**Sede Amministrativa: Università degli Studi di Padova**

Dipartimento di Scienze Medico-Diagnostiche e Terapie Speciali

*SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE MEDICHE, CLINICHE E SPERIMENTALI*

*INDIRIZZO "SCIENZE CARDIOVASCOLARI"*

*CICLO XXIV*

# *New tissue biomarkers of Antibody Mediated Rejection in Heart Transplantation*

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*…..…io son molte volte andando meco medesimo considerando, in proposito di quanto grande sia l'acutezza dell'ingegno umano; e mentre io discorro per tante e tanto meravigliose invenzioni trovate dagli uomini, si nelle arti come nelle lettere, e poi fo riflessione sopra il saper mio, tanto lontano dal potersi promettere non solo di ritrovarne alcuna di nuovo, ma anco di apprendere delle già ritrovate, confuso dallo stupore ed afflitto dalla disperazione.…..*

"Dialogo dei Massimi Sistemi",1632
Galileo Galilei

*A mio marito ed a mia figlia*

# 1 SUMMARY

**INTRODUCTION**

Heart transplant still represent the most important therapeutic strategy for end-stage cardiomyopathies. Humoral rejection was recognized as a distinct clinico-pathologic entity characterized by acute allograft rejection associated with the production of antidonor HLA antibodies and poor prognosis. For the first time on renal biopsy from patients with kidney function deterioration and absence of cellular rejection C4d was identified as an important diagnostic biomarker for antibody mediated rejection and was strongly correlated with the presence of circulating anti-HLA antibodies. In heart transplantation in the ISHLT working formulation of 2004 the diagnostic criteria of AMR were defined only as present or absent , recognizing grade 0 without histological and immunopathological signs of humoral rejection and grade 1 in the setting of both histological and immunopathological signs of humoral rejection. However the role of AMR remained controversial with different reported incidence between different centers. Even though we were able to obtain a better control of cellular rejection with a decreasing rate of positive Acute Cellular Rejection (ACR) requiring drug therapy modification, no concomitantly reduction in graft loss for cardiac allograft vasculopathy was observed. Recently the attention was mainly focused on humoral rejection and on our ability to detect it through immunohistopathological markers. The aims of the present thesis were summarized in three major objectives: 1) to study the pathophysiology of Antibody Mediated Rejection (AMR) in heart transplant recipients; 2) to assess the diagnostic ability of pathological features to identify AMR on monitoring post-transplant

endomyocardial biopsies (EMBs); 3) to clarify the prognosis of AMR in the early and late post-transplant period.

**MATERIALS AND METHODS.**

As C4d staining has been performed on a routine basis in heart transplant recipients at the University of Padua's Heart Transplantation Center since 2005, 1.452 consecutive EMBs from 131 patients (mean age 48.9±18.3) were reviewed. Donor mean age was 41,6±15,2 years and 15% had more than 60 year old**. Adult population.** To study survival in patients with C4d positivity on EMBs we evaluated 985 consecutive biopsies from a total of 107 adult patients (n=85, 79% of these were males) with a median age at the time of transplantation of 55 years (range 17-73 years). Our study population was divided into 4 groups on the basis of the patients' C4d, DSA, and graft function profile:

- C4d positive, DSA negative, without graft dysfunction (asymptomatic AMR) = group 1;
- C4d positive, DSA positive, without graft dysfunction (asymptomatic AMR) = group 2;
- C4d positive, DSA positive, presence of graft dysfunction (symptomatic AMR) = group 3
- C4d negative, DSA negative, without graft dysfunction, control group = group 0.

**Pediatric population.** We evaluated 226 consecutive biopsies from 24 transplant patients (mean age 8,1±6,3 years).

**The immunoperoxidase staining** was applied routinely both in paediatric and adult patients. The staining for C4d was performed retrospectively using affinity-purified antihuman C4d rabbit polyclonal antibody. The grading system was standardized and was defined in four grades: grade 0 = negative; grade 1 = weak staining with focal distribution, grade 2 = moderate staining with multifocal distribution; grade 3 = strong staining with diffuse pattern. We considered positive grades 2 and 3.The cut off value between grade 1

and grade 2 was 50% of intramyocardial capillaries involved. Immunostaining for C3d, was performed on paraffin embedded section and using a polyclonal antibody C3d.The grading system for C3d immunostaining was the same as for C4d.

**Histological features of AMR on EMBs.** Of the 1452 EMBs, 87 were positive for C4d staining (6% 87/1452) from 61 patients, 26 EMBs from 13 patients had repeated C4d positivity. Sixty-six patients staining negative for C4d were matched for pre-transplant diagnosis, time after transplantation age, and acute cellular rejection (ACR) grading (case-control study). Among the 61 C4d positive patients 9 were diagnosed as AMR. In this case-control study we also applied C3d staining. Two pathologists reviewed blindly all morphological features associated to humoral damage: endothelial swelling, interstitial edema, intracapillary mononuclear cells, intracapillary neutrophils, myocite damage, myocite necrosis, hemorrhage, intravascular microthrombi according to ISHLT 2005 classification.

**Donor specific Antibodies (DSA) Assessment.** Since the study of C4d has been mainly retrospective only in a subgroup of patients circulating anti HLA antibodies could be performed. In thirty-seven patient circulating Anti HLA antibodies were assessed and resulted 26 positive and 9 negative. IgG anti-HLA reactivity in the sera, obtain before transplantation and at the time of C4d positive detection on EMBs, was analyzed using bead-based screening assays, referred to as Luminex methodology. All sera tested positive at screening, were retested with Single Antigen beads in order to determine the antibody specificity.

**Contrast-enhanced transthoracic Doppler echocardiography.** We selected 19 patients who at the time of biopsy stained positive for C4d and in whom echocardiography was performed for Coronary Flow Reserve (CFR) evaluation using CE-TTE before and after

adenosine infusion. CFR was calculated as ratio of peak diastolic velocity(hyperemia) and peak diastolic velocity (basal).

**Statistical analysis**. . The comparison between C4d groups was conducted with the Fisher's exact test in case of categorical variables and the Kruskal-Wallis test for quantitative variables. The Kaplan-Meier method was applied to estimate the C4d groups' survival functions and the log–rank test was used to compare survival between groups. The Cox's regression model was used to estimate unadjusted and sex and age adjusted hazard ratios (HR). Sensitivity and specificity, were reported with 95% confidence interval calculated with the exact method

**RESULTS**

**Clinic-pathological profile of adult population**

The 22 patients in group 1 (61% of total C4d+ve) showed a 18 fold higher mortality risk compared to the C4d negative patients (95% CI 1.960 to 160.022). The 6 patients in group 2 had a 61 fold higher mortality risk (95% CI 3.399 to 1110.360). The 8 patients in group 3 had a 32 fold higher mortality risk (95% CI 5.884 to 179.432), overall $p<0.0001$ Overall the C4d positive patients showed a statistically significant reduction in survival compared to the C4d negative patients and this observation was maintained in the three different groups

When the asymptomatic (groups 1 and 2) and symptomatic patients (group 3) were compared with the control group an 18 and 26 fold increase mortality risk was observed, respectively.

**Clinic-pathological profile of pediatric population.** Seven patients (33%) showed a C4d +ve intra-graft capillary deposition. Of these 4 were positive for circulating donor specific

anti HLA antibodies; none were positive in the C4d negative group. One patient presented also graft dysfunction (14% of C4d+ve) within 3 months after transplantation. One patient with C4d positivity died for heart failure after 2 years since C4d positivity detection. The other five patients with C4d positivity are still alive at 2, 3, 4 , 14 years respectively. In the C4d positive group the rejection score was higher compare to C4d negative patients. (2.2 vs 0.2, p<0,05)

**Morphological parameters on Endomyocardial biopsy (EMB) to detect Asymptomatic AMR (AsAMR) and Symptomatic AMR (AMR).**

Of the 8 histological characteristic evaluated on Hematoxilyn Eosin , only two (endothelial swelling and interstitial edema) could be considered fair predictors of C4d capillary positivity in the light of their sensitivity: endothelial swelling with a 78.7 % sensitivity (its specificity was very poor 28.8%) and interstitial edema with a 77.1% sensitivity (its specificity was 31.8%).

Intracapillary macrophages had a sensitivity of 39.3% only and a specificity of 68.2%. The sensitivity and specificity of the histological parameters in EMBs of patients with C4d positivity (both asymptomatic and symptomatic) were similar to those observed in EMBs of patients affected by symptomatic AMR

ROC curve combining endothelial swelling and intra-capillary aggregates of macrophages did not improve the capacity to predict presence of circulating anti HLA antibodies.

**Immunostaining for C3d in the diagnosis of AMR.**

In the asymptomatic AMR group (52 EMBs of 52 patients) 31 were also positive for C3d (59,6%, 31/52). In the symptomatic AMR group (9 EMBs of 9 patients) 5 were also positive for C3d (55% 5/9). The sensitivity of C3d to predicted DSA was 42.3% and the

specificity of C3d was 56% for DSA. Combining C4d and C3d to predicting circulating DSA did not increase the sensitivity and specificity of C4d.

**Alloantibody profile in symptomatic AMR.**

Of the 9 symptomatic AMR patients, 5 had been followed up for C4d positivity on EMBs and circulating DSA. The results showed that MFI for class I and class II changed according to the treatment. High level of MFI (>10000) strongly correlated with diffuse and strong intensity of C4d staining; with MFI ranging between 1000-9000 C4d was variably detected; with MFI < 1000 C4d was always negative.

**Microvasculopathy and C4d**. Seven out of 19 patients, in whom we performed echostress(or where ECHO stress was reduced) had C4d positive immunostaining while 12 were negative (average age 50.1±8.7 years). In the group of patients with C4d positivity 4 were late symptomatic AMR (mean time after Htx 13.9±4.9 years) and three of them died after few months since the diagnosis of AMR. Three patients had asymptomatic AMR (mean time after HTx 5.3±3.4 years).

Coronary flow reserve (CFR) of patients with C4d+ was statistically significant reduced compared to C4d- group (1.28±0.48 vs 3.28±1.03 respectively, p=0.0297) suggesting a relation between humoral rejection and microvascular damage.

Even in pts without CAV at angiography C4d positivity identified a low value of CFR in the C4d+ group (p=0.0502).

**CONCLUSIONS.**

Our findings indicate that C4d+ve is an important marker for diagnosis of AMR , thus identifying asymptomatic AMR . C4d predicts worse prognosis and DSA and graft dysfunction further improve risk stratification. C4d+ve and DSA can be used as early mortality predictors in patients without signs of graft dysfunction.

AMR is a complex and ongoing phenomenon with different phenotypic features.

Morphological parameters alone, are not adequately sensitive and carry low positive likelihood ratio as screening tools for early asymptomatic AMR detection. Screening recommendations has been recently modified to include more sensitive tests such as C4d staining in the routine protocol to improve patient risk stratification.

The assessment of anti HLA antibodies, class I and II and their relation with C4d (complement activation)showed : 1) a difference in type of anti HLA-DSA in the early and late period post-transplant with class I anti HLA early appearance and class II anti HLA late onset after transplant 2) high levels of MFI correlate with C4d deposition on EMBs.

Endothelial damage and complement deposition produce microvascular remodeling leading to microvasculopathy with increased graft loss.

# 2 RIASSUNTO

**INTRODUZIONE**

Il trapianto cardiaco rappresenta ancora la più importante strategia terapeutica nei pezienti con scompenso cardiaco refrattario alla terapia medica. Nell'ultimo decennio la attenzione clinica è stata rivolta al rigetto umorale in seguito al dato che la percentuale di perdita del garft per rigetto cronico non è cambiata, nonostante la terapia immunosoppressiva controlli in modo ottimale il rigetto cellulo-mediato.

Il rigetto umorale viene definito come una entità clinico-patologica caratterizzata dalla presenza di anticorpi anti HLA (complesso maggiore di istocompatibilità) circolanti che provocano danno endoteliale sull'organo trapiantato con conseguente impatto sulla mortalità.

Per la prima volta in biopsie renali di pazienti con disfunzione del graft in assenza di rigetto cellulo-mediato, è stato evidenziato deposito di C4d sulla superficie endoteliale, sinonimo di attivazione del complemento , strettamente correlato con la presenza di anticorpi anti HLA circolanti nel siero. Le linee guida internazionali che definiscono i criteri patologici per la diagnostica del rigetto nel trapianto di cuore, solo nel 2004 per la prima volta definiscono il rigetto umorale come entità patologica distinta indicandone i criteri diagnostici istologici e immunopatologici maggiori, riconoscendo come grado 0 assenza di segni istologici e immunopatologici e grado 1 la presenza di segni istologici e immunopatologici di rigetto umorale.

Nell'ultima decade la letteratura internazione ha rivolto la propria attenzione alla ricerca del significato prognostico del rigetto umorale in relazione al rigetto cronico e alla ricerca di nuovi biomarcatori per la diagnosi precoce del rigetto umorale.

Gli obiettivi della presente tesi di dottorato possono essere riassunti in tre principali puntii: 1) studio della fisiopatologia del rigetto umorale o rigetto anticorpo mediato nel trapianto di cuore; 2) valutazione della capacità diagnostica dei criteri istopatologici definiti dalle linee guida internazioni del 2004, su biopsie endomiocardiche di monitoraggio post-trapainto; 3) definizione del significato prognostico del rigetto anticorpo mediato sia nella fase precoce che tardiva post-trapianto.

**MATERIALI E METODI.**

Dal 2005, nel nostro centro di riferimento per il trapianto cardiaco dell'Università di Padova, abbiamo studiato routinariamente il biomarcatore di attivazione del complemento (C4d) con tecnica immunoistotchimica su tessuto (biopsie endomiocardiche di monitoraggio). Abbiamo valutato 1452 biopsie endomiocardiche (BEM) da 131 pazienti (età media 48.9±18.3). **Popolazione adulta.** Per studiare la sopravvivenza di pazienti adulti che presentano una positività al C4d nel loro follow up, abbiamo studiato 985 BEM di 107 pazienti (79% maschi) con età al trapianto di 55 anni (mediana) range 17-73 anni. La nostra popolazione adulta è stata suddivisa il quattro gruppi sulla base del profilo del C4d nelle BEM, degli anticorpi circolanti anti HLA donatore specifici (DSA), e della disfunzione del graft (riduzione della frazione di eiezione, anomalie elettrocardiogarfiche):

- C4d positivo, DSA negativi, assenza di disfunzione del garft (rigetto anticorpo-mediato asintomatico)= gruppo 1
- C4d positivo, DSA positivi, assenza di disfunzione del graft (rigetto anticorpo-mediato asintomatico)= gruppo 2
- C4d positivo, DSA positivi, presenza di segni clinici di disfunzione del graft (rigetto anticorpo-mediato sintomatico)= gruppo 3.

- C4d negativo, DSA negativi, assenza di disfunzione clinica del graft (gruppo controllo )= gruppo 0.

**Popolazione pediatrica.** Abbiamo valutato 226 biopsie provenienti da 24 pazienti trapiantati (età media 8,1±6,3 anni).

**Tecnica immunoistochimica** è stata applicata routinariamente sia nella popolazione adulta sia nella popolazione pediatrica. La tecnica immunoistochimica è stata applicata sia retrospetticamente che prospetticamente usando anticorpo policlonale per C4d. Il grading per la valutazione della colorazione immunoistochimica è stata standardizzato in quattro gradi: grado 0= negativo; grado 1= debole intensità, focale coinvolgimento dei capillari; grado 2= moderata intensità e coinvolgimento multifocale dei capillari e grado 3= colorazione intensa e coinvolgimento diffuso dei capillari. Si considera positivo grado 2 e grado 3. E' stata inoltre eseguita l'immunoistochimica per C3d su materiale paraffinato applicando lo stesso schema di valutazione del C4d.

