independent risk factor for the development of de novo CAV and death (20). Late symptomatic AMR is associated with poor short-term prognosis: high mortality, fulminant forms of cardiac allograft vasculopathy (CAV) and persistent left ventricular (LV) dysfunction (89,129).

As underlined by a scientific statement from the American Heart Association, the widely used term of “hemodynamic compromise” lacks uniformity, and the specific criteria for dysfunction may range from decreased ejection fraction to cardiogenic shock requiring inotropic support (27). Cardiogenic shock is the most severe form of allograft dysfunction. The prognosis of AMR with cardiogenic shock is suspected to be poor but data are scarce in the literature.

In this retrospective cohort we aimed to analyze the characteristics, the treatment and the prognosis of patients treated for cardiogenic shock due to biopsy-proven AMR at our center.

III-B-2. Methods

III-B-2-a. Patients

We performed a retrospective, single center, observational study. We included all consecutive patients with (i) biopsy proven diagnosis of AMR and (ii) severe allograft dysfunction responsible for cardiogenic shock requiring inotropic support and / or

temporary mechanical circulatory support. Our institutional review board approved the protocol and informed consent was obtained.

III-B-2-b. Objectives

Our objectives were to analyze the characteristics, treatment and prognosis of patients treated for cardiogenic shock due to biopsy-proven AMR requiring inotropic support and / or temporary mechanical circulatory support at our center.

III-B-2-c. Definition of AMR and cardiogenic shock

The diagnosis of acute AMR was based on the presence of histological evidence of AMR on endomyocardial biopsies according to ISHLT guidelines (pAMR 1 to 3) (112). Cardiogenic shock was defined according to ESC guidelines on acute and chronic heart failure as hypotension (SBP \leq 90 mmHg) despite adequate filling status with signs of hypoperfusion (cold sweated extremities, oliguria, mental confusion, dizziness, narrow pulse pressure; laboratory measures: metabolic acidosis, elevated serum lactate, elevated serum creatinine) (1). All patients received inotropic support based on dobutamine infusion. In case of cardiac arrest or refractory cardiogenic shock with escalating inotropic requirements a temporary mechanical circulatory support – extracorporeal membrane oxygenation (ECMO) - was implanted usually through femoral vein and artery according to current standards (bridge to recovery strategy) (130).

III-B-2-d. Immunology

The detection of pre and post-transplant anti HLA antibodies was based on Luminex Single Antigen beads technology (One Lambda, Canoga Park, CA). The fluorescence of each bead is detected by a reader (LABscan) and recorded as the normalized Mean Fluorescence Intensity (MFI). Beads showing MFI > 500 were considered as a positive result. HLA typing of heart recipient was performed by low-resolution class I HLA-A, -B, and class II HLA-DRB1, -DQB1 PCR-SSO (LABType, One Lambda). Donors HLA-A, B, DR, DQ typing was performed by CDC (One lambda tissue-typing trays) and controlled by molecular biology.

Our routine monitoring protocol includes DSA quantification: #1 at 3, 6, 12 months post-transplant and then once a year in patients transplanted with pre-formed DSA and #2 every year in other patients.

III-B-2-e. Immunosuppressive protocol

Immunosuppressive therapy at the time of transplantation was based on an induction therapy with anti-thymocyte globulin (rabbit ATG, Thymoglobuline, Genzyme, Lyon, France). Prophylactic immunosuppressive treatment usually included calcineurin inhibitors, mycophenolate mofetil and corticosteroids with posology as recommended by ISHLT guidelines (115).

Treatment of AMR included intravenous methylprednisolone infusion (500 to 1000 mg per day for 3 days), 5 plasmapheresis (1 plasmapheresis on 5 consecutive days) and high dose IVIg (Privigen CSL Berhing Laboratories, Pennsylvania, USA – total dose = 2 g / kg administered during 4 consecutive days). When required, treatment could be intensified with more plasmapheresis, rabbit anti-thymocyte globulins (rATG, Thymoglobuline, Sanofi-Genzyme, Massasuchets, United States, 1.5 mg/kg per day during 3 days) and/or anti-CD20 therapy (Rituximab, Mabthera, Roche pharmaceuticals, Basel, Switzerland - 375 mg/m², 2 to 4 injections). Eculizumab (Soliris, Alexion Pharmaceuticals, Connecticut) could be used as a salvage therapy.

III-B-2-f. Endomyocardial biopsies (EMB)

EMBs were performed according to current standard using either right jugular vein or femoral approach. Biopsies were processed and examined according to current standards (2). Our routine EMB protocol includes 13 EMB during the first year, then every 4 months for 2 years, followed by every 6 months for 2 years. EMB after 5 years of transplantation are performed when clinically indicated. Standard serial sections were cut from formalin-fixed paraffin-embedded EMBs and stained with hematoxylin-eosin-safran for diagnosis. Immunofluorescence for C4d was performed on all reference and post-AMR EMBs. Only capillary staining for C4d was assessed. All EMB were retrospectively reviewed by an expert pathologist (blinded review, inclusion of control biopsies) and classified as cellular (0 to 3R) and AMR (pAMR 0 to 3) according to the ISHLT guidelines (112,113).

III-B-2-g. Cardiac allograft vasculopathy

At our institution, routine coronary angiograms are performed one year after heart transplantation and then every two years or in case of unexplained LV dysfunction. Staging of CAV was performed according to the recommended ISHLT nomenclature (35).

III-B-2-h. Statistical analyses

Statistical analyses were performed with Stata/IC 14.2 (StataCorp). Results are presented as median and inter-quartile range (IQR) for continuous variables and as number and percentage for categorical variables. Cumulative survival curves for the time-to-event analyses were constructed according to the Kaplan-Meier method. Statistical significance was determined if the null hypothesis could be rejected at the $p < 0.05$ level.

III-B-3. Results

III-B-3-a. Characteristics of included patients

Seventeen patients were diagnosed with AMR with cardiogenic shock requiring dobutamine infusion and/or ECMO support at our center. Their characteristics are detailed in Table 21. Patients were transplanted from 1994 to 2015. Rejections were diagnosed from 2008 to 2016. During this period of time, 740 heart transplantations were performed at our center (13 of 17 patients of our cohort were transplanted during this interval). 883 patients are currently followed in our transplant unit. The incidence of AMR induced cardiogenic shock could then be estimated around 1.7% of heart transplant recipients (=13/740).

Patients were mostly males ($n = 12/17$, 70 %). Median age at diagnosis was 55.7 years (17-69 years). Median time between heart transplantation and AMR was 21.5 months (10-64 months). Four patients were transplanted with MFI > 3,000 preformed DSA. Only 2 patients (12 %) had past history of biopsy proven AMR. All patients were treated with calcineurin inhibitors (CNI) and corticosteroids (CS); most of them received mycophenolate mofetil on top of CNI and CS (82 %) at the time of rejection. Eight patients (47%) required renal replacement therapy during the treatment of AMR induced cardiogenic shock.

III-B-3-b. Diagnosis and treatment of AMR

According to the inclusion criteria, all patients had severe graft dysfunction at the time of diagnosis of AMR. Median LVEF at diagnosis was 28 % (5 to 34 %). All patients had circulating anti-HLA DSA. Immunodominant DSA was a high-level class II de novo DSA in 76 % of cases (n = 13/17). Pre-formed DSA were present at the time of rejection in patients 1 and 2 but with lower MFI values compared to de-novo DSA. Median MFI of immunodominant DSA and cumulative MFI of DSA were respectively 10,895 (6,375-15,321) and 18,275 (11,193-33,907). All EMBs were performed for acute allograft dysfunction (for-cause EMBs). Histological analyses of EMB revealed features of AMR in all patients (pAMR 2: n = 3; pAMR1 (H+): n = 11; pAMR1 (I+): n = 3). Two patients had concomitant 2R3A acute cellular rejection and 5 patients 1R cellular rejection.

Treatments of AMR are detailed in Table 21. In brief, all patients received high dose IV corticosteroids pulses, plasmapheresis (5 to 15 plasmaphereses per patient), and high dose IVIg. Two patients died before the initiation of plasmapheresis and five before IVIg. On top of this standard regimen, 12 patients received add-on therapies: 10 patients were treated with rATG and 6 received at least one infusion of rituximab. Four of the six patients with “dobutamine infusion-only” group did not receive any complementary treatment.