**Valutazione dei criteri diagnostici morfologici di rigetto anticorpo mediato.** Delle 1452 BEM valutate, 87 erano positive per il C4d (6% 87/1452) in 61 pazienti, 26 BEM di 13 pazienti hanno mostrato un C4d positivo in più di un biopsia. Sessantasei pazienti negativi per il C4d sono stati selezionati e confrontati secondo i seguenti criteri: diagnosi pre-trapianto, età dopo il trapianto, grading del rigetto cellulo-mediato (studio caso-controllo). Tra i 61 pazienti positivi per il C4d 9 avevano rigetto anticorpo-mediato sintomatico, In questo studio caso-controllo abbiamo studiato anche il C3d. Due Patologi hanno rivisto "blindly" tutti i criteri istologici associati al rigetto umorale: edema endoteliale, edema interstiziale, cellule mononucleari intracapillari, neutrofili intracapillari, danno miocitario, necrosi miocitaria, emorragia,

microtrombi intravascolari in accordo con le linee guida internazionali "ISHLT working formulation " del 2004.

**Determinazione degli anticorpi anti HLA donatore specifici.** La valutazione degli anticorpi circolanti è stata eseguita su 37 casi, in virtù del fatto che il nostro studio è stata condotto anche retrospetticamente. E' stata utilizzata la tecnica Luminex per testare la reattività degli anticorpi anti HLA tipo IgG con tecnica "bead-based screening assay" da sieri ottenuti pre-trapianto e post-trapianto, quest'ultimo in concomitanza con la positività del C4d nelle BEM. Inoltre in una sottopopolazione di pazienti con rigetto umorale sintomatico gli anticorpi circolanti sono stati quantificati misurando la media di intensità di fluorescenza (MFI).

**Valutazione della riserva coronarica tramite tecnica ecocardiografica non invasiva.** Abbiamo selezionato 19 pazienti in cui al momento della biopsia endomiocardica di monitoraggio post-trapianto è stata eseguita ecocardiograficamente.La Riserva Coronarica valutata prima e dopo l'infusione di adenosina La riserva coronarica (CFR) è stata calcolata con rapporto tra picco di velocità diastolica (iperemia) e picco di velocità diastolica (basale).

**Analisi statistica.** Il confronto tra i vari gruppi C4d è stato calcolato con test Fisher nei caso di variabili categoriali e con test di Kruskal-Wallis per variabili quantitative. E' stato applicato il metodo di Kaplan-Meyer per studiare la sopravvivenza dei vari gruppi positivi per C4d. Test di regressione di Cox per stimare Hazard ratio. Sono state calcolate sensibilità e specificità dei vari parametri morfologici e riportate con intervallo di confidenza del 95%.

**RISULTATI.**

**Profilo clinico-patologico della popolazione adulta.**

Ventidue pazienti del gruppo 1 (61% del totale dei C4d positivi) mostano un rischio di mortalità 18 volte maggiore rispetto al gruppo di controllo (95% CI 1,960 – 160,022).. I sei pazienti del gruppo 2 hanno un rischio di mortalità 61 volte maggiore rispetto al gruppo di controllo (95% CI 3.399-1110.360). Gli 8 pazienti del gruppo 3 hanno un rischio di mortalità 32 volte maggiore rispetto al gruppo di controllo con una $p<0,0001$. Se si considerano nella totalità i pazienti C4d positivi mostrano una riduzione statisticamente significativa della sopravvivenza rispetto al gruppo C4d negativo. Si osserva un aumento del rischio di mortalità sia nei gruppi 1 e 2 (asintomatici) che nel gruppo 3 sintomatico

**Profilo clinico-patologico della popolazione pediatrica.** Sette pazienti (33%) mostrano una positività al C4d nelle BEM di monitoraggio. Di questi 4 presentavano anticorpi anti HLA circolanti; nessuno era positivo nel gruppo di controllo. Un paziente con positività al C4d ha presentato disfunzione del graft (14% dei C4d+) entro i 3 mesi dal trapianto. Un paziente C4d positivo è morto per scompenso cardiaco dopo circa 2 anni dalla comparsa della positività del C4d nelle BEM. Gli altri cinque pazienti con C4d + sono attualmente vivi a rispettivamente 2,3,4,14 anni di follow up. Nel gruppo C4d+ il "rejection score" per rigetto cellulare è maggiore rispetto al gruppo di controllo (2,2 vs 0,2, $p<0,05$).

**Parametri morfologici per la diagnosi di rigetto anticorpo-mediato sintomatico e asintomatico nelle biopsie endomiocardiche di monitoraggio.**

Degli 8 criteri morfologici valutati all'ematosillina ed eosina, solo due (edema endoteliale ed interstiziale) possono essere considerati predittivi di positività capillare al C4d. la sensibilità dell'edema endoteliale è del 78,8% e la sensibilità dell'edema interstiziale è del 77,1%. Accumulo intracapillare di cellule mononucleari hanno una sensibilità del 39,3%

nel predire la positività al C4d. La curva ROC mostra come i due criteri valutati assieme e riconosciuti essere i più importanti associati al danno anticorpo-mediato non aumentano la capacità di predite la presenza di anticorpi circolanti anti HLA.

**Immunoistotchimica per C3d nella diagnostica del rigetto umorale.** Nel gruppo degli asintomatici (52 BEM di 52 pazienti) 31 BEM sono positive anche per C3d (59,6%, 31/54). Nel gruppo dei sintomatici (9 BEM per 9 pazienti) 5 BEM erano positivi al C3d (55% 5/9). La sensibilità del C3d nel predire la presenza di anticorpi anti HLA circolanti è del 42,3% e la specificità del 56%. Combinando C4d e C3d la sensibilità e specificità nel predire la presenza di anticorpi circolanti non aumenta.

**Profilo anticorpale nei pazienti con rigetto anticorpo mediato sitomatico.** Dei 9 pazienti sintomatici della nostra popolazione studiata, 5 sono stati seguiti in follow up sia per C4d sia per anticorpi circolanti. I risultati mostrano che la quantità di anticorpo circolante (MFI) cambia in relazione al trattamento. Inoltre alti livelli di MFI >10000 correlano strettamente con grado 3 del C4d (diffuso coinvolgimento capillare con colorazione intesa), con MFI tra 1000-9000 il C4d ha una intensità ed una distribuzione variabile.; con MFI <1000 C4d è sempre negativo.

**C4d e microvasculopatia.** La riserva coronarica di pazienti con C4d + è ridotta rispetto al gruppo di controllo rappresentato da pazienti con C4d- (1,28±0,48 vs 3,28±1,03 rispettivamente, p=0,0297) suggerendo una relazione tra il rigetto anticorpo mediato e il danno microvascolare. Anche nei pazienti senza coronaropatia del graft i pazienti con C4d+ mostrano una riserva coronarica inferiore rispetto al gruppo di controllo (C4d-) (p=0,0502).

**CONCLUSIONI**

I nostri risultati dimostrano come il C4d sia un indicatore oltre che diagnostico anche prognostico per rigetto umorale e la sua positività è sinonimo di prognosi negativa anche in assenza di sintomi clinici . La valutazione di C4d, anticorpi circolanti e disfunzione del graft ci permette di stratificare il rischio di mortalità. I parametri morfologici da soli non sono sufficientemente sensibili e specifici per la diagnostica del rigetto umorale e dunque non sono adeguati per una valutazione di screening. Alla luce dei nostri risultati raccomandiamo il C4d come test di screening per individuare pazienti asintomatici.

Lo studio degli anticorpi circolanti hanno mostrato che alti livelli di anticorpi correlano con la positività capillare del C4d . Il danno endoteliale e il deposito di complemento determinano rimodellamento vascolare che porta a lungo termine

# 3 AIM OF THE PhD THESIS

Aims of the present PhD thesis were summarized in three major objectives: 1) to study pathophysiology of Antibody Mediated Rejection (AMR) in heart transplant recipients; 2) to assess the diagnostic ability of pathological features of AMR on monitoring post-transplant endomyocardial biopsies (EMBs); 3) to clarify the prognosis of AMR in the early and late post-transplant period. These main end points were further subdivided as follows:

- To Standardize immunostaining protocol technique on formalin fixed- paraffin embedded tissue to study complement activation on graft through the biomarker of activation of alterative pathway, C4d and to assess the feasibility, reproducibility of laboratory protocol and the grading scheme of immunstaining evaluation.
- to assess the diagnostic and prognostic significance of C4d positive capillary staining detected by the immunoperoxidase methodology performed on paraffin embedded tissue sections in adult and pediatric heart transplant recipients.
- to evaluate if morphological parameters detect signs of early subclinical or latent stages of AMR and their correlation with C4d staining in cardiac transplants recipients.
- to evaluate utility of C3d, another biomarker of complement activation, in identifying the different stages of Antibody Mediated Rejection
- to investigate whether the C4d positive staining on intramyocardial capillaries predict the levels of circulating anti HLA antibodies in symptomatic AMR
- to identify the role of AMR in the development of allograft vasculopathy.

# 4 INTRODUCTION

## *4.1 General concepts*

Heart transplant is most important therapeutic strategy for end-stage cardiomyopathies. Pioneer of heart transplant was Christian Barnard in 1967. Up to now more that 90 000 heart transplant have been performed worldwide and registered by International Society of heart and Lung Transplantation. In the last decade between 3600-3850 heart transplant have been registered per every year.

In our center at University of Padua the first heart transplant was performed in 1985 for Dilated Cardiomyopathy. after that more 700 heart transplant have been performed (average of 20 cases per year)

The most frequent cause of heart disease for adult transplant recipients is non ischemic cardiomyopathy and in the literature accounts for 53.3% of the casess compared with49% in our population.. Ischemic cardiomyopathies was the second most frequent cause of transplant with 37.7% recipients (33% in our study population)[1] .

The rejection in solid organ transplantation is clinically classified as hyperacute rejection, acute and chronic rejection. Hyperacute rejection begins within minutes to hours after host vessels are anastomosed to graft vessels and is mediated by preexisting antibodies in the host circulation binding antigens on endothelial cells (IgM and/or IgG against foreign HLA). Acute rejection compromise vascular and parenchymal injury mediated by T cells and antibodies that usually starts after the first week after transplantation. Chronic rejection is characterized by fibrosis and vascular abnormalities with loss of graft function occurring during long period after transplantation.

For the first time Caves et al. (1975)[2] proposed the use of endomyocardial biopsy to monitor rejection in cardiac transplant recipients. Historically, acute cellular rejection has been the most common cause of allograft dysfunction. In the early days after cardiac transplantation, more than 50% of patients had at least one significant episode of cellular rejection within the first year after transplantation [3]. The use of endomiocardial biopsy to detect cardiac rejection became of paramount importance to identify cellular rejection and apply most appropriate individual immunosupressive therapy. The multicenter study of Milles et al in the 1997 recognized that patients with hemodynamic failure and endomiocardial biopsies with low grades ISHLT biopsy score had worse outcome compared to negative patients (biopsy negative-rejection)[4]. Interestingly, throughout the years, 10 to 20% of patients have episodes of hemodynamic compromise with no evidence of cellular rejection in their endomyocardial biopsies. In such episodes often designated as noncellular rejection there is considerable evidence that the humoral arm of the immune system is responsible for the cardiac allograft dysfunction and injury.

Hammond et al.[5] were the first to recognize the importance of pure antibody-mediated cardiac allograft rejection. With a histological and immunofluorescence studies the Utah group was able to demonstrate vascular damage, in term of endothelial swelling with prominent endothelial nuclei, associated with capillary deposition of complement fragments and haemodynamic dysfunction. These episodes may be clinically even more severe than cellular rejection episodes[6] Humoral rejection was first recognized in kidney transplantation as a distinct clinico-pathologic entity characterized by acute allograft rejection associated with the production of antidonor reactive antibodies and poor prognosis[7]. This is poor response to traditional immunosopression.

Halloran et al[8] showed in pre-sensitized patients with renal dysfunction, circulating cytotoxic anti-donor human leukocyte antigen (HLA) class I antibodies and "atypical acute rejection" that typically occured a few weeks after transplantation . Another subsequent study demonstrated that 20-40% of patients with acute rejection have circulating anti HLA and they showed poor outcome[9, 10].. Tropkov K et al studied the relationship between histological findings and presence of anti-HLA antibodies and found that they were associated with neutrophils infiltration of the peritubular capillaries and fibrinoid necrosis of vessels However these changes were present only in a minority of cases. Unfortunately, immunofluorescence for IgG, IgM, C3, or fibrin is not as helpful as one might expect, revealing no statistically significant difference between AMR and ACR[11] . Identification of AMR in biopsy specimens has been problematic, because there are no morphologic feature described so far that carry either pathognomonic significance.

For the first time the group of Colvin RB evaluated renal biopsy from patients with kidney function deterioration and absence of cellular rejection and found that the C4d was an important diagnostic biomarker for Antibody mediated rejection and was strongly correlated with presence of circulating anti-HLA antibodies.[12].

An important problem that emerged is terminology describing this clinical entity.

Different terminology such as *vascular rejection, microvascular rejection,* and *humoral rejection are referring to the same entity* and the preferred terminology in the ISHLT-WF 2004 was Antibody mediated Rejection (*AMR)*[13, 14].

In the heart experience the 1990 working formulation of ISHLT well defined histological criteria for acute cellular rejection in six distinguished grades but humoral rejection was an option to record "…positive immunoflourescence, vasculitis or severe edema in absence of cellular infiltrate, recorded as additional required information." [15]. Only in

the working formulation of 2004 the diagnostic criteria of AMR were defined as present or absent , recognizing grade 0 without histological and immunopathological signs of humoral rejection and grade 1 in the setting of both histological and immunopathological signs of humoral rejection [13].

However it remained controversial with different reported incidence between different centers.

The Registry of The International Society of Heart and Lung Transplantation recently published, showed that post-transplant survival of adults is improving over the years[1] especially in the first year after transplantation On the contrary the mortality rate after one year since transplantation has improved only marginally for patients transplanted after 1992 without important improvement in the last two years[1]

## *4.2 Acute cellular rejection*

Endomyocardial biopsy after heart transplantation plays an important role in monitoring cardiac allograft rejection.in the first year after transplantation The method was introducted at Standford University in 1972 and remains an important tool to prevent early and late cardiac allograft rejection. The cardiac biopsy grading system was first standardized by ISHLT in 1990 and later revised in 2004 to introduce an easier to be applied internationally, more reproducible grading system The introduction of cyclosporine into clinical practice ushered in the modern era of transplantation modifying morbidity and mortality for Acute cellular rejection. Nevertheless, cyclosporine was not a panacea for transplantation. Drug levels needed for optimal immunosuppression cause

kidney damage. More immunosuppressive agents were discovered and introduced in clinical practice further improving acute cellular rejection control.

| 2004 | | 1990 | |
|---|---|---|---|
| Grade 0 R[a] | No rejection | Grade 0 | No rejection |
| Grade 1 R, mild | Interstitial and/or perivascular infiltrate with up to 1 focus of myocyte damage | Grade 1, mild A—Focal | Focal perivascular and/or interstitial infiltrate without myocyte damage |
| | | B—Diffuse | Diffuse infiltrate without myocyte damage |
| | | Grade 2 moderate (focal) | One focus of infiltrate with associated myocyte damage |
| Grade 2 R, moderate | Two or more foci of infiltrate with associated myocyte damage | Grade 3, moderate A—Focal | Multifocal infiltrate with myocyte damage |
| Grade 3 R, severe | Diffuse infiltrate with multifocal myocyte damage ± edema, ± hemorrhage ± vasculitis | B—Diffuse | Diffuse infiltrate with myocyte damage |
| | | Grade 4, severe | Diffuse, polymorphous infiltrate with extensive myocyte damage ± edema, ± hemorrhage + vasculitis |

[a]Where "R" denotes revised grade to avoid confusion with 1990 scheme.

From Stuart S J Heart and Lung Transplant 2005[13]

## *4.3 Cardiac Allograft Vasculopthy (Chronic Rejection)*

At the moment the most challenging problem in obtaining the long term successful outcome in cardiac transplantation is the development of Cardiac Allograft Vasculopathy (CAV), also known as graft coronary artery disease, accelerated graft arteriosclerosis and chronic rejection. This is a complex phenomenon worsened by the fact that older donor hearts are used more frequently, with the possibility for preexisting atherosclerotic lesions could be transported as "passive passengers" during the transplant surgery. Nonetheless, the chronic immunologic assault directed against the coronary endothelium, more generally to the vascular wall, , in conjunction with nonimmunologic factors, such as lipid perturbation, hypertension, viral infection, and possibly donor/ recipient genetic predisposition to the development of atherosclerosis, leads to coronary flow perturbation, particularly when preexisting atherosclerosis is present[16-21]. The significance

of cardiac allograft vasculopathy was first emphasized by the Stanford Team when they reported coronary intimal proliferation and obliterative lesions in the epicardial coronary arteries of long term canine and human heart transplant recipients [22, 23]

Indeed, allograft vascular disease in humans after heart transplantation was first reported in a short-term transplant survivor from Christiaan Barnard's Capetown experience. Thompson[23] detailed the autoptic findings of the longest surviving after heart transplant :coronary arteries had an unusual atheromatous appearance, with symmetrical, diffuse involvement of all vessels and in particular, small, more distal intramyocardial vessels. The first important serie has been describe by Costanzo and colleagues in 1998[24], who evaluated 5963 postoperative angiograms performed in 2609 heart transplant recipients from 39 institutions transplanted between January 1990 and December 1994. Angiographic diagnosis of allograft arteriopathy was classified as mild, moderate, or severe on the basis of degree of coronary involvement, primary-vessel stenosis, or branch-vessel stenosis. After 5 years of follow-up, coronary artery disease was present in 42% of the patients (mild in 27%, moderate in 8%, and severe in 7%)[24]. As we all know cardiac denervation at the time of heart transplantation usually prevents transplanted patients from experiencing angina, which is an important warning sign for ischemic heart disease. Because of this lack of early clinical symptoms, transplant patients with CAV typically have a late presentation of CAD with silent myocardial infarction, loss of allograft function or sudden death [25]. CAV shows no initial decrease in luminal diameter due to concentric vascular remodeling [26]. Only when the process is more advanced does the lumen undergoes narrowing and angiographic detection become possible.

Thus, angiography, although it is a good screening tool for CAD, often underestimates CAV, and in some patients with evenly distributed disease throughout the coronary tree,

CAV can be missed altogether [27]. Despite the poor sensitivity of angiography, it is widely used as screening tool to detect coronary disease.

Johnson et al[28] developed a classification system based on the different morphologies to help CAV to aid in its diagnosis using angiography. Briefly, type A lesions appear as discrete proximal tubular stenosis, type B as diffuse concentric middle or distal stenosis, with type B1 having an abrupt narrowing and type B2 having a smooth concentric tapering. Finally a type C angiographic appearance indicates irregular vessels with distal lesions and loss of small branches.

Another and more sensitive tool is intravascular ultrasonography (IVUS). IVUS is useful for detecting the extent of intimal thickening by imaging vessel wall structure rather than simply luminal diameter. Unfortunately, it is physically restricted to the larger epicardial arteries, and thus cannot be used to screen for CAV throughout the entire heart. One year after transplantation, IVUS detects CAV in 50% of patients whereas angiography detects disease in only 10%–20% of patients[26,27] .

From a pathological point of view the histolgical features of CAV and CAD are significantly different CAD is usually a focal, eccentric intimal proliferation and involves the proximal coronary vessels. Of importance in CAD is the deposition of calcium and disruption of elastic lamina. The initial lesion is represented by fatty streaks.

CAV is typically characterized as a diffuse concentric proliferation of the intima. Intramyocardial vessels are usually involved, and the process can even involve the coronary veins accounting for the name “vasculopathy”. The initial lesions are represented by smooth-muscle cells proliferation of the intima (see table 1)[22].

Table 1: Comparison between cardiac allograft vasculopathy and coronary artery disease

| Characteristic | Cardiac allograft vasculopathy | Coronary artery disease |
|---|---|---|
| **Vessel involvement** | All vessel types within the allograft. Mostly intramyocardial vessels | Proximal coronary vessels |
| **Plaque pattern** | Diffuse and concentric | Focal and eccentric |
| **inflammation** | Yes | Rarely |
| **Internal elastic lamina** | Intact | Disrupted |
| **Calcium deposition** | no | yes |

The pathophysiology of CAV although not completely understood, likely involve both of immunological and non immunological response for endothelial damage. There is substantial evidence that immunologic factors, including histocompatibility mismatch, acute rejection episodes and chronic inflammation, play a major role in CAV development. Nonimmunologic factors include cause of donor brain death, cytomegalovirus (CMV) infection, age, sex, obesity, dyslipidemia, hyperhomocysteinemia (HHcy), diabetes mellitus, hypertension, smoking and ischemia–reperfusion injury.[29]

Recently, it has been documented that statins through their pleiotropic and anti-inflammatory effects may improve vascular function. In 1995, Kobashigawa and associates84 showed that treatment with pravastatin (40 mg/d) for 1 year, lowered mean LDL and triglyceride levels, raised HDL levels and reduced intimal thickening and cardiac rejection accompanied by hemodynamic improvement ($p = 0.002$). In this trial, patients treated with pravastatin had a lower incidence of CAV and improved survival ($p = 0.025$)[30]..

By definition CAV involves the entire coronary tree , affecting not only the epicardial but also the distal intramyocardial segments. Microvascular tree is composed of three categories in terms of functional and anatomical characteristics: large arteries (diameter >500 micron), small arteries (diameter between 100 and 500 micron), and arterioles (diameter<100 micron). The major part of the coronary vessel resistance is assigned to arterioles and small arteries, which are referred to as resistance vessels [31] .

To evaluate the function of intramyocardial vessels and their remodeling after heart transplantation , coronary flow reserve (CFR) has been recently proposed as reliable diagnostic tool by our group and clinicopathological correlations on monitoring endomyocardial biopsy have been performed. The coronary flow reserve is a functional parameter which measures the rate between peak diastolic velocity after infusion of adenosine and peak basal diastolic velocity.

Echocardiography is performed for CFR evaluation using CE-TTE before and after adenosine infusion, with an ultrasound system. CFR is measured in the distal portion of the LAD, first obtaining a modified foreshortened two chamber view or, if a distal LAD flow recording is not feasible, using a low parasternal short-axis view of the base of the heart and performing. Adenosine infusion occurred at a rate of 0.14 mg/kg/min for 5 min. CFR in the LAD is calculated, as the ratio of hyperemic to basal diastolic flow velocity.[32-37].

Histological evaluation was performed on endomyocardial biopsy and evaluated for tangential cuts. In the literature there are different proposed diametes for what can be regarded as microvessels (Table 2) . An artery is considered normal if the intima comprised a single layer of flat endothelial cells, the internal elastic lamina was intact, the media showed no fibrosis, and the adventitia was not thickened

[38, 39] and when thickening of tunica media or proliferation of intima layer occur microvasculopathy is assigned. Heimann et al elaborated a diagnostic schedule of micorvasculopathy in posttransplant biopsies based on four grades, A and B without stenosis , C and D progressive lumen stenosis[39]

Table 2. Definition of microvessels and microvasculopathy.

| **Author** | **Microvasculature evaluation (diameter μm)** | **Microvasculapathy definition** |
|---|---|---|
| **Drakos SG et al JACC 2010**[40] | < 300 | Microvascular density (number of microvessels/tot tissue analysis area) |
| **Escaned J et al Circulation 2009**[41] | <100 | Arteriolar density, capillary density and arteriola obliteration AOI [1-(LA/TAA)]x100 |
| **Heimann et al Circulation 2007**[39] | 10-20 | Luminal radius/medial thickness <1 |

LA: luminal area
TAA: total arteriolar area

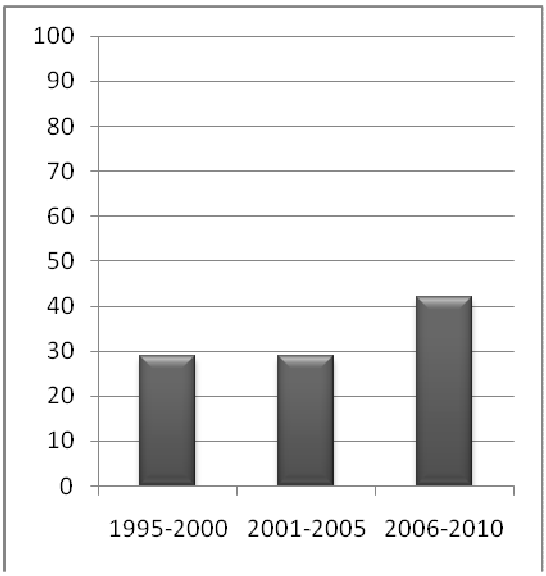
Our experience showed the relation between rejection score and reduction of CFR in absence of CAV. Although micovascular damage in early period post-transplant without epicardial vessels damage was ascribed to immunomediated mechanism. This results[37] were in keeping with that reported by Heimann[39].

Significant microvasculopathy after heart transplant affects > 40% of cardiac transplant recipients within the first post-transplant year and it was associated with both poor overall survival and reduced freedom from fatal cardiac events , independently of epicardial transplant Vasculopathy[39]. Early graft vascular lesions seemed to be confined mostly to small coronary arteries , supporting the hypothesis that microvasculopathy is an immune-mediated phenomenon, similar to epicardial CAV, often involving microvessel early and before involvement of major epicardial segments, and influencing outcomes[42].

## *4.4 Antibody Mediated Rejection*

in the last ten years, the antibody mediated rejection in renal and heart transplantation represented the most important research topic reported in the literature . If we considered all the heart transplant papers in the last 5 years AMR accounted for about 40% of all the reported studies (see figure 1)

Figure 1. Heart AMR Papers in the literature




More recently since we were able to obtain a better control of cellular rejection with a decreasing rate of positive ACR requiring drug therapy on EMBs without reducing concomitantly graft loss for coronary graft vasculopathy drove the attention back to humoral rejection and to our ability to detect it through immunohistopathological markers.

Clinically we recognize acute and chronic form of antibody rejection and both starts with binding of alloantibodies to endothelial cells of blood vessels in the graft.

### 4.4.1 Acute antibody mediated rejection

Acute antibody mediated rejection is classified according to the concomitant presence of four criteria[43] (table 3)

Table 3. Diagnostic criteria for Acute Antibody Mediated Rejection

| |
|---|
| Clinical evidence of acute graft dysfunction |
| Histological evidence of acute tissue injury:<br>that is, neutrophils, macrophages or thrombi in capillaries necrosis |
| Immunopathological evidence for the action of antibodies:<br>that is, complement component 4d (C4d) on intramyocardial vessels |
| Serological evidence of HLA-specific antibodies or other donor-specific antibodiesat the time of biopsy |

Acute antibody mediated rejection has been observed in conjunction with all of the immunosuppressive therapies that are currently used to promote graft acceptance, and it has a poorer prognosis than pure T-cell-mediated acute rejection[44] . The pathological features of hyperacute rejection and acute rejection are similar, includes aggregation of macrophages or neutrophils on capillaries, presence of microthrombi and fibrin on the vessels associated with parenchimal or myocardial injury. However some biopsies are negative for histological characteristics in the presence of C4d positivity. The first organ that recognized acute antibody mediated rejection was kidney but other organs showed the presence of that rejection in particular in heart. The work in hear transplantation has been widely facilitated by all the data and knowledge obtained in kidney which is far in advance in the study of the humoral rejection in renal biopsies. The first important step of clinical and pathological definition of antibody mediated rejection was the National Conference that was held at the National Institute of Health in April 2003 to assess knowledge regarding humoral rejection in solid organ transplantation [45].

The incidence of acute antibody mediated rejection in kidney transplantation occurs in 6.7% of kidney-transplant recipients [45, 46] and is present in 32% of renal biopsies from

patients who have been diagnosed with acute rejection[47-49]. In the last decade late graft losses due to antibody and pathologic features of chronic antibody mediated rejection related to C4d and donor specific antibodies (DSA) were recognized [50-52] (BANFF 2010).

Several studies on heart transplantation document the occurrence of AMR. It was at the end of eighties that Elizabeth Hammond first published her fundamental work on Humoral rejection in Heart transplantation identifying patients prospectively examined by routine immunofluorescence on endomyocardial biopsies in whom accumulation of immunoglobulin and complement in the microvasculature could be detected. Subsequently many works documented the presence of AMR within three months since transplantation[5, 53, 54]. The term of vascular rejection was at that time introduced since the vascular endothelium was recognized as the target of activation and damage in the absence of acute cellular rejection. Suspected prevalence of pure AMR in heart transplantation ranges between 7-18%, 68% of these presented early after transplantation and only 13% presented symptoms in the long term. Moreover 23% of cases with ACR had also AMR ( first evidence of mixed rejection).[55] Also in heart transplantation the histological features as intracapillary accumulation of macrophages or neutrophils, in the presence of endothelial swelling or denudation and recognized microthromi, myocardial necrosis and presence of fibrin when the process was ongoing or severe were recognized.

### 4.4.2 Chronic Antibody Mediated Rejection

In the kidney, several works support the hypothesis that a subset of cases of chronic rejection might be mediated by alloantibodies. Circulating HLA-specific antibodies are common in patients with long-term organ allografts. In a large multicentre trial, HLA-

specific antibodies were detected in 21% of patients with renal allografts and 14–23% of patients with heart, liver or lung allografts [56] . Of 2,278 renal-allograft recipients who were followed prospectively, graft failure at 1 year occurred more frequently in patients who developed alloantibodies than in those who did not (8.6% versus 3.0%). Regele H et al[51] showed for the first time that the presence of C4d in the peritubular capillaries of renal allografts precedes the development of chronic allograft glomerulopathy. It also showed that C4d deposition correlates with lamination of the peritubular capillary basement membrane. Several studies have reported that de novo antibodies that are specific for graft class I and class II HLA molecules are a risk factor for premature graft loss as a consequence of renal and cardiac chronic arteriopathy [57, 58].

In the heart transplant some works showed the direct relation of C4d deposition on intramyocardial capillaries with circulating anti-HLA specific antibodies.[59] Also our experience confirmed the onset of AMR many months or years after transplantation. The antibody-mediated rejection has been shown to be associated with a significantly worse survival and to predispose patients to coronary vasculopathy. Poelz et al[60] showed that complement deposition of C4d fragment on endomyiocardial biopsies obtained from pts during the first year after heart transplantation was associated with development of CAV.. Antibody-mediated rejection occuring later after transplantation (months to years)was symptomatic less frequently (13%) than in the early phase. [61].