III-B-3-c. ECMO support

Eleven patients presented with refractory cardiogenic shock requiring ECMO support at the diagnosis of AMR or during follow-up (LV decline despite treatment). Eight patients died under ECMO support after a median time of circulatory support of 13 days (3 to 30 days). ECMO was successfully weaned in two patients after respectively 13 and 45 days of support. The remaining patient died shortly after total artificial heart surgery.

↓ *Table 21: Characteristics of included patients.*

ACR means Acute Cellular Rejection, AMR: Antibody-Mediated Rejection, ATG: anti-thymocyte globulin, CAV: Cardiac Allograft Vasculopathy, DSA: donor-specific anti-HLA antibodies, EF: Ejection Fraction, EMB: Endomyocardial Biopsy, ID: Immunodominant, LVEF: Left Ventricular Ejection Fraction, MFI: mean fluorescence intensity, MI: Myocardial Infarction, MOF: multi-organ failure, yr: year, TAH: Total Artificial Heart, AZA: azathioprine, CS: corticosteroids, CYA: Cyclosporine A, EVL: Everolimus, MMF: Mycophenolate mofetil, TAC: Tacrolimus, TAH: total artificial heart.

Patient	1	2	3	4	5	6	7	8	9
Baseline characteristics									
Gender	F	M	F	M	M	M	F	M	M
Age at diagnosis of AMR	18	57	55	69	58	54	20	53	19
Transplant → AMR (months)	5	28	77	6	12	170	10	41	21
CAV stage	NA	NA	0	NA	0	0	NA	0	0
History of ACR ≥ 2R (n)	0	0	0	0	0	0	1	0	0
History of AMR (n)	0	2	0	0	0	0	0	0	0
Preformed DSA	Yes	Yes	No	No	No	No	Yes	No	No
Immunosuppression at the time of AMR									
	TAC/MMF/CS	CYA/CS	CYA/CS	CYA/MMF/CS	CYA/MMF/CS	CYA/AZA/CS	TAC/MMF/CS	CYA/MMF/CS	CYA/MMF/CS
MMF daily dose	2000	0	0	500	1000	0	1000	1000	3000
Diagnosis of AMR									
<i>Cardiac allograft dysfunction</i>									
Cardiac allograft dysfunction	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
LVEF	20	34	30	32	30	25	15	25	30
ECMO support	Yes	No	Yes	No	No	No	Yes	Yes	Yes
<i>Immunology</i>									
MFI of ID DSA	22,370	10,490	6,740	5,282	6,746	13,076	15,578	4,691	10,867
ID DSA type	de novo	de novo	de novo	de novo	de novo	de novo	preformed	de novo	de novo
ID DSA class	II	II	II	II	II	II	II	II	II
Nbr of DSA (class I / class II)	4/4	2/1	1/4	2/4	0/1	1/3	0/3	0/1	2/1
Cumulative MFI of DSA	153,136	12,195	15,931	12,171	6,746	26,769	34,197	4,691	19,792
<i>Histology and IHC</i>									
ACR	1R1A	0R	0R	2R3A	0	0	1R1B	1R1A	2R3A
pAMR	2	1 (H+)	2	2	1 (H+)	1 (I+)	1 (H+)	1 (I+)	1 (H+)
Treatment									
Plasmapheresis	10	5	2	0	7	10	10	0	5
IVIg	Yes	Yes	No	No	Yes	Yes	Yes	No	Yes
ATG	Yes	No	Yes	No	No	Yes	Yes	No	Yes
Rituximab	2	4	No	No	No	No	No	No	2
Others	Eculizumab Splenectomy	-	-	-	-	-	-	-	-
Renal replacement therapy									
	No	Yes	Yes	No	No	Yes	No	No	Yes
Outcomes									
Survival	Death	Death	Death	Death	Death	Death	Death	Death	Death
Survival (days)	307	34	3	0	25	33	32	4	51
Cause of death	Sudden death	Cardio/septic shock	Cardio shock	Refractory cardiac arrest	Septic shock	Hemorrhagic stroke	TAH surgery	Cardio shock	Cardio shock
CAV worsening	No	NA	NA	NA	NA	NA	NA	NA	NA
LVEF normalization	Yes	No	No	No	No	No	No	No	No

Patient	10	11	12	13	14	15	16	17
Baseline characteristics								
Gender	M	F	F	M	M	M	M	M
Age at diagnosis of AMR	62	57	64	43	63	17	29	63
Transplant → AMR (months)	36	84	12	15	9	1.5	77	64
CAV stage	1	0	0	0	NA	NA	1	1
History of ACR ≥ 2R (n)	0	0	0	0	0	0	0	0
History of AMR (n)	0	0	0	1	0	0	0	0
Preformed DSA	No	No	No	Yes	Yes	No	No	No
Immunosuppression at the time of AMR	CYA/MMF/ CS	CYA/MMF/ CS	CYA/ MMF/CS	CYA/MMF/ CS	CYA/MMF/ CS	CYA/MMF/ CS	CYA/MMF/ CS	CYA/MMF/ CS
MMF daily dose	1500	1000	500	1000	1000	500	1500	2000
Diagnosis of AMR								
<i>Cardiac allograft dysfunction</i>	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
LVEF	20	15	25	30	32	30	28	15
ECMO support	Yes	Yes	No	Yes	No	Yes	Yes	Yes
<i>Immunology</i>								
MFI of ID DSA	19,363	14,031	19,781	4,884	4,243	11,301	2,333	15,236
ID DSA type	de novo	de novo	de novo	preformed	preformed	de novo	de novo	de novo
ID DSA class	II	II	II	I	I	I	II	II
Nbr of DSA (class I / class II)	2/4	1/2	2/5	3/1	1/0	1/0	0/2	1/2
Cumulative MFI of DSA	63,880	33,811	38,831	10,869	4,243	11,301	4,577	32,562
<i>Histology and IHC</i>								
ACR	0R	0R	1R1A	1R1B	0	0	0	0
pAMR	1 (H+)	1 (H+)	1 (H+)	1 (H+)	1 (H+)	1 (I+)	1 (H+)	1 (H+)
Treatment								
Plasmapheresis	0	5	5	12	9	5	6	10
IVIg	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ATG	Yes	Yes	No	Yes	No	Yes	No	Yes
Rituximab	No	2	No	No	No	No	2	1
Others	-	-	-	-	-	-	-	-
Renal replacement therapy	Yes	Yes	No	No	No	Yes	Yes	No
Outcomes								
Survival	Death	Death	Alive	Alive	Alive	Death	Death	Death
Survival (days)	5	34	365	958	509	15	47	41
Cause of death	MOF	Cardio/septic shock	-	-	-	MOF septic shock	MOF	MOF
CAV worsening	NA	NA	No	Yes (0→2)	No	NA	NA	NA
LVEF normalization	No	No	Yes	Yes	Yes	No	No	No

III-B-3-d. Survival after AMR

Survival after AMR is described in Figure 36. In hospital and one-year mortality were 76 % and 82 % respectively. Causes of death are detailed in Table 21. The leading cause of death was refractory cardiogenic shock but infectious complications during hospitalization in the intensive care unit and multiple organ failure (MOF) were common. Among patients discharged from hospital, one patient presented a sudden cardiac death at home 10 months after AMR whereas the last EMB was considered as normal and coronary angiography performed after AMR revealed smooth coronary arteries (CAV 0).

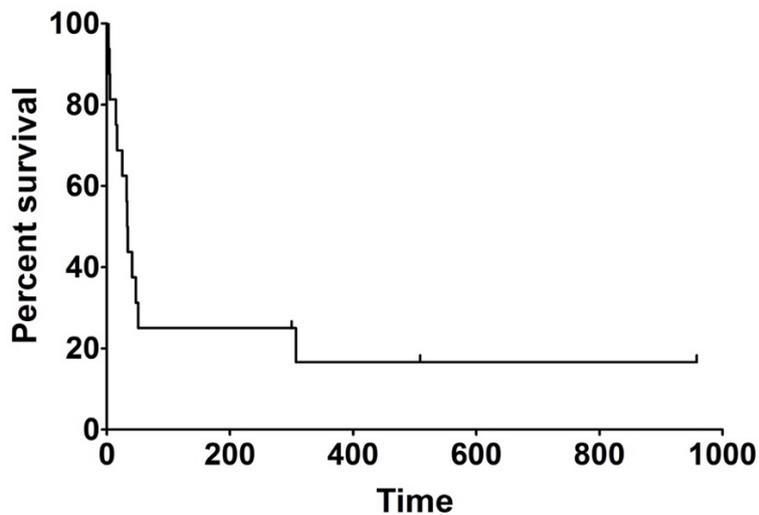


Figure 36: Survival after antibody-mediated rejection with cardiogenic shock.