These results are in keeping with those obtained in renal transplantation[62, 63]. According to these different experiences in different organs it has been proposed putative stages of AMR (see table 4) [45]

Table.4: Putative stages of humoral response to an organ graft

| |
|---|
| I: Latent humoral response<br>Circulating antibody[1] alone (but without biopsy findings or graft dysfunction) |
| II: Silent humoral reaction (accommodation vs. prerejection state)<br>Circulating antibody1 + C4d deposition (but without histologic changes or graft dysfunction) |
| III: Subclinical humoral rejection[2] Circulating antibody1 + C4d deposition + tissue pathology<br>(but without graft dysfunction) |
| IV: Humoral rejection<br>Circulating antibody1 + C4d deposition + tissue pathology + graft dysfunction |

[1]Circulating antibody to HLA or other antigens expressed on donor endothelial cells.
[2]May differ among organs, as the ability to detect particularly mild degrees of graft dysfunction varies among organs.

According to this model, the first stage for antibody mediated response was the development of de novo circulating anti HLA antibodies (stage 1, latent AMR ). In many circumstances and for unknown reasons, the antibodies do not always elicit AMR. In the next stage (stage II silent AMR) the circulating antibodies activated the complement cascade on the endothelium of the graft, with C4d deposition on the capillaries. At these stage there is no evidence of pathological and clinical features. In the stage III (subclinical AMR) there are circulating antibodies that activate complement in the graft with C4d deposition on capillaries and develope tissue injury with evident histological features on the surveillance biopsies, but without clinical symptoms.

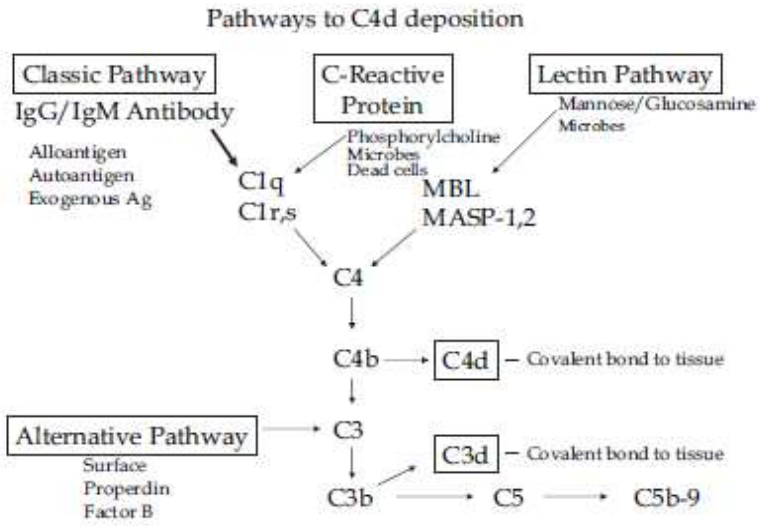
Finally, in stage IV (humoral rejection) in addition to C4d deposition and pathological features, clinical symptoms of graft dysfunction occurs, which are in kidney proteinuria and increase BUN , in the heart Ejection Fraction (EF) reduction and ECG abnormalities..

The new emerging concept of Accomodation was considered to explain the early stages of asymptomatic AMR illustrated previously. This phenomenon was first described in AB0-incompatible kidney allograft [64] In HLA-mismatched grafts, alloantibodies can be found in the absence of clinical graft dysfunction thereby fitting the definition of accommodation. Patients with circulating HLA-specific antibodies have a greater likelihood of subsequent graft loss, indicating that, if accommodation occurs, then this is either transient or insufficient to prevent further antibody mediate rejection[41].

### 4.4.3 AMR: Mechanisms of antibody mediated rejection

Complement has been rediscovered as a potent mediator and diagnostic indicator of inflammation and rejection in organ transplants and it is one of the major effector mechanism of humoral immunity. It recognizes three major mechanism of activation: classical pathway, which is activated by antibodies; alternative pathway, which is activated through microbial cell surface without presence of antibodies; and the lectin pathway , which is activate by plasma lectin binding to mannose residues on microbes.

Figure 1. Complement activation scheme.




Activation of complement via the classical pathway is initiated by C1. C1 is a protein complex, including C1q which can bind to several targets of interest in transplantation. These include antibodies IgM and certain subclasses of IgG, apoptotic blebs, and C-reactive protein[65-67]. When C1q interacts with one of those targets, the protease function of C1r/C1s is activated. C1s cleaves C4 yielding two fragments C4a and C4b. C4a is a soluble mediator.

C4b undergoes a structural change, following proteolytic cleavage of C4b, the split product C4d remains covalently bound to target structures for the presence of thioester covalent bound, whereas larger fragments such as C4c (C3c and probably also the terminal complex C5b-9), go into solution. C4d is the stable (target-bound) remnant of

classical complement activation and can reveal humoral attacks against endothelial cells that escape detection by other methods.C4 can also be activated by mannose binding lectin which is structurally similar to C1q.

C4b continues the complement cascade by binding to C2, which is then cleaved by C1 s or MASP to generate C2b that diffuses away and C2a that remains associated with C4b. C4b and C2a together form the classical pathway C3 convertase. C3 also can be directly activated by the alternative pathway of complement. When regulation is surmounted and C3b is deposited together with components of either the classical or alternative C3 convertase, it forms a complex, the C5 convertase, that is capable of cleaving C5. It is at this point that the complement cascade intensifies its proinflammatory effects. The larger fragment, C5b, initiates assembly of the terminal components of complement (C5b-C9) into a pore-forming structure, the membrane attack complex (MAC). MAC is capable of causing lysis of cells.

To avoid injury to autologous tissues, complement is regulated at multiple levels. Most proximally, the enzymatic subcomponents of C1 can be bound by C1 inhibitor and removed from C1q. More extensive array of regulators has evolved to control the more prevalent C4 and C3. Fragments of the structurally related C4 and C3 molecules are controlled by a group of six regulators of complement activation : membrane cofactor protein (MCP; CD46), CR1 (CD35) and CR2 (CD21), decay-accelerating factor (DAF), C4 binding protein (C4 bp), and factor H. These regulators work by different mechanisms. DAF functions by dissociation of the classical and alternative C3 convertases. Other regulators (MCP, CR1, C4 bp and factor H) function as cofactors for Factor I allowing it to cleave C4b and C3b into biologically inactive fragments.[68]

### 4.4.4 AMR: C4d as a diagnostic biomarker

***Kidney experience***

Immunofluorescence studies of complement have been most valuable in advancing the diagnosis and understanding of hyperacute rejection [69,70, 71]. Localization of immunoglobulin and complement in samples of hyperacutely rejected renal allografts led to the concept that hyperacute rejection is caused by high titers of donor- specific antibodies activating complement. In contrast, acute rejection is usually not accompanied by readily detectable amounts of immunoglobulin or of most complement components. The primary purpose for performing immunofluorescence studies on clinical biopsy specimens is to assist diagnostic and treatment decisions. Patients with circulating antibody to donor HLA antigens often have biopsy samples that exhibit neutrophil infiltration and microvascular injury, but have no antibody deposition detectable by immunofluorescence [8, 10, 11]

Feucht and coworkers[72 73, 74] introduced the idea that staining for C4d may be a useful marker for acute renal allograft injury. As a marker, C4d has the advantages of sensitivity and specificity over C1q or C3, that were previously the most commonly used markers of complement deposition. The use of monoclonal or polyclonal immunofluorecesce or immoperoxidase antibodies specific for C4d accomplishes two goals: first, it increased the specificity by eliminating background staining of inactivated C4. Second, it increases the sensitivity because the C4d is covalently bound to tissues and has a longer half life on tissues than C1q, IgM or IgG. [75].

The prevalence of capillary C4d on renal graft biopsies depends on the clinical situation of the recipients. In the setting of 'delayed graft function', especially when pre-sensitized recipients were involved, about 50% of biopsies showed either diffuse or focal staining of interstitial, peritubular capillaries [73]. In grafts with acute rejection, 30% of biopsies were found to be positive[47, 48]. In grafts with a strict definition of chronic rejection, 60% of biopsies showed capillary C4d.

In the report of BAFF 2007 Conference of renal allograft pathology, the grading of C4d was recognized as important biomarker for Antibody Mediated Rejection in renal allograft transplant[76].

Figure 2. Scoring of C4d staining on renal biopsies

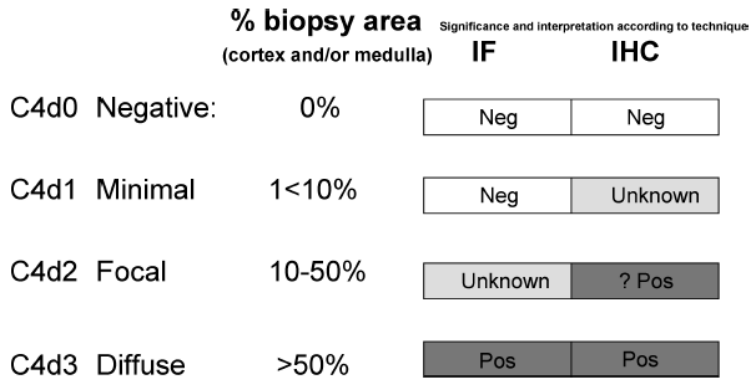
**Table 2:** Scoring of C4d staining (% of biopsy or 5 high-power fields)

| | | |
|---|---|---|
| C4d0: | Negative: | 0% |
| C4d1: | Minimal C4d stain/detection: | 1<10% |
| C4d2: | Focal C4d stain/positive: | 10–50% |
| C4d3: | Diffuse C4d stain/positive: | >50% |

In renal transplantation the scoring system for C4d staining was well defined (see figure 2)[74]. In the update of renal allograft pathology scoring system the relation of C4d by immunofluorescence technique and immunoperoxidase technique has been defined. Immunohistochemistry (IHC) on paraffin section is usually less sensitive than immunofluorescence in cryopreserve tissue (i.e. diffuse staining on IF can be seen as focal on IHC). Diffuse positive C4d by IF or IHC is highly correlated with circulating antidonor antibody. Focal positive C4d by IHC is possibly equivalent to diffuse positive IF,

and should be retested on IF, if possible. However, for focal positive C4d by IF and for minimal C4d by IHC, the clinical significance is unknown. (See figure 3)[74].

Figure 3. Significance and interpretation of C4d staining according to technique.




***Heart experience***

It was at the end of eighties that Elizabet Hammond first published her fundamental work on Humoral Rejection identifying pts prospectively examined by routine immunofluorescence of EMBs in whom accumulation of immunoglobulin and complement in the microvasculature could be detected [5]. The term of vascular rejection was at that time introduced since the vascular endothelium was recognized as the target of activation and damage in the absence of acute cellular rejection. It was recognized that also a mixture of acute cellular rejection and Vascular rejection could coexists in some pts. The outcome of pts with vascular rejection was reported to be significantly worse than that of pts with cellular rejection or those with the mixture type.[77]

Immunofluorescence was proposed as the technique to be applied on a routine base on all EMBs for the first month after transplant and a panel of antibodies for complement

activation and immunoglobulins suggested (IgG, IgM, Complement C3 and C1q, fibrinogen, albumin, HLA-DR)[5, 77-79].

Other reported completely different results suggesting that IF positivity could not be considered as a diagnostic sign of graft dysfunction in the absence of cellular rejection, or be used to direct treatment appropriately, or even carry a prognostic value. Technical aspects for IF had been raised as well [80]. Because of difficulties in collecting frozen samples and applying immunofluorescence in a standardized and reproducible manner by many laboratories all round the world the attention of the international transplant community was mainly focused on cellular rejection which could be assessed on Haematoxylin Eosin staining on paraffin samples and antibody mediated rejection discharged as irrelevant. The efforts were mainly devoted to grading cellular rejection and reaching a consensus on standardization of ACR nomenclature[13, 15]. More recently the fact that we were able to obtain a better control of the cellular rejection with a decreasing rate of positive ACR EMBs without reducing concomitantly graft loss for coronary graft vasculopathy drove the attention back to humoral rejection and to our ability to detect it through immunohistopathological markers.

The work in heart transplant pts has been widely facilitated by all the data and knowledge obtained from the kidney community which is far in advance in the study of the humoral rejection in renal biopsies. They have addressed this issue since many years and translated their data into the Banff Classification that is worldwide accepted and applied. Mainly from their experience came the proposed sequence of stages for antibody-mediated rejection with four grades [43] recognizing that rejection after solid organ transplantation is a continuum phenomenon in humoral as it is in the cellular rejection. As such we have recognized progressive stages of antibodies-mediated rejection from

accommodation phases or asymptomatic ones to rejection, symptomatic phases and graft loss.

Antibodies that are specific for graft antigens might be produced at any time after engraftment from days to months or years and be responsible for fixation of complement resulting in tissue injury and coagulation.

The ISHLT 2004 revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection [13] proposed as diagnostic criteria for AMR, **an integrated multidisciplinary approach** : 1) evidence of histologic features for AMR (myocardial capillary injury with endothelial swelling and intravascular macrophage accumulation with possible interstitial edema and presence of neutrophils) and 2) positive immunofluorescence or positive immunoperoxidase staining (positive CD68, C4d) for AMR. 3) circulating antidonor antibody 4) graft dysfunction . The consensus meeting recommended that screening should have not be advocated at that time, but every endomyocardial biopsy should have been assessed for critical histologic evaluation for features suggestive of antibody-mediated rejection.

Pathologically, antibody –mediated rejection can be recognized by myocardial capillary injury with endothelial-cell swelling and intravascular macrophage accumulation. Interstitial edema and hemorrhage can be present together with neutrophils in and around capillaries. Intravascular thrombi and myocyte necrosis without cellular infiltration can also be identified. When these features are seen in the presence of unexplained cardiac dysfunction, typically early onset of hemodynamic compromise and myocardial dysfunction, it was proposed that immunostaining could be performed by immunofluorescence or immunohistochemistry as follows:

a)Immunoglobulin (IgG, IgM and/or IgA) plus complement deposition (C3d, C4d and/or C1q) in capillaries by immunofluorescence on frozen sections; and/or b) CD68 staining of macrophages within capillaries (CD31- or CD34-positive) by immunohistochemistry ; and c) C4 d staining of capillaries by paraffin immunohistochemistry. It was only recommended at that time that patients with hemodynamic compromise of unexplained origin should undergo assessment for circulating antibodies.

Currently there are no standardized , reliable histopathologic markers to detect allograft antibody deposition in cardiac transplants which could identify and follow-up patients at risk for humoral rejection. To detect histologically antibody deposition in cardiac allografts, investigators have used indirect immunofluorescence of tissue immunoglobulins (IgM and IgG) and complement (C3, C1q). This methodology seems to show a poor correlation between fluorescence and the actual presence of anti-donor–specific antibodies and graft dysfunction, especially as fluorescence is commonly observed in biopsies from patients without antibodies.

Dramatic improvement in our ability to diagnose humoral rejections in solid organ transplants has been provided by **C4d staining.**

Review of the literature has revealed heterogeneity of diagnostic criteria, significance and overall frequency and detection methodologies: pts populations differ in demographics, immunologic characteristics of donor and recipient, therapies; diagnostic criteria of inclusion differ significantly; the definition of clinical allograft dysfunction is not standardized and can be based on echo, need for inotropic support , cardiac catheterization.

Skepticism on the morphological criteria identified at light microscopy is produced by data from the literature.