Time from AMR is indicated in days.

III-B-3-e. AMR recurrences, graft function and CAV

Patients discharged from hospital recovered allograft function evaluated by left ventricular ejection fraction (LVEF) during follow-up (n = 4). Two patients were diagnosed with at least one recurrence of AMR without symptoms or allograft dysfunction (subclinical AMR).

Results of pre and post-AMR coronary angiography are detailed in Table 21. One patient with recurrence of AMR presented progression of CAV (CAV grade 2, 6 months after AMR), whereas his coronary angiogram performed 3 months before AMR was normal (CAV 0).

III-B-4. Discussion

We described a cohort of 17 patients treated for cardiogenic shock due to AMR, including 11 patients requiring ECMO support. AMR was mostly due to high-level de novo class II DSA. Our results suggest that AMR with cardiogenic shock is a rare condition associated with very poor short-term prognosis (in-hospital mortality = 76 %). Major causes of deaths were refractory allograft dysfunction and infectious complications. Patients discharged from hospital recovered allograft function, but one case of rapidly progressive CAV and one case of sudden cardiac death occurred during follow-up.

Acute allograft dysfunction is a well-known risk factor of death in patients diagnosed with AMR. Our purpose was not to compare sub-clinical AMR to symptomatic AMR. We aimed to point out the particularly poor prognosis of CS due to AMR to emphasize the need for an early-standardized aggressive immunosuppressive protocol in this high emergency context.

The poor prognosis of cardiogenic shock due to AMR observed in our cohort suggests that an early intensification of immunosuppressive treatment should strongly be considered. Whereas most centers would treat symptomatic AMR similarly, the treatment of AMR resistant to conventional therapies is highly controversial. Rituximab and bortezomib have been used for the treatment of cardiac allograft AMR. However, the literature in cardiac allograft AMR is mostly based on case reports (131). A randomized trial in acute kidney AMR failed to show any additional effect of rituximab in patients receiving plasmapheresis, IVIg, and steroids (132). Similarly, bortezomib was not proven to be superior to placebo for the treatment of late kidney AMR (133). Since all of these drugs only prevent the production of new DSA and are not able to remove circulating antibodies, their use in the high emergency context of AMR with cardiogenic shock is questionable. Complement activation is the predominant effector pathway of acute AMR (27). Therefore, the blockade of antibody-mediated complement activation is an attractive target in the context of severe AMR. Eculizumab is a C5 inhibitor that is approved for use in paroxysmal nocturnal hemoglobinuria and has been used as rescue therapy in the treatment of kidney AMR resistant to conventional therapies (134) with mixed results (135). A human plasma-derived C1 inhibitor that is approved for use in patients with hereditary angioedema has been tested in a phase 2b double blind randomized trial for the treatment of kidney AMR

with promising results (136). The prompt use of complement inhibitors could have the advantage of immediate effects by stopping cellular injury, providing a therapeutic window to allow conventional treatment modalities to be effective (134). Only one patient of our cohort was treated with eculizumab (patient 1) and experience in cardiac transplantation is very limited. The significant cost associated with the use of eculizumab might limit its use.

Despite the single center design of our study, significant discrepancies do exist in the treatment of patients. Their prognosis could greatly benefit from a standardized immunosuppressive protocol. We believe that corticosteroids pulses, plasmapheresis and IVIg remain the basic treatment. The high rate of infections complications and the poor effect on allograft function we observed with ATG and rituximab emphasized the need for an immediately effective and well-tolerated drug. In this way, the use of complement inhibitor should be discussed as soon as possible. However, the lack of a standardized treatment protocol and the small number of patients in our study limits how strong any association between immunosuppressive treatment and outcomes can be made.

Several factors may have worsened the prognosis of our patients beyond the severity of AMR. First, more than one-third of our patients had mixed rejection. This condition reflects a complex interplay between cellular and humoral processes and is associated with worse outcomes (137). Secondly, almost half of our patients required renal replacement therapy, a well-known risk factor for poor outcomes in cardiogenic shock (138). Finally, the effect of timing of AMR might be important. Most of our patients suffered from late AMR (> 12 months post-transplant), a condition that has been associated with poor outcomes (20,89).

Graft dysfunction during AMR was an independent predictor of progression of CAV after AMR in a large cohort (20). Fulminant form of cardiac allograft vasculopathy has been described following late AMR (89). Rapidly progressive forms of CAV might have been underdiagnosed in our cohort since only 3 patients (18 %) had a coronary angiogram after AMR, including one case of rapidly progressive CAV (patient 13). We suggest that in case of persistent allograft dysfunction despite aggressive immunosuppressive regimen, a coronary angiogram should be discussed to rule out an ischemic participation to the persistent allograft dysfunction.

Animal models of AMR underline the early endothelial and platelets activation following the passive transfer of donor specific antibodies (139). In our cohort, all patients were under anti-platelets agents (low dose aspirin) but only patients under ECMO support received curative anticoagulation. However, as underlined by an AHA scientific statement, systemic anticoagulation is probably required in severe AMR to reduce the risk of intra-vascular thrombosis (20).

Refractory cardiogenic shock due to AMR is considered a contra-indication to cardiac retransplantation at our institution. Survival after retransplantation for acute rejection has been reported to be as low as 30 % at one year (140). The analysis of the UNOS database suggests that retransplantation under ECMO support is also associated with poor prognosis. These critical patients could be considered for a bridge-to-transplant strategy with long term ventricular assist devices since survival after retransplantation for patients bridged has improved considerably over time (141). Timing is crucial in this strategy. On the one hand, cardiac allograft recovery after AMR can take weeks. On the other hand, long term ECMO support is associated with potentially life-threatening complications and poor outcomes (142).

We acknowledge some notable limitations in our study. First, the small sample size of our cohort is a major limit. Second, this cohort represents a single center experience with very similar treatments used. It would be interesting to analyze data from others centers with different protocols. Finally, despite exhaustive research, retrospective data collection might have induced selection bias.

III-B-5. Conclusion

Cardiogenic shock due to AMR occurred mostly in patients without history of biopsy proven AMR who developed high-level de novo class II DSA. Our results suggest that AMR with cardiogenic shock has a very poor short-term prognosis despite aggressive immunosuppressive therapies and mechanical circulatory support. Despite a significant incidence of infectious complications, an early intensification of immunosuppressive therapies should strongly be considered in case of cardiogenic shock due to AMR. Among patients discharged from hospital one case of rapidly progressive CAV and one sudden cardiac death occurred during follow-up.

Chapter III-C: Conclusion of the clinical studies.

These clinical data allow us to better understand the clinical events of two populations at high immunological risk (Figure 37).

On the one hand, an exclusively **post-transplant** strategy based on the management of pre-formed DSA using plasmapheresis and IVIg was associated with **favorable** post-transplant outcomes, with no impact on post-transplant survival, but at the cost of an **increased incidence of subclinical AMR** and bleeding complications.

On the other hand, since graft dysfunction is so advanced in AMR-related **cardiogenic shocks**, usual and **widely accepted therapies** (plasma exchange, IVIg) are **poorly effective** and an early intensification of immunosuppression is mandatory in these patients (Figure 37).

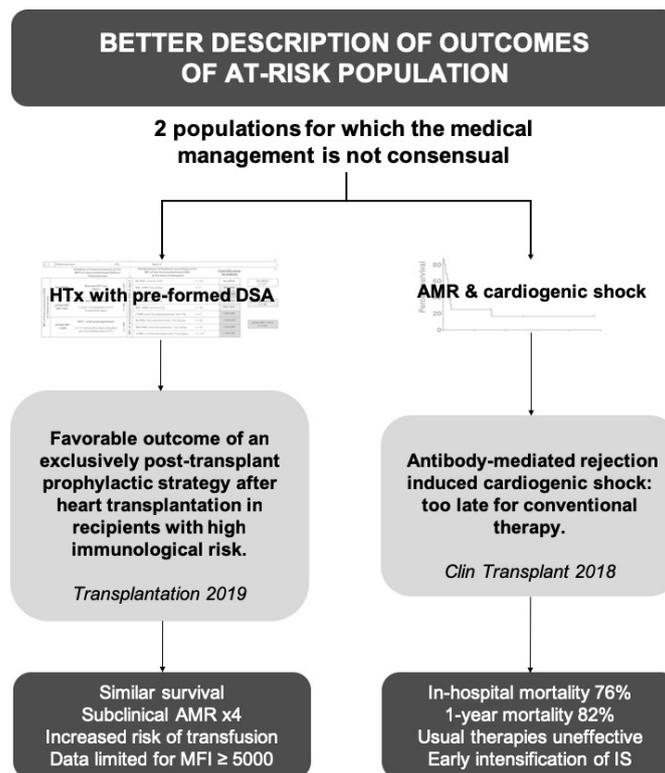


Figure 37. Summary of the results of clinical studies.

Conclusion

Allosensitization and antibody-mediated rejections are **major risk factors** for the development of graft-specific pathologies, which are the **leading cause of late mortality** after heart transplantation.

The **standardization** of the pathologic **diagnosis of AMR**, the improvement of the **risk stratification** of allosensitization- and AMR-related complications and the better **description of the clinical events** in populations at high-immunological risk are all key points which might allow an improvement in the clinical management in heart transplantation.

To answer these questions, we applied three complementary approaches: a **molecular**, an **epidemiological** and a **clinical** approach. Our results provide new information in the field of alloimmunization in heart transplantation that could contribute to the evolution of clinical practices.

Discussion of results and future directions

Molecular biology as a companion to the pathologic diagnosis of rejection

Our results raise the question of the place of molecular biology in the field of heart allograft rejection. Molecular biology has often been described as the new gold standard for the diagnosis of rejection, neglecting the central role of pathology in diagnosing rejections and guiding therapies. We believe that pathologic and molecular results should be analyzed as a whole since each approach brings valuable and additional information allowing an in-depth histo-molecular characterization of rejection. We believe that the results from our molecular analyses make it possible to apprehend **molecular biology as a companion to the pathologic diagnosis of allograft rejection**. The good agreement between the unsupervised molecular diagnosis based on RT-MLPA gene expression profiles and the pathologic diagnosis is an important point. The **discrepancies** between these two diagnoses are just as much important, both for the cases of histological non-rejection classified as molecular rejection (biopsy-negative rejection with a high prevalence of allograft dysfunction), and for the cases of histological rejections classified as molecular non-rejection (low-grade rejections). Molecular biology has also allowed us to **validate in-situ a histological classification** of microvascular inflammation. Its extension, categorized into four classes, correlated with the degree of molecular activity of AMR. These two studies illustrate how **pathologic analysis and molecular biology can jointly** help to standardize the diagnosis of AMR, a necessary first step towards improving the management of this pathology.

The pursuit of research aimed at standardizing pathologic diagnosis is a priority objective. The contribution of molecular biology as a companion to pathology is an important area of research. International kidney allograft rejection classifications now incorporate these molecular tools in the diagnosis of allograft rejection, particularly for AMR (40). Despite its satisfactory diagnostic performance, RT-MLPA suffers from the limitation of the number of transcripts of interest that can be analyzed in the same sample. Despite its cost, we believe that the molecular biology technique developed by NanoString technologies is a promising technique that might be applicable in clinical practice. This technique allows not only the use of FFPE EMB, but also offers the

possibility of simultaneously analyzing several hundred transcripts of interest, to provide an absolute gene expression and do without any amplification step during the procedure. We have already begun to apply this technique to FFPE BEMs in a retrospective case-control study with encouraging initial results. We are currently performing a multicenter study with a centralized review of pathology to validate a restricted molecular signature of each type of allograft rejection based on NanoString analyses.

Integrative epidemiology

The unit in which I work has developed over time a **multidisciplinary and transversal** scientific approach by mixing clinical and statistical investigations. The mathematical tool is used in an **integrative** thought process coupled with multiple questions, intuitions, and clinical investigations. We have called this approach **integrative epidemiology**. Our approach uses the statistical and epidemiological tool as a means of first-line investigation, generating new hypotheses and new directions of research. This approach, applied to large multicenter cohorts of very well phenotyped patients, allowed us to better describe the epidemiology, natural history, and risk factors of two important complications of alloimmunization, namely allograft rejections and CAV.

Although our results provide new insights into the epidemiology, natural history, and determinants of allograft rejection and CAV, they do not yet allow us to achieve an individual risk stratification of events that would allow the development of personalized medicine in heart transplantation. Consideration of the notion of futile tests is of primary importance in our field. What is the probability that a protocol biopsy performed at 8 months after transplant is abnormal when the 8 previous biopsies were normal? What is the probability that a coronary angiography performed 5 years after transplantation will show evidence of CAV when the 2 previous coronary angiograms were normal, and the patient has no risk factors for developing the disease? The application of mixed models to assess these issues will allow us to access the individual prediction level. As previously discussed, these models allow us to understand the longitudinal character of the data and to introduce random effects. We are currently applying mixed logistic models to the risk of rejection modeling in the first year. The first results are promising. We will now continue to characterize an optimal model that allows access to an individual stratification of the risk of rejection. We are also working on the

development of joint models, which associates a mixed model with a Cox model. We wish to apply this approach to graft coronary artery disease in order to individually define the likelihood of finding abnormalities in a coronary angiogram based on the results of previous coronary angiography and the risk factors for disease development.

Clinical studies

The analysis of the results of the application of a prospective protocol for the treatment of pre-formed DSA at the time of the transplant that we report is a first step towards a better definition of the optimal management of these patients. We were able to show that the risk of AMR was particularly high in patients with high MFI values on the day of the transplant, especially if MFI did not significantly decrease after specific treatment. Intensification of immunosuppression might be indicated in these very high-risk patients. Comparing the experience of other centers could help define optimal management of such patients. We are currently working towards this goal with the team of Dr. J. Kobashigawa and J. Patel of Cedars-Sinai Medical Center, the world's leading heart transplant center. This team has developed an innovative approach to cardiac transplantation with a very high immunological risk by combining prolonged complement inhibitor therapy (Eculizumab) with the standard of care including polyclonal induction, high dose infusion of IVIg, and a triple basal immunosuppression. The first results seem very encouraging. I will have the opportunity to continue the comparison between the cohorts of our two centers during a postdoctoral year that I will perform within this prestigious team.

Bibliography

1. Ponikowski P, Voors AA, Anker SD et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *European Journal of Heart Failure* 2016;
2. Crespo-Leiro MG, Metra M, Lund LH et al. Advanced heart failure: a position statement of the Heart Failure Association of the European Society of Cardiology. *European Journal of Heart Failure* 2018; 20: 1505.
3. Fang JC, Ewald GA, Allen LA et al. Advanced (stage D) heart failure: a statement from the Heart Failure Society of America Guidelines Committee. *Journal of Cardiac Failure* 2015; 21: 519.
4. Rose EA, Gelijns AC, Moskowitz AJ et al. Long-term use of a left ventricular assist device for end-stage heart failure. *The New England Journal of Medicine* 2001; 345: 1435.
5. Shah KB, Starling RC, Rogers JG et al. Left ventricular assist devices versus medical management in ambulatory heart failure patients: An analysis of INTERMACS Profiles 4 and 5 to 7 from the ROADMAP study. *The Journal of Heart and Lung Transplantation [Internet]* 2017; [cited 2018 Feb 1] Available from: <http://linkinghub.elsevier.com/retrieve/pii/S1053249817321423>
6. Filippatos GS, Colvin MM, Casey DE, Butler J, Bozkurt B. 2017 ACC/AHA/HFSA Focused Update of the 2013 ACCF/AHA Guideline for the Management of Heart Failure. 2017; : 129.
7. Khush KK, Cherikh WS, Chambers DC et al. The International Thoracic Organ Transplant Registry of the International Society for Heart and Lung Transplantation: Thirty-fifth Adult Heart Transplantation Report-2018; Focus Theme: Multiorgan Transplantation. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2018; 37: 1155.
8. 2017 annual report of “Agence de la Biomédecine” [Internet]. Available from: <https://www.agence-biomedecine.fr/annexes/bilan2017/donnees/organes/03-coeur/telechargement/FC6.gif>
9. 2017 annual report from the French National Agency for Biomedicine - heart

transplantation. [Internet]. [cited 2019 Jul 3] Available from: <https://www.agence-biomedecine.fr/annexes/bilan2017/donnees/organes/03-coeur/synthese.htm>