1) **Heterogeneity of inclusion criteria** of different studies: Some papers select pts excluding those with ACR. Other excluded only the >3A(2R). This would exclude from possible evaluation of relevant subgroup of pts with the mixed form. Kfoury reported on 801 stable pts classified according to the presence of various pattern of rejection in their data base , with ACR490/801 pts (61%), 190mixed /801 (24%) and 118/801 AMR (15%), excluding all the symptomatic pts [54].

   Recently with introduction of C4d staining Fedson showed that there was no correlation between cellular rejection and any pattern or location of C4d staining [81]. One third of the pts showed mixed form of rejection also in a recent paper from Wu [82].

**2) Correlation between C4d and DSA. Is C4d a marker of alloantibodies deposition on tissue?**

First paper from Rex Smith presented 85 EMBs from 38 pts reaching the conclusions that C4d+ has a good correlation with the presence of circulating alloantibodies. Evaluation on 9 pts out of the 38( 23%) was carried out since they had positive DSA and tissue samples with 25 EMBs assessed. 21/25(84%) had EMBs C4d positivity and DSA positivity, 4/25(16%) DSA positive were C4d-. But 2 become C4d positive in following EMBs. 53/60 were DSA - and C4d – as well(88%) which means per converse that 7/60 (12%)had C4d positivity in the absence of detectable DSA. Arterioles were positive both in the DSA + and DSA- negative thus suggesting not to evaluate the arteriolar positivity at difference with

Chantranuwat paper which showed a correlation with capillary staining and arterioles and venules. In the absence of DSA, capillaries are unlikely to stain diffusely for C4d in HTEMBs with diagnosis of post-transplant ischemic injury. Only 2EMB were DSA positive and C4d neg. C4d be positive in only 1 case with DSA-. **C4d+ capillary staining and ischemic injury** was assessed on 33 cases 5/7 were DSA+ and C4d+ while 25/26 DSA- showed that also capillary C4d was negative (1/26 DSA- was C4d positive.) [59].

The second one is from Gupta who reported the evaluation of DSA routinely at the time of clinical rejection(not better specified). C4d was evaluated on 1frozen fragment to correlate C4d staining with ACR , retrospective DSA crossmatched and detection of post-Tx DSA. C4d was reported as positive or negative without specification of the grading system. 14C4d positive pts, had more commonly retrospective positive crossmatch for DSA (39% versus only 2% in the 53 negative for C4d; and detection of postTx DSA in 13/67 pts studied ( in 12/14 C4d+ (92%) and only 1/53 for C4d- (2%)). The authors suggested that pts with ACR who also had a positive C4d staining should be assessed for the presence of DSA [83].

Cleveland is the only center on which IF was performed on 4 fragments. A large cohort of pts were studied 330 w+ith 1511 EMBs . In 38pts with C4d positive staining, DSA was also evaluated. DSA was detected in 95% (18/19) of those with a double positivity C4d+/C3d+ versus only 35%(6/17 ) of C4d+/ C3d -, ( 24/34pts assessed ).C4d/C3d positivity showed a better correlation with DSA than C4d alone and with Graft dysfunction detected present in 84%(16/19)C4d +/C3d + and only in 5%(1/17) with C4d+ alone [84].

Cano assessed the relation of C4d deposition in Quilty Effects in 17 EMBs from 11 pts (128 EMBs from 42pts). Eleven EMBs(65%) from 8 pts showed C4d deposition in the endocardium in a linear pattern. In 7/11(64%) of C4d endocardial positivity C4d was

detected also in interstitial capillaries(3diffuse, 4 focal) in 4/11 C4d capillaries was negative suggesting a pathogenic relationship between the QE and complement attivation. QE +C4d deposition could indicate a better adaptation of the graft to the host. [85]

2) **Correlation of IF versus ICH for C4d in four papers. A total of 278 pts and 700 EMBs have been evaluated for this correlation (Table4).**

**Table 4. Correlation between IP and IF**

| Authors | N0. PtS | No. EMBs | IF/ICH | Gold Standard | Sensitivity | Specificity | Concordant rate |
|---|---|---|---|---|---|---|---|
| Chantranuwat 2004[86] | 166 | 315 | IF=1fragment<br>ICH=2/3fragments | IF | 84% | 93% | IF>ICH<br>IF2+/3+=30EMB<br>ICH2+/3+=18EMB |
| Rex Smith 2005[59] | 38 | (85)38 | IF=1fragment<br>ICH= all EMB | IF | 100% | 96% | |
| Fedson 2008[81] | 34(4) | 400(4) | IF=1fragment<br>ICH =all EMB | ICH | 1 positive both at IF and ICH;<br>3 negative both at IF and ICH | | |
| Miller[87] 2010 | 70 | 296 | IF=1fragment not reevaluated<br>ICH=all EMB | IF | 23 IF>ICH; 34 ICH>IF;<br>ICH1+ >IF=23 vs 11<br>ICH2+<IF=3 vs 6<br>ICH3+>IF= 8 vs 6 | | Kappa value<br>0.81 |

The first paper is from Chantranuwat[84] reporting on 315 frozen endomyocardial biopsies with IF staining for C4d, in which 280 were negative and 35 were positive. IF was considered the gold standard. The extent of IP and IF staining was graded as 0 to 3+ as: 0 (negative/rare positivity), 1+ (focal; <1/3 of vessels), 2+ (multifocal; 1/3 to 2/3 of vessels), and 3+ (diffuse; >2/3 of vessels). Staining intensity (1+, weak [barely visible at low power]; 2+, strong [clearly visible at low power]) as well as the location (capillary, venule, or arteriole)and the number of positive vessels from every usable high-power field (HPF) and type of positive vessels were evaluated (table 5) . In **34 biopsies**, IP criteria of C4d

2+/3+,which is considered positive and which correspond to more than 10 to 20 positive vessels per 10 high-power fields, detected 25.0% (1/4), 18.2% (2/11), and 84.2% (16/19) of 1+, 2+, and 3+ IF-positive biopsies, respectively, without false positives. Considering C4d IF 3+ as positive IP resulted in 84.2% (16/19) sensitivity and 93.0% specificity (40/43). Overall 19 EMB out of 34 (55%)with IF grade 1, 2 and 3 were positive at IP with grade 2 and 3. Correlation between intensity and distribution for IP was made on 43 EMBs . Among 43 EMBs with C4d+ capillary positivity , C4d grade 2 positivity corresponded to the presence about 20 capillaries. More data are required to support a correlation between intensity and distribution on IP. Of relevance is that IF which represents the gold standard is evaluated on 1 fragment while the IP is assessed on two/three fragments. Focal, multifocal and diffuse could be different on one fragments or two/three fragments. Diffuse on one fragment could be multifocal in two/three. Ischemic injury did not produce positive IF or IP staining , no false positive capillary staining. IP positivity in venules and arterioles actually tended to parallel capillary positivity. Thus it is not necessary to exclude arterioles and venules or to distinguish the strongly stained capillary from the weakly stained capillaries during the examination of IP C4d positivity. Six cases out of 19 (30%) C4d IF3+ biopsies showed positive features for Humoral Rejection providing evidence that 3+ IF, diffuse pattern , has the strongest correlation with the clinicopathologic entity of cardiac humoral rejection. No reference to DSA has been made. The second paper , from Rex Smith represents the first correlation between C4d and alloantibodis using 85 tissue samples from 38pts. Since then we accepted that capillary positive staining is highly correlated with the presence of DSA. There was a small paragraph comparing C4d staining in frozen and paraffin-embedded tissue on 38 EMBs. Thirteen out of 38(32%) were IF and IP C4d+. In 25 IF negative EMB only one C4d+ on IP.

No definition of IF or IP staining pattern or other comments were presented regarding IF and IP. Other pattern of staining, including plasma, arteriolar, endothelial, ischemic myocardial , were also identified but they did not correlate with the presence of alloantibodies as did the capillaries. In another paper from Fedson designed to define pathological criteria for diagnosis AMR and to correlate ICH C4d patterns with intracardiac hemodynamics (symptomatic pts?) only four EMBs were evaluated for both IF and ICH with only one C4d+. The authors suggested that since routine surveillance for AMR is not currently standard practice IHC staining for C4d is a feasible alternative to immunofluorescence since can be performed on formalin fixed tissue collected for grading of cellular rejection. IHC C4d endothelial staining correlates with higher intracardiac pressures and lower cardiac index and is independent of cellular rejection grades. However the authors stated clearly that the methods of IHC C4d staining need to be standardized to allow for robust studies of both desensitization and treatment protocols for AMR, which should lead to decreased morbidity and mortality [81]. A more recent paper by Miller (85) *(submitted in 2010)* presented 296 EMBs from 70 patients in which the positivity for C4d was 53/296 with IF and 65 with IP. The discordant rate was 19% similar to that of 17% presented by Chantranuwat (84). The kappa value was 0.88 for the first EMB assessed and 0.81 if considering 0and 1+ as negative and ? 2+/3+ as positive. IF was >IP in 23 cases while IP was >IF in 34 cases. Major discrepancies were detected only in 11%of the cases. (IP1+ >IF 23 vs 11; IP2+ <IF 3vs6;IP 3+>IF 8 vs 6.

Table 5. Grading systems applied for C4d IF and ICH

| Authors | Technique | Grading | Capillary staining | No.Fragments | Positive |
|---|---|---|---|---|---|
| Chantranuwat 2004 (84) | IF/ICH | 0-3+++ | 0=neg.<br>1=focal (<1/3)<br>2=multifocal(1/3-2/3)<br>3=diffuse(>2/3) | IF=1<br>ICH=2/3 | 2 and 3 |
| Beletskaya LV 2006 (86, 87) | IF | 0-3+++ | 5-15% capillaries =slight ;<br>Up to 25% moderate;<br>>25% severe rejection | IF=all | all |
| Poelz 2005(58) | ICH | 0-3+++ | 0=neg.; 1=few capillaries<br>2=>50% of all capillaries or partial staining of most capillaries<br>3=markedly deposition on all capillaries | ICH=all fragments | 2 and 3 |
| Behr 1999(51) | IF | 0-3+++ | 0=neg.<br>1=few capillaries<br>2=segmental staining of capillaries<br>3=all capillaries marked deposition | IF=1 | >2 |
| Rex Smith 2005(57) | IF | Not graded | C4d +<br>C4d- | IF=1 | positivity=diffuse positive staining |
| Rodriguez 2005(59) | IF | 0-4++++ | ND | IF one fragment | >2 in linear pattern |
| Tan 2009(82) | IF | 0-3+++ | 0=neg ; 1=?<br>2= focal <50%<br>3=diffuse>50% capillaries | IF=All fragments | 3= diffuse linear pattern |
| Moseley 2010(88) | ICH | 0-3+++ | 0=neg.<br>1=weak and few areas(focal)<br>2=moderate, several areas (multifocal)<br>3= strong and diffuse | ICH=all fragments | >2 |

| Authors | Technique | Grading | Capillary staining | No.Fragments | Positive |
|---|---|---|---|---|---|
| Holt 2008(89) | ICH | 0-3+++ | 0=neg<br>1=>25%<br>2=25-75%<br>3=>75% | ICH= all EMB | 2 and 3 |
| Suggs (2008)(90) | IF | 0-3+++ | 0=neg<br>1=weak<br>1+=2<br>2+=4<br>1+/2+=3<br>3+=6 | Not stated | Not stated |
| Galambos (2008)(91) | IP | Neg-pos | Neg<10 cap+/10hpf<br>Pos>10 cap /10hpf | All fragments | >10 cap/10hpf |
| Gupta (2009)(81) | IF | Neg-pos | Positive=strong linear staining of capillaries | One fragment | Yes or no |
| Fedrigo (2010)(92) | IP | 0-3+++ | 0=neg<br>1= focal weak<br>2=multifocal and moderate<br>3=diffuse and strong | All fragments | 2-3 |
| Miller (2010)(85) | IPvsIF | Ip0-3+++ | 0=absent or focal<br>1+=weak<br>2+=intermediate<br>3+=strong diffuse | IF-1fragment from archives<br>IP-all fragments | 2-3 |
| Arias (2010)(93) | IP | Different grading schemes used | A: fedson<br>B: galambos<br>C:poelz,Chantranuwat<br>D: Solez/Banff: 0=neg,1 =minimal (1-<10%cap,2=focal (10-50%cap;3=difuse (>50%cap) | 3-5 fragments=all EMBs | See above for Galambos; Poejz; Chantranuwat; Solez:2-3 |
| Revelo (2011)(94) | IF | 0-3+++ | 0=neg<br>2-3=strong positive | 1 fragment | Not stated |

### 3) Grading system (table5)

**For papers with ICH (IP)** the prevalent grading system is that proposed by Chantranuwat (84): The extent of IP and IF staining was graded as 0 to 3+ as: 0 (negative/rare positivity), 1+ (focal; <1/3 of vessels), 2+ (multifocal; 1/3 to 2/3 of vessels), and 3+ (diffuse; >2/3 of vessels). Staining intensity (1+, weak [barely visible at low power]; 2+, strong [clearly visible at low power]) as well as the location (capillary, venule, or arteriole)and the number of positive vessels from every usable high-power field (HPF) and type of positive vessels were evaluated . Positive staining was considered C4d2+ and C4d3+ which includes a large interval comprehensive of multifocal and diffuse (multifocal; 1/3 to 2/3 of vessels) (diffuse; >2/3 of vessels). Holt in 2008 proposed a grading of 4 step with more precise cutoff from 0 neg to 1<25% capillary involved ; 2 25-75% ; and 3 >75% capillary involvement[88]. Poelz proposed a grading from 0 to C4d3+, with C4d2++>50%or partial staining of most capillaries. 16/64 EMBs(25%) from 17pts were considered as positive(C4d2+/c4d3+++). (3/5 positive pts(with at least 2EMBs positive in the first year) developed CAV versus 1/12 C4d negative pts)[60].

**For IF** nearly all groups except one from Cleveland and one from Moscow is usually assessing C4d on one frozen fragment. Behr assessed 1 Frozen fragments and 4 IP fragments for HE in 155 EMBs from 56 pts, grading for C4d in 4 steps (0=neg. ; C4d1+ only few capillaries,; C4d 2++ segmental staining of capillaries; C3+++ all capillaries with C4d deposition. C4d was compared with traditional markers and no correlation was found between C4d staining and the traditional marker (c1q, C3c, IgM ) with the exception of fibrin. The outcome showed 9 deaths in the 56 pts within the first 3 months and 5 death

/graft losses were from C4d+++; 1 death/graft losses in C4d 1+; 3 death/graft losses in C4d- allowed for conclusion that C4d+ intensity correlates with clinical outcomes[19].

Rex Smith also assessed 1fragment at IF and correlated C4d + with the presence of DSA. C4d positivity was not graded but define as diffuse positive staining. The author reported that only C4d capillaries positivity correlated with DSA [59].

Tan performed immunoflorescence on 4 fragments evaluated semiquantitatively with a 0-3 scale. (diffuse pattern defined as >50% capillaries involved, focal when >50% (distribution patterns were on a 0-2 scale). Location was capillaries and perymyocytes. A mean of the IF score was assessed per pts. Positivity was assigned when >50% of capillaries involved. A correlation between IF intensity and distribution is reported (Table 5) .Correlation between diffuse pattern and hemodynamics . No correlation with other patterns (perimyocytes)[84]. Beletskaya[89, 90] performed immunofluorescence on 4 fragments evaluated semiquantitatively with a 0-3 scale (from 0 to 25% of capillary involvement for immunoglobulin and complement fragments). Locations were capillaries, interstitium, sarcolemma. In 61 out of 63 EMBs from 22 pts signs of humoral type rejection (slight, medium and severe) as fixation of IgG, IgM and complement components (C3, C4d) were identified suggesting the rheumatoid course of the process and that C4d could be viewed as diagnostic measure.