10. Lund LH, Khush KK, Cherikh WS et al. The Registry of the International Society for Heart and Lung Transplantation: Thirty-fourth Adult Heart Transplantation Report-2017; Focus Theme: Allograft ischemic time. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2017; 36: 1037.
11. Shah KB, Flattery MP, Smallfield MC et al. Surveillance Endomyocardial Biopsy in the Modern Era Produces Low Diagnostic Yield for Cardiac Allograft Rejection. *Transplantation* 2015; 99: e75.
12. Vaughn GR, Jorgensen NW, Law YM et al. Outcome of antibody-mediated rejection compared to acute cellular rejection after pediatric heart transplantation. *Pediatric Transplantation* 2018; 22.
13. Coutance G, Van Aelst L, Hékimian G et al. Antibody-mediated rejection induced cardiogenic shock: too late for conventional therapy. *Clinical Transplantation* 2018; : e13253.
14. ISHLT. International Thoracic Organ Transplant (TTX) Registry Data Slides. 2017 Annual report. Adult heart transplantation statistics. [Internet]. [cited 2019 Jan 30] Available from: https://ishltregistries.org/downloadables/slides/2018/heart_adult.pptx
15. Kobashigawa JA, Tobis JM, Starling RC et al. Multicenter intravascular ultrasound validation study among heart transplant recipients: outcomes after five years. *Journal of the American College of Cardiology* 2005; 45: 1532.
16. Potena L, Masetti M, Sabatino M et al. Interplay of coronary angiography and intravascular ultrasound in predicting long-term outcomes after heart transplantation. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2015; 34: 1146.
17. Hirohashi T, Uehara S, Chase CM et al. Complement Independent Antibody-Mediated Endarteritis and Transplant Arteriopathy in Mice. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2010; 10: 510.
18. Hirohashi T, Chase CM, Della Pelle P et al. A novel pathway of chronic allograft rejection mediated by NK cells and alloantibody. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society*

of Transplant Surgeons 2012; 12: 313.

19. Topilsky Y, Gandhi MJ, Hasin T et al. Donor-specific antibodies to class II antigens are associated with accelerated cardiac allograft vasculopathy: a three-dimensional volumetric intravascular ultrasound study. *Transplantation* 2013; 95: 389.
20. Clerkin KJ, Restaino SW, Zorn E, Vasilescu ER, Marboe CC, Mancini DM. The effect of timing and graft dysfunction on survival and cardiac allograft vasculopathy in antibody-mediated rejection. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2016; 35: 1059.
21. Paul P, Picard C, Sampol E et al. Genetic and Functional Profiling of CD16-Dependent Natural Killer Activation Identifies Patients at Higher Risk of Cardiac Allograft Vasculopathy. *Circulation* 2017;
22. Ambrosi P, Kreitmann B, Riberi A, Lambert M, Pankert M, Habib G. Chronic heart failure in heart transplant recipients: Presenting features and outcome. *Archives of Cardiovascular Diseases* 2016; 109: 254.
23. Loupy A, Toquet C, Rouvier P et al. Late Failing Heart Allografts: Pathology of Cardiac Allograft Vasculopathy and Association With Antibody-Mediated Rejection. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2016; 16: 111.
24. Clemmensen TS, Koefoed-Nielsen P, Jensen L-A et al. Donor-specific antibodies are associated with micro- and macrovascular coronary disease, restrictive myocardial damage, and poor outcome in heart-transplanted patients. *Clinical Transplantation* 2017;
25. Loupy A, Lefaucheur C. Antibody-Mediated Rejection of Solid Organ Allografts. *N Engl J Med* 2018; In Press.
26. Chih S, Tinckam KJ, Ross HJ. A survey of current practice for antibody-mediated rejection in heart transplantation. *American journal of transplantation: official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2013; 13: 1069.
27. Colvin MM, Cook JL, Chang P et al. Antibody-mediated rejection in cardiac transplantation: emerging knowledge in diagnosis and management: a scientific statement from the American Heart Association. *Circulation* 2015; 131: 1608.
28. Kobashigawa J, Colvin M, Potena L et al. The management of antibodies in

heart transplantation: An isHLT consensus document. *The Journal of Heart and Lung Transplantation* [Internet] 2018; [cited 2018 Feb 1] Available from: <http://linkinghub.elsevier.com/retrieve/pii/S1053249818312920>

29. 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. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2013; 32: 1147.

30. 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. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2017; 17: 42.

31. Crespo-Leiro MG, Zuckermann A, Bara C et al. Concordance among pathologists in the second Cardiac Allograft Rejection Gene Expression Observational Study (CARGO II). *Transplantation* 2012; 94: 1172.

32. Halloran PF, Potena L, Van Huyen J-PD et al. Building a tissue-based molecular diagnostic system in heart transplant rejection: The heart Molecular Microscope Diagnostic (MMDx) System. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2017;

33. Afzali B, Chapman E, Racapé M et al. Molecular Assessment of Microcirculation Injury in Formalin-Fixed Human Cardiac Allograft Biopsies With Antibody-Mediated Rejection. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2016;

34. Costanzo MR, Dipchand A, Starling R et al. The International Society of Heart and Lung Transplantation Guidelines for the care of heart transplant recipients. *The Journal of heart and lung transplantation: the official publication of the International Society for Heart Transplantation* 2010; 29: 914.

35. 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.

36. Nwakanma LU, Williams JA, Weiss ES, Russell SD, Baumgartner WA, Conte JV. Influence of pretransplant panel-reactive antibody on outcomes in 8,160 heart transplant recipients in recent era. *The Annals of Thoracic Surgery* 2007; 84: 1556.
37. Raess M, Fröhlich 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. *Transplant International: Official Journal of the European Society for Organ Transplantation* 2013; 26: 1097.
38. Stastny P, Lavingia B, Fixler DE, Yancy CW, Ring WS. Antibodies against donor human leukocyte antigens and the outcome of cardiac allografts in adults and children. *Transplantation* 2007; 84: 738.
39. 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 International: Official Journal of the European Society for Organ Transplantation* 2016; 29: 63.
40. Haas M, Loupy A, Lefaucheur C et al. The Banff 2017 Kidney Meeting Report: Revised diagnostic criteria for chronic active T cell-mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials. *American Journal of Transplantation* 2018; 18: 293.
41. Loupy A, Haas M, Solez K et al. The Banff 2015 Kidney Meeting Report: Current Challenges in Rejection Classification and Prospects for Adopting Molecular Pathology. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2017; 17: 28.
42. Patel R, Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. *The New England Journal of Medicine* 1969; 280: 735.
43. Stites E, Le Quintrec M, Thurman JM. The Complement System and Antibody-Mediated Transplant Rejection. *Journal of Immunology (Baltimore, Md.: 1950)* 2015; 195: 5525.
44. Cole DS, Morgan BP. Beyond lysis: how complement influences cell fate. *Clinical Science (London, England: 1979)* 2003; 104: 455.
45. 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.

46. Duong Van Huyen J-P, Tible M, Gay A et al. MicroRNAs as non-invasive biomarkers of heart transplant rejection. *European Heart Journal* 2014; 35: 3194.
47. Di Francesco A, Fedrigo M, Santovito D et al. MicroRNA signatures in cardiac biopsies and detection of allograft rejection. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2018; 37: 1329.
48. Van Aelst LNL, Summer G, Li S et al. RNA Profiling in Human and Murine Transplanted Hearts: Identification and Validation of Therapeutic Targets for Acute Cardiac and Renal Allograft Rejection. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2016; 16: 99.
49. Holweg CTJ, Potena L, Luikart H et al. Identification and classification of acute cardiac rejection by intragraft transcriptional profiling. *Circulation* 2011; 123: 2236.
50. Bodez D, Hocini H, Tchitchek N et al. Myocardial Gene Expression Profiling to Predict and Identify Cardiac Allograft Acute Cellular Rejection: The GET-Study. *PLoS One* 2016; 11: e0167213.
51. Halloran PF, Venner JM, Madill-Thomsen KS et al. Review: The transcripts associated with organ allograft rejection. *American Journal of Transplantation* 2018; 18: 785.
52. Parkes MD, Aliabadi AZ, Cadeiras M et al. An integrated molecular diagnostic report for heart transplant biopsies using an ensemble of diagnostic algorithms. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2019; 38: 636.
53. Bayliss J, Maguire JA, Bailey M et al. Increased vascular endothelial growth factor mRNA in endomyocardial biopsies from allografts demonstrating severe acute rejection: a longitudinal study. *Transplant Immunology* 2008; 18: 264.
54. Pérez EC, Shulzhenko N, Morgun A et al. Expression of Fas, FasL, and soluble Fas mRNA in endomyocardial biopsies of human cardiac allografts. *Human Immunology* 2006; 67: 22.
55. Breinholt JP, Vallejo JG, Gates CM et al. Myocardial pro-inflammatory cytokine expression and cellular rejection in pediatric heart transplant recipients. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2008; 27: 317.