5) **Correlation with C4d and C3d**

Tan correlated C4d and C3d with HLA serology and graft function for the diagnosis of AMR.

Diffuse positive capillary pattern for both C4d/C3d correlated well with DSA95%, graft dysfunction 84%, mortality 42%. Moreover combined C4d /C3d had a sensitivity of 100%

and a specificity of 99% for the pathological diagnosis of AMR. C4d or C3d positivity not always results in graft dysfunction because of the presence of complement regulatory proteins CD55 and Cd59 . The proportion of pts with CD55 and CD59 staining was highest in C4d+/C3d- pts without graft dysfunction. C4d/C3d is diagnostically more useful than C4d alone in the evaluation of AMR since can identify more precisely pts with DSA and Graft dysfunction[82].

Rodriguez in 2005 reported his experience on 665 EMBs and 166 pts. One fragments alone with IF with a staining score of 0-4+. Location and pattern. 10% of the pts and 4% of EMBs showed C4d 2+or > for C4d and C3d, but this latter has been reported as more intense. Of the 20 pts with EMB positive for C4d/C3d 5(25%) showed hemodynamic dysfunction, 15(75%) no graft dysfunction. Of this 8 had C4d/C3d +, 3 only C4d and 4 only C3d. In the 5 symptomatic, 3 has DSA.Deposits of C4d is a late event occurring between 60- and 1 63 months after transplantation supporting the concept of subgroup of AMR in late follow-up [61].

Holt in a pediatric population on 74 EMBs in 15 pts on Paraffin embedded tissue. The grading scale was for both C4d and C3d 0-3+ with 0 neg, 1+ >25% capillaries, 2+ 25-75% , 3+ >75%. C3d was detected in 100% of EMBs with a distribution of 7/74(9%) , 2++ 18/74(24%), 3+++ 49/74 (66%) while the C4d was positive in only 6/74 EMB(8%) with 3 C4d1+, 1 C4d2+, 2 C4d3+. Of these 6, 4 were symptomatic and 2 asymptomatic. C4d was absent in all episodes of rejection and low cellular rejection. C3d and C4d staining did not correlate with rejection episodes thus not supporting the usefulness of routine surveillance for AMR with C3d and C4d. No DSA assessed [88].

Mosely C4d and C3d in CAV development C4d and C3d on paraffin embedded tissue with Chantranuwat classification for the scoring . 0= neg ; 1 weak, 2 moderate and 3+ strong.

O-1 beeing negative and 2-3 considered positive. Deposition was reported on capillaries and arterioles not on the interstitium . 282 EMBs evaluated at 1 week, 4 weeks , 8 weeks, 6 mo,1 yr and 2yrs.There was a constant deposition of C4d and C3d throughout the 2 yrs with only 4% negative EMBs (12/282) for C4d and 5%(15/280) C3d negative. This is in accordance with Fedson who reported that they could detect costant deposition throughout the first 18 mos post-TX with very few (13%) completely negative pts. Similar to the paper by Beltskaya on IF who reported presence of Complement split product deposition of C4d on 61/63 EMBs from 22HTx pts. In Moseley paper pts were divided in CAV-free (26pts) and CAV positive pts(26pts). C4d was detected in less EMBs than C3d accounting for 54/148 EMBs (36%) in CAV –FREE and 61/134(46%) in CAV +. C3d was present in 66/143(46%) and 80/137(58% in CAV+. Dual staining was present in 25-143EMBs of Cav-free(17%) and 39/134 (29%) of CAV+. Dual negativity was present in 43/143 in CAV-Free and 33/134 in CAV+. C4d deposition was not associated with the development of CAV while C3d was positively associated with it. Limitation no DSA detection [91].

**6) C4d /C3d and ischemic injury**

Baldwin IF on frozen fragment and HE on Fixed-Paraffin embedded biopsies. 33 consecutive pts evaluated 14/33(42%) were positive for C3d or C4d complement split products. 9 pts had C4d/C3d in diffuse capillary pattern and pericapillary moderate-to strong. 2 pts presented C4d + with a weak stain in the absence of C3d. 5pts had C4d + and focal IgM. 8/of 9 C4d/C3d staining had moderate to severe ischemic damage.3/6 of the EMBs that had C4d or C3d alone had mild ischemia . 11/15 EMBs (73%) with detectable complement deposition had ischemic injury. 8/18(44%)of EMBs without C4d or C3d

deposition had ischemic injury). Ischemic changes are associated with the activation of complement. Complement activation may in turn promote tissue injury . At difference with this result is the observation from Chantranuwat on 35/36 EMB with necrotic fibers which did not showC4d capillary staining by If or IP. “harvesting Injury should not give false positive results with diffuse capillary staining [92].

**6) Coexistence with ACR and C4d as Marker of Humoral Rejection**

Maria Crespo reported an early experience on 445 Tx and the assessment of Humoral rejection. In 12 Pts (2.7%) the suspicion of HR was raised since there was severe hemodynamic dysfunction in the absence of cellular rejection . In six of the 12 pts archive frozen material were available for C4d staining and the results showed a good correlation with HR clinical diagnosis while testing for the traditional Immunoglobulin and Complement (IgM, IGG, C1q, C3 and fibrin) was of no value for the diagnosis of HR [93].

De Gouveia also reported a brief experience with 25HTx and 112 EMBs on frozen fragment, She found 13 C4d EMBs (HR), 31 ACR , 7 EMBs with both ACR and C4d(Mixed) and in 1 HR was in a pt with CAV. The conclusion was for a routine use of C4d as screening supporting the concomitant presence of ACR and C4d [94]

**Table 6 Outcomes in Antibody Mediated Rejection**

| Authors | NoPts | No EMBs | IF/ICH | %C4d+ | DSA | Graft dysfunction | CAV | Mortality |
|---|---|---|---|---|---|---|---|---|
| Bayliss 2008(98) | 76 | 152 | IF(1Fr) | 23/152 (15%) | NA | 8/23 (35%C4d+) | C4d+Pts=RR>5 Time than C4d- | NA |
| Behr 1999(51) | 56 | 155 | IF(1fr) | 40/56 (71%) | NA | C4d+++ correlates with Graft Dysfunction | NA | 5/9pts C4d+(55%); Intensity of staining correlates with mortality |
| Poelz 2005(58) | 17 | 64 | IP | 16/64 (25%) | NA | NA | CAV 3/5 C4d+ vs 1/12 C4d- Specificity = 85%; sensitivity =75%8;, CAV+ (4PtS)= 4 Cd4+; CAV- (8PTS)=No C4d+ | NA |
| Casarez (<18yrs) 2007(99) | 103 | 358 | IF(1Fr) | 36/103 (32%) | NA | AMR 17/36(47%)No AMR 22/75(29%) | AMR=7/36(19%) noAMR=14/75 (18.7%) | AMR =14%; No AMR=33% |
| Kfoury 2009 (100) | 960 | 26365 | IF(1fr) | NA | NA | NA | NA | Mortality in 13% at 7 yrs Incremental risk of mortality with more severe AMR scores (Hazard Ratio> 14 V4+V5 vsV1) |
| Kfoury 2009 (101) | 822 | 22325 | IF(1fr) | 118 AMR; 193Mixed ; 490 CR | NA | NA | NA | CVmortality : AMR or stable Mixed vs CR(AMR 21%- MR 18% CR12%) |
| Wu 2009(80) | 129 | ND | IP | 43 Pts | NA | NA | Freedom from CAV (AsyAMR 52%; TxAMR 68%; Control 79%) | Survival at 5 yrs=ns (AsyAMR86%; TxAMr68%; Controls 79) |

| Authors | NoPts | No EMBs | IF/ICH | %C4d+ | DSA | Graft dysfunction | CAV | Mortality |
|---|---|---|---|---|---|---|---|---|
| Moseley 2010(88) | 43 | 282 | IP | C4d+= 115/280 (41%); C3d= 146/280 (52%) | NA | NA | C4d not associated with CAV; C3d associated with CAV | NA |
| Fedrigo (2010) (92) | 107 | 985 | IP | C4d+=56/985(5.7%) 36/107 pts (34%) | Yes (14/36pts) C4d=39% DSA+ | AMR=8/107pts (7,5%)8/14C4d+ DSA+(57%) | 23%pts C4d+ with CAV 27%pts C4d-with CAV | Survival at ten years: C4dneg 96% C4d+62% Mortality Risk: C4d+=18;C4d+&DSA+=61;AMR=32-fold risk(p=0.0001) |
| Arias (2010) (93) | 44 | 88 | IP | C4d=33/88 (37.5%);25/44pts(56%) | NA | Good or poor outcomes | NA | At 15-24 months post HTx outcomes was not associated with C4d+(C4d+vsC4d-p=0.64) |
| Revelo (2011) (94) | 422 | 3,712 | IF | NA | NA | NA | NA | Predictive model of Cv mortality (p=0.0001: C4d &C3d ($R^2$ =0.93);C3d,C4d,HLA-DR, fibrin ($R^2$ =0.988):C3d,C4d,HLA-DR,fibrin,IgG,IgM ($R^2$ =0.989)mortality;p<0.001:C4d &C3d ($R^2$ =0.93) |
| Rodriguez (2005) (59) | 165 | 665 | IF | 9.6% (16/165) | Yes (3/16pts with C4d=19% DSA+) | 3% (5/165) | 7/16 pts with C4d (44%) | 4/16 pts with C4d (25%) |
| Tan (2009) (82) | 330 | 1511 | IF | 11.5%(38/330)pts | Yes (24/36 pts with C4d=67% DSA+) | 5% (17/330); C3d+ correlates with graft disfunction | NA | 8/19 pts with C3d+ C4d+ (42%) |

## 7) Outcome, CAV and mortality (Table 6)

Three papers reported first experience with C4d in single or dual cases[95-97] suggesting the usefulness of C4d staining on paraffin embedded material and the possibility of EMBs screening for AMR also in the long term. Bayliss reported on 152 EMBs in 76 HTx correlating the expression of VEGF with outcome and development of CAV. IF on 1 fragment only 23/152 (15%) were C4d positive. Graft dysfunction was present in 8/23 (35%) of C4d +. No assessment of DSA was performed. These results are in line with the reported incidence of 1/3 of C4d positive and graft dysfunction. The population was divided between 26 CAV + pts and and 50 CAV free pts.

ACR was present in a minority of cases (3/26) and (5/50). Pts with C4d were at five time greater risk of of developing CAV than pts without AMR (98).

Behr 56 Ht and 155 EMBs within 3mo. IF on one fragments. C4d compared with traditional markers for complement deposition and IG . C4d and clinical outcomes : the clinical status of the pts correlated well with the intensity of C4d++; 9 death within 3months in the 56 pts assessed. 5/9 death (55%) had C4d positivity , no correlation with the old markers. Other 3 pts with C4d+++ showed reduced ventricular function[51].

Poelz small numbers EMBs were subdivided in four group according to the intensity of C4d staining (0-3+++). Capillary C4d staining was correlated to intimal index (0.25) and was higher in C4d positive than negative pts. No C4d staining was detected in negative control (15EMB). 16/64(25%) were positive and 9/16 were after 3mo. Three /five C4d positive pts versus 1/12 C4d-negative revealed significant CAV with a specificity of 85%, and sensitivity 75% . All pts with CAV (4pts) showed at least 1 EMBs positive for C4d, no

negative C4d pts(8) presented CAV. The presence of C4d is associated with the development of CAV[58].

Kfoury try to correlate the severity of AMR with cardiovascular mortality . Histological and immunopathologic findings of AMR varied in severity. An incremental risk of mortality was observed with more severe AMR. A vascular score was assigned to each EMBs according to both histology and IF. IF was performed on one fragments (IgG, M,A, C1q, C3d , C4d, fibrin, HLA-DR. The authors reported that hemodynamic compromise was not encountered frequently enough to enable to perform any statistical evaluation. However, when present it was overwhelming due to AMR (98%) rather than to cellular rejection suggesting that AMR should be viewed as spectrum of variable severity rather than as only positive or negative. Limitations no DSA and no clinical correlation symptomatic or asymptomatic pts. Surveillance AMR is performed on all EMBs in the first 8/12 weeks no later has been reported. Since 2003 also C4d has been added [98].

Kfoury reported the necessity of early screening for AMR since 85% of pts had their first and 54% their third episode within 3 months. This will allow also the detection of asymptomatic AMR and may prompt changes in therapies. The study was conducted on 375 cardiac recipients (43%) with positivity for AMR [54].

Kfoury always on 869 HT reported on cellular rejectors, stable mixed cellular and asymptomatic AMR rejectors in the first 12 weeks after transplantation. 118 AMR , 193 as mixed and 490 as cellular. Excluded were hemodynamic compromise AMR or ACR (101).

Mortality occurred in 12.6% of cellular rejectors, 21.2% of AMR,18% of mixed rejectors. There was no difference in mortality between stable MR and asymptomatic AMR.

Casarez evaluated a pediatric population 358 EMBs from 103 pts. 36 Grafts (32%) had evidence of HR. 75 were negative. 17/36 (47%) of HR developed failure over a 3yr time. HR negative pts developed failure in 22/72(29%). HR was diagnosed on EMBs at HE if positive IF performed also on subsequent EMB until negativity occurred. IF was routinely performed at 1 and 2weeks and 6 mos and 1yrs as surveillance . Only one frozen fragments for IG and complement split products. ICH was performed with CD68/CD34 and C4d . No DSA assessment , no specification of C4d.36/111Pts (35%)had at least 1 HR positive EMBs. Mortality rate was 33% for HR versus 14% in nonHR.HR developed at 111 days. TCAV developed in similar manner for the two group(19%) and this is different from the experiences reported for Adult pts. In the time period between 3 mo and 3 yrs the survival rate was inferior for HR than nonHRpts. Suggestion is to screen for histological evidence of HR. On suspicion IF should be performed [99].

In Moseley paper pts were divided in CAV-free (26pts) and CAV positive pts(26pts). C4d was detected in less EMBs than C3d accounting for 54/148 EMBs (36%) in CAV –FREE and 61/134(46%) in CAV +. C3d was present in 66/143(46%) and 80/137(58% in CAV+. Dual staining was present in 25-143EMBs of Cav-free(17%) and 39/134 (29%) of CAV+. Dual negativity was present in 43/143 in CAV-Free and 33/134 in CAV+. C4d deposition was not associated with the development of CAV while C3d was positively associated with it. Limitation no DSA detection[88].

**Asymptomatic versus symptomatic pts.**

Wu try to assess the significance of untreated asymptomatic AMR. They evaluated the outcomes at 5yrs between asymptomatic and symptomatic pts. Pts with ACR >3A were excluded. Forty- three pts were selected and 86 pts served as controls: 21 Asymptomatic and 22 Treated AMR. AMR was diagnosed on HE according to severity based on the

degree of cellular swelling, interstitial hemorrhages and interstitial edema. Being a retrospective study no DSA post-transplant could be assessed. ICH could be additionally performed for C4d and CD68.Actuarial survival at 5 yrs was not statistically different between the three groups. (ASAMR 86%, TXAMR 68 Controls 79%). Freedom from CAV was lower in TxAMR (68%) and ASAMR 52% versus controls 79%. At difference with Hammond study which found a significant difference in the time to development of CAV between pts with ACR , mixed and AMR alone.(different population , no C4d performed at that time ) [82].