56. Tsang H-F, Xue VW, Koh S-P, Chiu Y-M, Ng LP-W, Wong S-CC. NanoString, a novel digital color-coded barcode technology: current and future applications in molecular diagnostics. *Expert Review of Molecular Diagnostics* 2017; 17: 95.
57. Bobée V, Ruminy P, Marchand V et al. Determination of Molecular Subtypes of Diffuse Large B-Cell Lymphoma Using a Reverse Transcriptase Multiplex Ligation-Dependent Probe Amplification Classifier. *The Journal of Molecular Diagnostics* 2017; 19: 892.
58. Mareschal S, Ruminy P, Bagacean C et al. Accurate Classification of Germinal Center B-Cell-Like/Activated B-Cell-Like Diffuse Large B-Cell Lymphoma Using a Simple and Rapid Reverse Transcriptase-Multiplex Ligation-Dependent Probe Amplification Assay: A CALYM Study. *The Journal of molecular diagnostics: JMD* 2015;
59. Stewart S, Winters GL, Fishbein MC et al. Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. *The Journal of heart and lung transplantation: the official publication of the International Society for Heart Transplantation* 2005; 24: 1710.
60. Adam B, Afzali B, Dominy KM et al. Multiplexed color-coded probe-based gene expression assessment for clinical molecular diagnostics in formalin-fixed paraffin-embedded human renal allograft tissue. *Clinical Transplantation* 2016; 30: 295.
61. Smith RN, Adam BA, Rosales IA et al. RNA expression profiling of renal allografts in a nonhuman primate identifies variation in NK and endothelial gene expression. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2018; 18: 1340.
62. Eldering E, Spek CA, Aberson HL et al. Expression profiling via novel multiplex assay allows rapid assessment of gene regulation in defined signalling pathways. *Nucleic Acids Research* 2003; 31: e153.
63. Fedrigo M, Leone O, Burke MM et al. Inflammatory cell burden and phenotype in endomyocardial biopsies with antibody-mediated rejection (AMR): a multicenter pilot study from the AECVP. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2015; 15: 526.
64. Aubert O, Loupy A, Hidalgo L et al. Antibody-Mediated Rejection Due to

Preexisting versus De Novo Donor-Specific Antibodies in Kidney Allograft Recipients. *Journal of the American Society of Nephrology: JASN* 2017; 28: 1912.

65. Halloran PF, Pereira AB, Chang J et al. Microarray diagnosis of antibody-mediated rejection in kidney transplant biopsies: an international prospective study (INTERCOM). *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2013; 13: 2865.

66. Sellarés J, Reeve J, Loupy A et al. Molecular diagnosis of antibody-mediated rejection in human kidney transplants. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2013; 13: 971.

67. Fishbein MC, Kobashigawa J. Biopsy-negative cardiac transplant rejection: etiology, diagnosis, and therapy. *Current Opinion in Cardiology* 2004; 19: 166.

68. Tremblay-Gravel M, Racine N, de Denus S et al. Changes in Outcomes of Cardiac Allograft Vasculopathy Over 30 Years Following Heart Transplantation. *JACC. Heart failure* 2017; 5: 891.

69. Rizeq MN, Masek MA, Billingham ME. Acute rejection: significance of elapsed time after transplantation. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 1994; 13: 862.

70. Coutance G, d'Orio V, Belin L et al. Favorable outcome of an exclusively posttransplant prophylactic strategy after heart transplantation in recipients with high immunological risk. *Transplantation* 2018;

71. Stehlik J, Starling RC, Movsesian MA et al. Utility of long-term surveillance endomyocardial biopsy: a multi-institutional analysis. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2006; 25: 1402.

72. Neylan JF. Immunosuppressive therapy in high-risk transplant patients: dose-dependent efficacy of mycophenolate mofetil in African-American renal allograft recipients. U.S. Renal Transplant Mycophenolate Mofetil Study Group. *Transplantation* 1997; 64: 1277.

73. Beniaminovitz A, Itescu S, Lietz K et al. Prevention of rejection in cardiac transplantation by blockade of the interleukin-2 receptor with a monoclonal antibody. *The New England Journal of Medicine* 2000; 342: 613.

74. Cantarovich M, Giannetti N, Barkun J, Cecere R. Antithymocyte globulin induction allows a prolonged delay in the initiation of cyclosporine in heart transplant patients with postoperative renal dysfunction. *Transplantation* 2004; 78: 779.
75. Kobashigawa JA, Miller LW, Russell SD et al. Tacrolimus with mycophenolate mofetil (MMF) or sirolimus vs. cyclosporine with MMF in cardiac transplant patients: 1-year report. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2006; 6: 1377.
76. Hamour IM, Burke MM, Bell AD, Panicker MG, Banerjee R, Banner NR. Limited utility of endomyocardial biopsy in the first year after heart transplantation. *Transplantation* 2008; 85: 969.
77. Imamura T, Kinugawa K, Nitta D et al. Late rejection occurred in recipients who experienced acute cellular rejection within the first year after heart transplantation. *International Heart Journal* 2015; 56: 174.
78. Chen CK, Manlhiot C, Conway J et al. Development and Impact of De Novo Anti-HLA Antibodies in Pediatric Heart Transplant Recipients. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2015; 15: 2215.
79. 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. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2011; 11: 312.
80. Pober JS, Jane-wit D, Qin L, Tellides G. Interacting mechanisms in the pathogenesis of cardiac allograft vasculopathy. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2014; 34: 1609.
81. Mehra MR. Contemporary concepts in prevention and treatment of cardiac allograft vasculopathy. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2006; 6: 1248.
82. Langstraat M, Musters KJS, Manintveld O, Masetti M, Potena L. Coronary artery disease in heart transplantation: new concepts for an old disease. *Transplant International* : n/a.
83. Watanabe T, Seguchi O, Yanase M et al. Donor-Transmitted Atherosclerosis

Associated With Worsening Cardiac Allograft Vasculopathy After Heart Transplantation: Serial Volumetric Intravascular Ultrasound Analysis. *Transplantation* 2017; 101: 1310.

84. Sato T, Seguchi O, Ishibashi-Ueda H et al. Risk Stratification for Cardiac Allograft Vasculopathy in Heart Transplant Recipients - Annual Intravascular Ultrasound Evaluation. *Circulation Journal: Official Journal of the Japanese Circulation Society* 2016; 80: 395.

85. Prieto D, Correia P, Baptista M, Antunes MJ. Outcome after heart transplantation from older donor age: expanding the donor pool. *European Journal of Cardio-Thoracic Surgery: Official Journal of the European Association for Cardio-Thoracic Surgery* 2015; 47: 672.

86. Harris J, Teuteberg J, Shullo M. Optimal low-density lipoprotein concentration for cardiac allograft vasculopathy prevention. *Clinical Transplantation* 2018; 32: e13248.

87. Mehra MR, Uber PA, Ventura HO, Scott RL, Park MH. The impact of mode of donor brain death on cardiac allograft vasculopathy: an intravascular ultrasound study. *Journal of the American College of Cardiology* 2004; 43: 806.

88. Johansson I, Andersson R, Friman V et al. Cytomegalovirus infection and disease reduce 10-year cardiac allograft vasculopathy-free survival in heart transplant recipients. *BMC infectious diseases* 2015; 15: 582.

89. 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: The Official Publication of the International Society for Heart Transplantation* 2015; 34: 1050.

90. Deckers JW, Hare JM, Baughman KL. Complications of transvenous right ventricular endomyocardial biopsy in adult patients with cardiomyopathy: a seven-year survey of 546 consecutive diagnostic procedures in a tertiary referral center. *Journal of the American College of Cardiology* 1992; 19: 43.