# 5 MATERIALS AND METHODS

As C4d staining has been performed on a routine basis in heart transplant recipients at the University of Padua's Heart Transplantation Center since 2005, 1.452 consecutive EMBs from 133 patients (mean age 48.9±18.3) were reviewed. Donor mean age was 41,6±15,2 years and 15% had more than 60 year old.

In our population more frequent indication to heart transplantation was the non ischemic cardiomyopathy (see figure 1)that consist of dilated cardiomyopathy, hypertrophic and restrictive cardiomyopathy and arrhythmogenic right ventricular cardiomyopathy, in particular in this group the distribution was 74% (n=48) of dilated cardiomyopathy, 11%(n=7) of Hypetrophic cardiomyopathy, 11% (n=7) of arrythomogenic right ventricle cardiomyopathy and 4% (n=3) of restrictive cardiomyopathy.

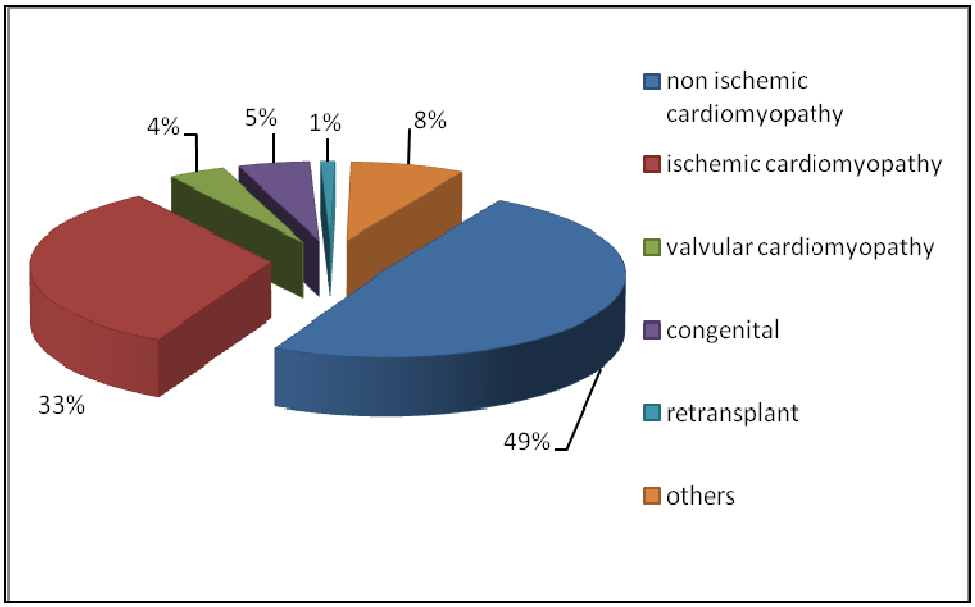



*Figure 1. Indication o transplantation during period 2005-2010.*

## *5.1 Adult population*

To study survival in patients with C4d positivity on EMBs we evaluated a total of 107 adult patients (n=85, 79% of these were males) with a median age at the time of transplantation of 55 years (range 17-73 years) participated in the study. We evaluated 985 consecutive biopsies from 107 transplant patients (median=8 biopsies per patients). EMBs were performed in accordance with the protocol schedule [100] and graded for acute cellular rejection using the 1990 ISHLT biopsy grading scale, as indicated elsewhere [13] until 2004 and 2005 ISHLT scale after that date [15]. After the first year EMBs were performed at one year interval

Sixteen (15.0%) of adult patients were transplanted less than a year earlier, while 55 (51.4%) had undergone transplantation 1-5 years earlier. The remaining 36 (33.6%) had undergone transplantation more than 5 years earlier. After receiving information about the aims and procedures of this study the patients signed informed consent forms. C4d staining on EMBs has been routinely performed in our centre as part of our post-transplant monitoring protocol since 2004.

Hematoxylin and eosin biopsy stains taken before 2004 were re-graded using the 2005 ISHLT scale. A rejection score, based on a modification of the ISHLT classification, was assigned as follow: 1R=1, 2R=2, 3R=3. The following scores were calculated for each patient: the total rejection score (TRS) calculated taking into consideration the total number of scores registered during the follow-up; severe rejection score (sev TRS) calculated taking into consideration the scores equal to or above 2R. All the scores were normalized for the total number of biopsies performed in each patient by dividing each score by the total number of EMBs performed during the study period.

In accordance with the ISHLT revised consensus criteria, AMR was diagnosed on the basis of evidence of graft dysfunction, circulating anti-donor antibodies (DSA), histological evidence of acute capillary injury, CD 68 intracapillary positivity and C4d capillary positivity on endomyocardial biopsies[101, 102]. However NIH classification of AMR proposed sequence of stages of AMR: latent, subclinical and clinical[45]. C4d immunostaining was carried out during routine surveillance controls**,** performed in accordance with the protocol described above. In the presence of C4d positivity, DSA were also determined at the same time.

Our study population was divided into 4 groups on the basis of the patients' C4d, DSA, and graft function profile:

- C4d positive, DSA negative, without graft dysfunction (asymptomatic AMR) = group 1;
- C4d positive, DSA positive, without graft dysfunction (asymptomatic AMR) = group 2;
- C4d positive, DSA positive, presence of graft dysfunction (symptomatic AMR) = group 3
- C4d negative, DSA negative, without graft dysfunction, considered the control group = group 0.

## *5.2 Pediatric population.*

In our Cardiac surgery unit forty-nine pediatric patients underwent heart transplantation and we selected 24 patients that underwent EMBs after 2004 (median age at the transplantation 12 years; range 2months-17 years) . Three of them were excluded for ABO incompatible HTx and two had perioperative complication and died.

We evaluated 226 consecutive biopsies from 21 transplant patients (median 10,7 EMBs per patients). Three (14%) of these were transplanted less then one year, while 4 (19%)

had undergone transplantation 1-5 years earlier. The remaining 14 (67%) had undergone transplantation more than 5 years earlier.

EMBs were performed in accordance with the protocol schedule, graded for acute cellular rejection using the 1990 ISHLT biopsy grading scale and calculated rejection score as indicated previously for adult population. As we described for adult population the diagnosis for AMR following histological and immunopathological criteria. In the presence of C4d positivity, circulating anti-donor antibodies (DSA) were also determined at the same time.

## *5.3 Histological features of AMR on EMBs*

Of the 1452 EMBs 87 were positive for C4d staining (6% 87/1452) from 131 patients, 26 EMBs from 13 patients had repeated C4d positivity. Sixty-one positive C4d EMBs were selected after the exclusion 4 C4d positive EMBs which presented mixed rejection with ACR >2R. Sixty-six staining negative for C4d were matched for pre-transplant diagnosis, time after transplantation age, and acute cellular rejection (ACR) grading, thus a total of 127 EMBs were considered (61 C4d positive, 66 C4d negative). Among the 61 C4d positive EMBs 9 were diagnosed as AMR on the basis of the three criteria: histological/immunohistochemical positivity, presence of circulating DSA and presence of graft dysfunction. Among C4d positive EMBs 35 presented with ACR<= 1R (57% EMBs) in the C4d negative EMBs 22 presented with ACR <0 1R (33% EMBs). It was found that 29.1% (n=37) of the EMBs were collected within 3 months of heart transplantation , 27.6% (n=35) between 3 and 12 months, and 43.3% (n=55) after 12 months. There were 26 EMBs from 13 patients who had C4d capillary positivity at time intervals longer than 2

weeks and investigated separately. New C4d positive events were considered separately when positivity appeared at time intervals longer than 2 weeks.

Right ventricular endomyocardial biopsy specimens were retrieved following the standard surveillance monitoring EMBs protocol [100] using the percutaneous transcatheter method. The specimens were immediately placed in a formalin fixative solution and embedded in paraffin. Paraffin blocks were serially cut (up to 60 sections) into 3-4 μm thick sections. Three slides with three sections for each biopsy specimen were stained with hematoxylin and eosin and read by cardiac pathologists using the2005 ISHLT classification system [13, 103]. The histological characteristics of AMR, assessed in accordance with the revised ISHLT grading system (1) were evaluated qualitatively as present or absent (Table 1).Two pathologists (MF and AA) evaluated independently all the slides included in this study. When discrepancy was found the slides were reviewed by the two pathologists and definitive agreement was reached.

Table 1 Histological characteristic of Antibody Mediated rejection

| **Histological parameters** | **Definition** |
|---|---|
| **Endothelial swelling** | Swelling of cytoplasm and nuclear enlargement with luminal protrusion or hobnail |
| **Intracapillary mononuclear cells** | Accumulation of aggregates of mononuclear cells within capillaries which appear enlarged |
| **Intracapillary leucocytes** | Accumulation of polynucleated cells within capillaries which appear enlarged |
| **Edema** | As interstitial enlargement and myocytes dissociation |
| **Hemorrage** | Interstitial red cells |
| **Microthrombi** | Intravascular thrombi with fibrin deposition or platelets aggregates |
| **Myocyte injury** | Cytoplasm vacuolization or myofilament loss |
| **Myocite necrosi** | Coagulative or colliqutive necrosi |

## *5.4 Immunohistochemistry (IHC) staining technique*

We performed C4d staining protocol to apply routinely on endomyocardial biopsies to monitor heart transplant both adult and pediatric population. The protocol is as following: All the histologic section were cut ex novo before applying immunostaining. This is an important step to perform the best quality of the immunostaing of C4d on endomayocardial biopsies.

The immunoperoxidase stainig for C4d was performed using affinity-purified antihuman C4d rabbit polyclonal antibody (Cat. No : BI-RC4, Biomedica Gruppe, Vienna). Prior to

immunostaining sections were retrieved using an EDTA solution on pressure-cook (Pascal, DakoCytomatic).

These were incubated with anti-C4d antibody at a 1/50 dilution at room temperature for 1 hour, with antirabbit EnVision (Dako Corporation, Hamburg, Germany) for 30 minutes and, finally, with peroxidase diaminobenzidine (DAB). for 5 minutes. Sections were counterstained with Mayer's hematoxylin for 1 minute, dehydrated in alcohol and mounted with medium mounting (Eukitt, Bioptica).

In accordance with Bohmig et al.'s scoring system in renal transplant patients and Chantranuwat et al.'s in heart transplant recipients, C4d staining intensities and patterns were used to categorize our patients into 4 groups [86, 104] as follows:

grade 0 = negative; grade 1 = weak staining with focal distribution, grade 2 = moderate staining with multifocal distribution; grade 3 = strong staining with diffuse pattern. The cut off value between grade 1 and grade 2 was 50% of intramyocardial capillaries. (Figure 1).

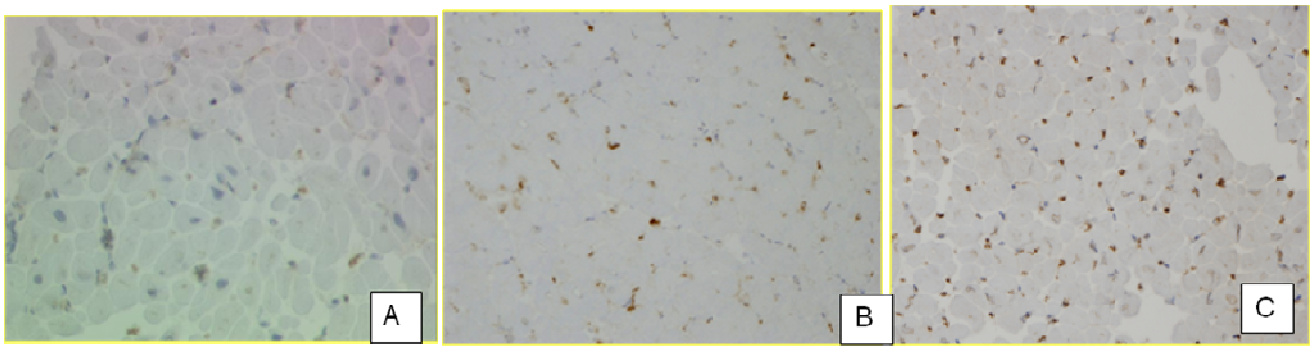



**Figure 1**. *Grading scheme of C4d staining : grade 1 (A) = weak staining with focal distribution, grade 2 (B) = moderate staining with multifocal distribution; grade 3 (C) = strong staining with diffuse pattern*

Immunostaining for C3d, another marker of activation of complement, was performed retrospectively on paraffin embedded section and has been used a polyclonal rabbit antihuman C3d (dil 1/500,Dako)and was retrieved with microwave using citrate solution. The grading system of C3d immunostaining was the same of C4d described previously.

Immunohistochemical (IHC) staining of CD68, a monocyte/macrophage marker, was performed routinely and retrospectively on paraffin sections of each biopsy, all of which were evaluated by light microscopy. Monoclonal mouse CD 68 , clone KP1 (dil 1/100, Dako), was retrieved with microwave using a citrate solution. To distinguish if CD68 was intracapillary and/or extracapillary staining with CD 34 prediluted antibody (Immunotech). was performed

## *5.5 Standardization of the immunohistochemistry method of C4d.*

Before applied the methodology and grading of C4d staining we tested the quality and reproducibility together with other laboratories of Europe and United States and to obtain the standardization of the method.

Three laboratories of United States, Cleveland clinic , Ohio US (Rodriguez ER) Mayo clinic, Rochester Minnesota US (Dogan A) Columbia university,New York US (Marboe C) and four European centers ,Copenhagen (Andersen K) Harefield, UK (Burke M) Padova, Italy (Angelini A) Pompidou, Paris ( Brunevald P) participated to the standardization of immunoperoxidase staini g of C4d on formalin fixed paraffin embedded endomyocardial biopsies . Core laboratory was Cleveland clinic directed by prof ER Rodriguez. All participants send to prof ER Rodriguez the selected specimen that showed the histologic and immunopathologic hallmarks for AMR. The laboratories of Cleaveland assembled all the specimens in only one single paraffin embedded block (figure 2) . All laboratories

received the block and performed the test of IHC C4d blindly. The paraffin block had 13 specimens , two of them were negative controls.

Our center applied the protocol of immunostaining of C4d described previously.

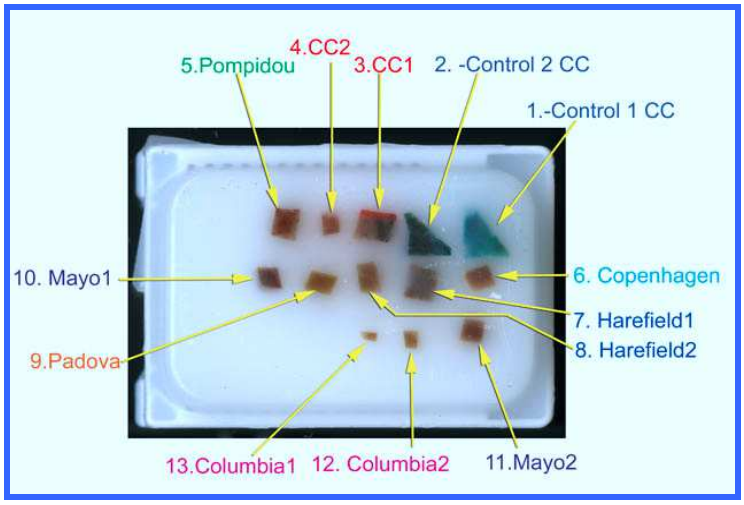



*Figure 2. Paraffin-embedded block to test the immunostain of C4d*

## *5.6 Donor specific Antibodies (DSA) Assessment*

Since the study of C4d has been mainly retrospective only in a subgroup of patients circulating anti HLA antibodies could be performed. In thirty-seven patient circulating Anti HLA antibodies were detected 26 were positive and 9 were negative.