91. Weckbach LT, Maurer U, Schramm R et al. Lower frequency routine surveillance endomyocardial biopsies after heart transplantation. *PLoS One* 2017; 12: e0182880.

92. Pham MX, Teuteberg JJ, Kfoury AG et al. Gene-expression profiling for rejection surveillance after cardiac transplantation. *The New England Journal of Medicine* 2010;

362: 1890.

93. Deng MC, Eisen HJ, Mehra MR et al. Noninvasive discrimination of rejection in cardiac allograft recipients using gene expression profiling. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2006; 6: 150.

94. Khush KK, Patel J, Pinney S et al. Non-invasive Detection of Graft Injury after Heart Transplantation Using Donor-Derived Cell-Free DNA: a Prospective Multi-Center Study. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2019;

95. 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. *Journal of the American College of Cardiology* 2014; 63: 1224.

96. De Vlaminck I, Valantine HA, Snyder TM et al. Circulating cell-free DNA enables noninvasive diagnosis of heart transplant rejection. *Science Translational Medicine* 2014; 6: 241ra77.

97. Eurotransplant. 2017 Statistical report. [Internet]. 2018; [cited 2019 Jul 3] Available from: https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=3&cad=rja&uact=8&ved=2ahUKEwjrmuD6-pfjAhVEQhoKHWTRAKcQFjACegQIAxAC&url=http%3A%2F%2Fwww.eurotransplant.org%2Fcms%2Fmediaobject.php%3Ffile%3D803150%2B020288%2BStatistical%2BReport%2B2017%2B%2528online%2529131.pdf&usg=AOvVaw1U-4EpkFaOSvGZ2mKtgo_w

98. López-Sainz Á, Barge-Caballero E, Barge-Caballero G et al. Late graft failure in heart transplant recipients: incidence, risk factors and clinical outcomes: Heart failure after heart transplantation. *European Journal of Heart Failure* 2018; 20: 385.

99. PCSK9 Inhibition After Heart Transplantation [Internet]. Available from: <https://clinicaltrials.gov/ct2/show/NCT03537742?term=cardiac+allograft+vasculopathy&cond=heart+transplantation&rank=24>

100. Cholesterol Lowering With EVOLocumab to Prevent Cardiac Allograft Vasculopathy in De-novo Heart Transplant Recipients (EVOLVD). [Internet]. Available from:

<https://clinicaltrials.gov/ct2/show/NCT03734211?term=heart+transplantation&cond=cardiac+allograft+vasculopathy&rank=5>

101. Proust-Lima C, Philipps V, Lique B. Estimation of Extended Mixed Models Using Latent Classes and Latent Processes: The R Package LCMM. 2017;
102. Allen NB, Siddique J, Wilkins JT et al. Blood pressure trajectories in early adulthood and subclinical atherosclerosis in middle age. *JAMA* 2014; 311: 490.
103. Gill TM, Gahbauer EA, Han L, Allore HG. Trajectories of disability in the last year of life. *The New England Journal of Medicine* 2010; 362: 1173.
104. Song M, Hu FB, Wu K et al. Trajectory of body shape in early and middle life and all cause and cause specific mortality: results from two prospective US cohort studies. *BMJ (Clinical research ed.)* 2016; 353: i2195.
105. Nagin DS, Odgers CL. Group-based trajectory modeling in clinical research. *Annual Review of Clinical Psychology* 2010; 6: 109.
106. Chemouny J-M, Suberbielle C, Rabant M et al. De Novo Donor-Specific Human Leukocyte Antigen Antibodies in Nonsensitized Kidney Transplant Recipients After T Cell-Mediated Rejection. *Transplantation* 2014;
107. Hausmann D, Erbel R, Alibelli-Chemarin MJ et al. The safety of intracoronary ultrasound. A multicenter survey of 2207 examinations. *Circulation* 1995; 91: 623.
108. Mehra MR, Dipchand A, Ensminger SM 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.
109. Lund LH, Edwards LB, Kucheryavaya AY et al. The registry of the International Society for Heart and Lung Transplantation: thirty-first official adult heart transplant report--2014; focus theme: retransplantation. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2014; 33: 996.
110. Geft D, Kobashigawa J. Current concepts for sensitized patients before transplantation. *Current Opinion in Organ Transplantation* 2017; 22: 236.
111. Kransdorf EP, Kittleson MM, Patel JK, Pando MJ, Steidley DE, Kobashigawa JA. Calculated panel-reactive antibody predicts outcomes on the heart transplant waiting list. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2017; 36: 787.

112. Berry GJ, Angelini A, Burke MM et al. The ISHLT working formulation for pathologic diagnosis of antibody-mediated rejection in heart transplantation: evolution and current status (2005-2011). *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2011; 30: 601.
113. Stewart S, Winters GL, Fishbein MC et al. Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. *The Journal of heart and lung transplantation: the official publication of the International Society for Heart Transplantation* 2005; 24: 1710.
114. Kobashigawa J, Zuckermann A, Macdonald P et al. Report from a consensus conference on primary graft dysfunction after cardiac transplantation. *The Journal of Heart and Lung Transplantation* 2014; 33: 327.
115. Costanzo MR, Dipchand A, Starling R et al. The International Society of Heart and Lung Transplantation Guidelines for the care of heart transplant recipients. *The Journal of heart and lung transplantation: the official publication of the International Society for Heart Transplantation* 2010; 29: 914.
116. Schaffer JM, Singh SK, Reitz BA, Oyer PE, Robbins RC, Mallidi HR. Heart transplant graft survival is improved after a reduction in panel reactive antibody activity. *The Journal of Thoracic and Cardiovascular Surgery* 2013; 145: 555.
117. Lefaucheur C, Loupy A, Hill GS et al. Preexisting donor-specific HLA antibodies predict outcome in kidney transplantation. *Journal of the American Society of Nephrology: JASN* 2010; 21: 1398.
118. Cole RT, Gandhi J, Morris A et al. De Novo DQ Donor-Specific Antibodies Are Associated with Worse Outcomes Compared to Non-DQ DSA Following Heart Transplantation. *The Journal of Heart and Lung Transplantation* 2016; 35: S42.
119. Gandhi MJ, DeGoey SR, Bundy K et al. Effect of pretransplant human leukocyte antigen antibodies detected by solid-phase assay on heart transplant outcomes. *Transplantation proceedings* 2011; 43: 3840.
120. Wu GW, Kobashigawa JA, Fishbein MC et al. Asymptomatic antibody-mediated rejection after heart transplantation predicts poor outcomes. *The Journal of heart and lung transplantation: the official publication of the International Society for Heart Transplantation* 2009; 28: 417.
121. Tambur AR, Lavee J. Incorporating human leukocyte antibody results into clinical practice. *The Journal of Heart and Lung Transplantation: The Official*

Publication of the International Society for Heart Transplantation 2016; 35: 851.

122. Loupy A, Lefaucheur C, Vernerey D et al. Complement-binding anti-HLA antibodies and kidney-allograft survival. *The New England Journal of Medicine* 2013; 369: 1215.

123. Lefaucheur C, Viglietti D, Bentelejewski C et al. IgG Donor-Specific Anti-Human HLA Antibody Subclasses and Kidney Allograft Antibody-Mediated Injury. *Journal of the American Society of Nephrology: JASN* 2016; 27: 293.

124. Reinsmoen NL, Patel J, Mirocha J et al. Optimizing transplantation of sensitized heart candidates using 4 antibody detection assays to prioritize the assignment of unacceptable antigens. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2016; 35: 165.

125. Ius F, Verboom M, Sommer W et al. Preemptive treatment of early donor-specific antibodies with IgA- and IgM-enriched intravenous human immunoglobulins in lung transplantation. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2018;

126. Ius F, Sommer W, Kieneke D et al. IgM-Enriched Human Intravenous Immunoglobulin-Based Treatment of Patients With Early Donor Specific Anti-HLA Antibodies After Lung Transplantation. *Transplantation* 2016; 100: 2682.

127. Hachem RR, Yusef RD, Meyers BF et al. Anti-human leukocyte antigen antibodies and preemptive antibody-directed therapy after lung transplantation. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2010; 29: 973.

128. Kfoury AG, Hammond MEH, Snow GL et al. Cardiovascular mortality among heart transplant recipients with asymptomatic antibody-mediated or stable mixed cellular and antibody-mediated rejection. *The Journal of heart and lung transplantation: the official publication of the International Society for Heart Transplantation* 2009; 28: 781.

129. 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.

130. ELSO. ELSO Guidelines for Cardiopulmonary Extracorporeal Life Support. Extracorporeal Life Support Organization, Version 1.3. 2013;

131. Garrett HE, Duvall-Seaman D, Helsley B, Groshart K. Treatment of vascular rejection with rituximab in cardiac transplantation. *The Journal of heart and lung transplantation* 2005; 24: 1337.
132. Sautenet B, Blancho G, Böhler 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. *Transplantation* 2016; 100: 391.
133. Eskandary F, Wahrmann M, Mählbacher J, Böhmig GA. Complement inhibition as potential new therapy for antibody-mediated rejection. *Transplant International* 2016; 29: 392.
134. Yelken B, Arpalı E, Görcin S et al. Eculizumab for Treatment of Refractory Antibody-Mediated Rejection in Kidney Transplant Patients: A Single-Center Experience. *Transplantation Proceedings* 2015; 47: 1754.
135. Burbach M, Suberbielle C, Brochériou I et al. Report of the inefficacy of eculizumab in two cases of severe antibody-mediated rejection of renal grafts. *Transplantation* 2014; 98: 1056.
136. Montgomery RA, Orandi BJ, Racusen L et al. Plasma-Derived C1 Esterase Inhibitor for Acute Antibody-Mediated Rejection Following Kidney Transplantation: Results of a Randomized Double-Blind Placebo-Controlled Pilot Study. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2016; 16: 3468.
137. Kfoury AG, Miller DV, Snow GL et al. Mixed cellular and antibody-mediated rejection in heart transplantation: In-depth pathologic and clinical observations. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2016; 35: 335.
138. Marenzi G, Cosentino N, Marinetti A et al. Renal replacement therapy in patients with acute myocardial infarction: Rate of use, clinical predictors and relationship with in-hospital mortality. *International Journal of Cardiology* 2017; 230: 255.
139. Kuo H-H, Fan R, Dvorina N, Chiesa-Vottero A, Baldwin WM. Platelets in early antibody-mediated rejection of renal transplants. *Journal of the American Society of Nephrology: JASN* 2015; 26: 855.
140. Radovancevic B, McGiffin DC, Kobashigawa JA et al. Retransplantation in 7,290 primary transplant patients: a 10-year multi-institutional study. *The Journal of*

Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation 2003; 22: 862.

141. Khan MS, Mery CM, Zafar F et al. Is mechanically bridging patients with a failing cardiac graft to retransplantation an effective therapy? Analysis of the United Network of Organ Sharing database. *The Journal of Heart and Lung Transplantation: The Official Publication of the International Society for Heart Transplantation* 2012; 31: 1192.

142. Xie A, Phan K, Tsai Y-C, Yan TD, Forrest P. Venoarterial extracorporeal membrane oxygenation for cardiogenic shock and cardiac arrest: a meta-analysis. *Journal of Cardiothoracic and Vascular Anesthesia* 2015; 29: 637.

Supplementary appendix

S1. Data collection procedures

Baseline clinical data on the donors and recipients from Paris and Leuven were obtained from the national registry CRISTAL (Agence de la Biomédecine, French National Agency for Organ Procurement) and Eurotransplant registry, respectively. Codes were used to ensure strict donor and recipient anonymity and blinded assays. Informed consent was obtained from the participants at the time of transplantation. Anonymized data from these registries are prospectively entered by dedicated staff at specific time points for each patient (day of listing, day of transplant and at hospital discharge) and are updated annually thereafter. Longitudinal data from the day of transplantation to 10 years post-transplantation were retrieved based on a complete review of medical charts and the collection of clinical events. This retrospective collection of data was performed by cardiologist of each center and research assistants. We performed an extensive evaluation of potential nonimmune (classical) and immune (antibody-related) risk factors that might be associated with CAV. We recorded the following data: 1) donor cardiovascular profile, including age at donation, gender, cause of death (including vascular, traumatic, anoxia or other), donor comorbidities including hypertension, diabetes mellitus, dyslipidemia, tobacco and alcohol consumption, body mass index, renal function, medical treatment, the presence of a cardiac arrest during initial management, CMV serology and coronary angiograms, 2) recipient pre-transplant cardiovascular profile and general characteristics including age at transplantation, gender, ethnicity, hypertension, diabetes mellitus, dyslipidemia, tobacco and alcohol consumption, body mass index, using of mechanical cardiac support or implanted devices, the presence of an ECMO support at the time of transplant, primary heart disease, 3) transplantation characteristics including cold ischemic time, type of transplantation (isolated heart transplantation vs combined transplantations) gender mismatch, weight mismatch, CMV mismatch, 4) immunosuppressive therapies including the type of induction therapy and 1-year immunosuppressive regimen (type of calcineurin inhibitors, mycophenolate mofetil, everolimus, azathioprine, corticosteroids), 5) post-transplant

158

cardiovascular profile including treated hypertension, treated diabetes mellitus, LDL-c, tobacco consumption, body mass index, statins therapy, and renal function evaluated at 1-year post-transplant, 6) immunologic parameters including the number of HLA A-B-DR mismatches (and A-B-DR-DQ when available) and the presence of preformed or de-novo DSA at the time of transplant and/or during the first year post-transplant, DSA characteristics (class, MFI of immunodominant DSA), 6) histological parameters of endomyocardial biopsies (EMB) retrospectively reclassified according to ISHLT 2004 and 2013 guidelines for cellular rejection and AMR (Berry JHLT, Steward JHLT). CRISTAL database networks (official website: <https://www.sipg.sante.fr/portail/>) have been approved by the National French Commission for Bioinformatics Data and Patient Liberty: CRISTAL: CNIL, registration number: 363505, validated April 3, 1996. Our unified prospective European database complies with the European Union chart concerning data collection and protection (General Data Protection Regulation) and received an agreement from the National French Commission for Bioinformatics Data and Patient Liberty (2206319 v 0).

S2. Circulating donor-specific antibodies

All patients included in the European derivation cohort were screened for the presence of circulating anti-HLA antibodies in sera collected before HTx, at the time of HTx and 12 months after HTx. Banked sera were retrospectively reassessed for anti-HLA antibodies between November 2017 and March 2018, using Luminex technology, at the reference laboratory of histocompatibility of Paris (Saint-Louis Hospital). An expert immunologist (JLT) interpreted the results and determine whether each tested serum contained donor-specific anti-HLA antibodies (DSA). Antibodies against the HLA-A, HLA-B, HLA-Cw, HLA-DR, HLA-DQ, and HLA-DP epitopes were tested using single-antigen flow bead assays (One Lambda, Inc., Canoga Park, CA, USA) on a Luminex platform. All beads with a normalized MFI of greater than 500 were considered positive. In patients with multiple anti-HLA DSA, the immunodominant anti-HLA DSA (the one with the highest MFI) was selected for analysis. HLA typing of the recipients was performed via molecular biology (Innolipa HLA typing kit, Innogenetics, Belgium). For all heart transplant donors, tissue typing was initially performed using the microlymphocytotoxicity technique with One Lambda Inc. tissue typing trays at transplantation and was confirmed by molecular biology.

S3. Pathology

Endomyocardial biopsies were performed, processed and examined according to current standards. The routine EMB protocol from the European derivation cohort included 13 protocolar EMBs during the first year (every 10 days from day 15 to M2, every month from M2 to M6 and every month and a half from M6 to M12). Additional biopsies were performed in case of clinical indication. Serial sections were cut from formalin-fixed paraffin-embedded EMB specimens and stained with hematoxylin, eosin and saffron for diagnosis. Immunofluorescence for C4d was performed (frozen section; C4d monoclonal Quidel Corporation 1/100; Polyclonal rabbit anti mouse FITC Dako). Only capillary staining for C4d was assessed. The EMBs were classified as cellular (0 to 3R) or ABMR (pAMR 0 to 3) according to the ISHLT guidelines.

For the purpose of this study, a systematic retrospective analysis of all abnormal EMBs (i.e. ACR \geq 1R1B and/or pAMR \geq 1), for-cause EMBs and pre-specified protocolar EMBs (first, M1, M3, M6 and M12) was performed by expert pathologists, independently of the initial pathological diagnosis, clinical, and CAV data in the French derivation cohort.