IgG anti-HLA reactivity in the sera, obtain before transplantation and at the time of C4d positive detection on EMBs, was analyzed using bead-based screening assays, referred to as Luminex methodology. All sera tested positive at screening, were retested with Single Antigen beads in order to determine the antibody_specificity. Specifically, LABScreen Mixed kit and LabScreen panel reactive antibodies (PRA) screening tests (One Lambda, CA, USA) were used. The former simultaneously detects Classes I and II antibodies using microbeads coated with purified Classes I and II human leucocyte antigens (HLA), and the latter establishes the PRA percentage and specificity for both classes. The tests were carried out following the guidelines provided by the manufacturer and analyses were

performed using One Lambda software (HLA Visual Version 3.3). In order to limit any lot-to-lot variability in the assay kits, only Luminex products from a single lot were used. In accordance with the manufacturer's guidelines, results above a cut-off value of 3.0 were considered positive. When it was not possible to identify HLA specificity or when the ratio value was greater then 10, the Single Antigen Kit (One Lambda) was also used [105, 106].

Mean Fluorescence Intensity (MFI)

Each serum reactivity is assesed by the fluorescence signal for each HLA coated bead after correction for non- specific binding to the negative control bead. All data is normalized to the results obtained using negative control serum. The reactivity of a serum in screening is calculated based on the fluorescence value which equals the value of Class I or Class II coated bead minus the value of negative control bead. For antibody identification by Single Antigen beads, the normalized fluorescent value for each HLA coated bead equals the value of that bead divided by the value of negative control bead.

## *5.7 Cardiac Allograft Vasculopathy (CAV) assessment*

Ninety-five out of the 107 adult patients and 16 out of 21 pediatric patients has been underwent annual coronary angiography after heart transplantation. CAV was assessed in accordance with the criteria established by Gao et al. [107]. The diagnosis of CAV was based upon any of the following features at annual coronary angiography: (i) discrete lesions resulting in > or =10% narrowing of major graft vessels, or (ii) diffuse, concentric narrowing of whole vessels, including their branches. The coronary angiograms were obtain one year after transplantation.

## *5.8 Contrast-enhanced transthoracic Doppler echocardiography*

Echocardiography was performed for CFR evaluation using CE-TTE before and after adenosine infusion, with an ultrasound system (Sequoia C256, Acuson, Mountain View, CA) connected to a broadband transducer with second harmonic capability (3V2c). All studies were continuously recorded on 0.5-in. (1.27 cm) S-VHS videotape. Briefly, CFR was measured in the distal portion of the LAD, first obtaining a modified foreshortened twochamber view or, if a distal LAD flow recording was not feasible, using a low parasternal short-axis view of the base of the heart[33, 35]. If the angle between color flow and the Doppler beam was >20◦, angle correction was performed using the software package included in the software unit. Administration of the contrast agent (Levovist, Schering AG, Berlin, Germany) was performed both before and during adenosine intravenous administration . Cross-sectional area of the distal sample vessel remains constant at rest and during adenosine-mediated hyperemia, due to the endotheliumindependent vasodilation caused by the drug.

## *5.9 Pilot study on reproducibility of ISHLT2004 classification system*

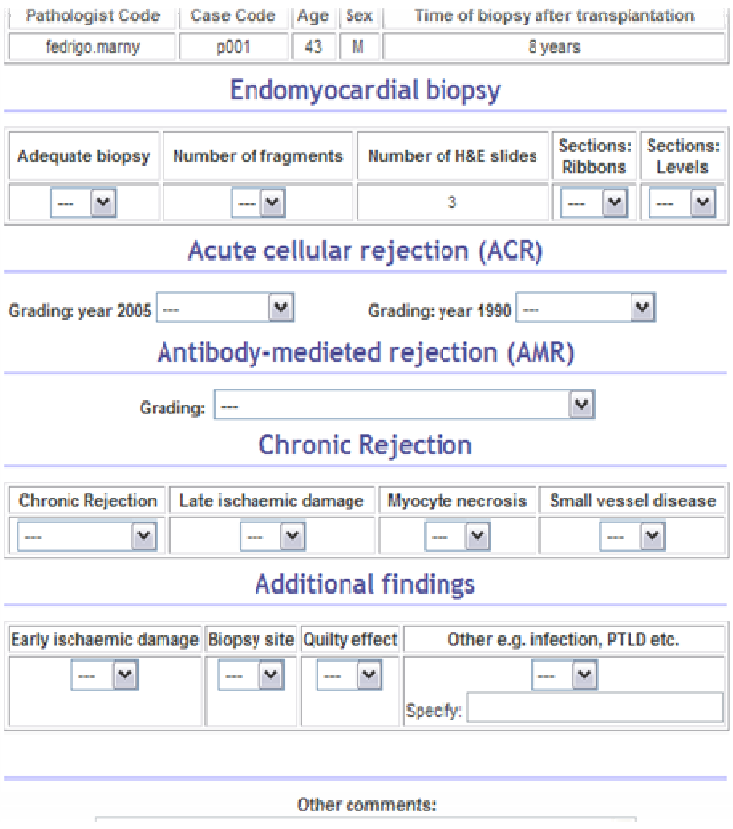
Twenty-cases preselected by two pathologists (MB,AA) were digitalized and inserted into an Aperio System for evaluation. Eighteen pathologists from 16 different heart transplant centres throughout Europe participated to the study. Cardiac pathologists from any centre in Europe performing heart transplants, volunteered to participate in this project. The inclusion criteria for individual pathologists were that they were:

- Working in/associated with a centre with an active heart transplant programme

- Regularly evaluating endomyocardial biopsies for allograft rejection either as Lead Pathologist for their centre or on a transplant biopsy rota
- Using the new 2004 ISHLT classification to assess Acute celluar rejection and Antibody mediated rejection.

A call for voluntary participation was send out through the membership circulation list of the AECVP and through personal contacts already existing between pathologists involved in European heart transplant programs. The 20 cases included different diagnosis and pathological features to cover all the aspects of the classifications as each grade of ACR, early ischemic lesions, Quilty lesions, late ischemic lesions, AMR. A respond form was designed by two pathologists (MB,AA) and inserted in the webpage of the study to collect data electronically (see respond form Fig.3). The evaluation was carried out blindly and the agreement in the diagnoses was analyzed.

*Fig. 3 Template of electronic response form*



## *5.10 Statistical Analysis*

Qualitative data were expressed as counts and percentages, while quantitative data were expressed as medians and ranges (minimum and maximum), as they were not normally distributed. The comparison between C4d groups was conducted with the Fisher's exact test in case of categorical variables and the Kruskal-Wallis test for quantitative variables. The Kaplan-Meier method was applied to estimate the C4d groups' survival functions and the log–rank test was used to compare survival between groups. The Cox's regression model was used to estimate unadjusted and sex and age adjusted hazard ratios (HR) with the 95% confidence intervals (95% CI) considering the C4d occurrence a time dependent covariate in the post-transplantation period.

Sensitivity and specificity, were reported with 95% confidence interval calculated with the exact method. Positive and negative likelihood ratio with 95% confidence interval were calculated according to Deeks JJ and Altman DG [108]. Liklihood rations are alternative statistics for summarizing diagnostic accurancy, which summarize how many times more(or less) likely patients with disease are to have that particular result than patients without the disease. A likelihood ration grater than 1 indicates that test result is associated with the presence of the disease, whereas a likelihood ratio less than 1 indicates that the test result is associated with absence of disease. Liklihood ratios above 10 and below 0.1 are considered to provide strong evidence to rule in or rule out diagnoses respectively in most circumstances. The area under the curve (AUC) for endothelial swelling and Intracapillary macrophages in predicting C4D and DSA positivity was estimated with multivariate logistic regression analysis.

The interobserver reliability has been assessed by means of the analysis of variance, through a random-effects linear model, and weighted kappa statistics, which is regarded as a common method for measuring inter-rater reliability. The strength of agreement was defined according to the degree of pathologist agreement as poor <0.00, slight 0.21-0.40, fair 0.21-0.40, moderate 0.41-0.60, substantial 0.61-0.80 and almost perfect with values ranging between 0.81-1.00 [109, 110]

All the tests were two-tailed and a p value of 0.05 was considered statistically significant.

The statistical analyses were performed using SAS 9.1.3 for Windows (SAS Institute Inc., Cary, NC, USA).

# 6 RESULTS

## *6.1 Standardization of the immunostaining method for C4d on EMBs.*

- Aim: To Standardize immunostaining protocol technique on formalin fixed- paraffin embedded tissue to study complement activation on graft through the biomarker of activation of alterative pathway, C4d and to assess the feasibility, reproducibility of laboratory protocol and the grading scheme of immunstaining evaluation.

The results of immunohistochemistry performed on all the specimen tested for C4d are reported in Fig 1 Picture A and B correspond to the control cases in which the staining was focal and weak (100x of the original magnification). In picture B a high magnification is also reported (320x of the original magnification) to show the linear C4d staining on intramyocardial capillaries. C and D: Cleveland specimen in which the C4d staining was present in more than 50% of capillaries and the intensity of staining was strong. E: Pompidou (Paris), specimen, the c4d was present only in the sera of interstitium (false negative). F Copenhagen specimen, C4d stained diffuse and strong in all the capillaries (100x of the original magnification). G and H Harefield UK specimens: there was a linear strong and diffuse staining for C4d (125x of original magnification).J Padua specimen: showed multifocal and strong staining (125x of the original magnification). K and I Mayo clinic specimen: diffuse and multifocal staining for C4d (125x of original magnification). L, Columbia University specimen with a beautiful strong diffuse staining of C4d of the longitudinal cut section of capillaries.

Data of the C4d on feasibility and reproducibility from different centres are reported in Table 1

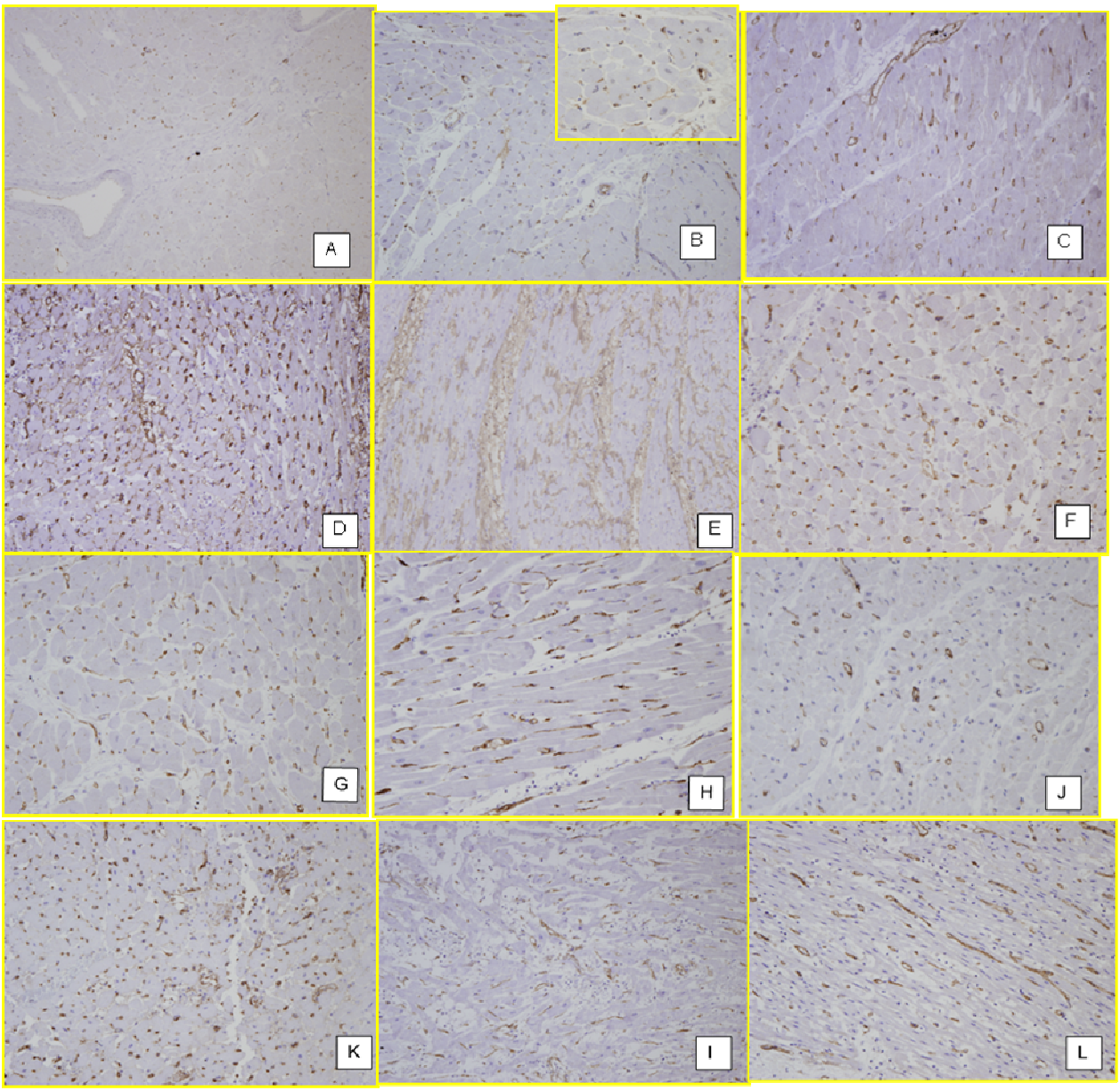



*Figure 1 Results of reproducibility of C4d protocol in our center.*

Table 1. Quality control of protocol for C4d staining

| **Center** | **Padua quality control: C4d staining on intramyocardial capillaries** | |
|---|---|---|
| | Intensity staining | Pattern of distribution of C4d staining |
| **Cleveland -Control 1** | 1+ | focal |
| **Cleveland -Control 2** | 1+ | focal |
| **Cleveland1** | 3+ | diffuse |
| **Cleveland2** | 3+ | multifocal/diffuse |
| **Pompidou** | neg | serum |
| **Copenhagen** | 3+ | diffuse |
| **Harefield 1** | 3+ | diffuse |
| **Harefield 2** | 3+ | diffuse |
| **Padova** | 2+ | multifocal |
| **mayo 1** | 3+ | diffuse |
| **mayo 2** | 3+ | diffuse |
| **Columbia 1** | 3+ | diffuse |
| **Columbia 2** | 3+ | diffuse |

## *6.2 Clinico-pathological profile of AMR in adult population*

- Aim:to assess the diagnostic and prognostic significance of C4d positive capillary staining detected by the immunoperoxidase methodology performed on paraffin embedded tissue sections in adult and pediatric heart transplant recipients.

Of the 985 consecutive cardiac allograft biopsies performed, fifty-six (5.7%) from 36 out of 107 patients (34%) were found to have C4d deposits. Fourteen (39%) of these were also positive for DSA. Five patients were found to be positive to DSA within the first year of transplantation. Of the 14 positive to C4d immunostaining and DSA, 8 (57%) had signs of allograft dysfunction. On the basis of our criteria, 22 of the patients fell into group 1, 6

into group 2, 8 into group 3 and 71 into the control group (group 0). Groups 1 and group 2 represented the asymptomatic patients and group 3 the symptomatic ones.

Patients in group 1 became positive to C4d following transplantation after a median time of 2.2 months (range 0.37-121.40), those in group 2 after 0.70 months (range 0.43-25.17) and those in group 3 after 112.42 months (14.10-251.87 ) (see Table 2).

Table 2. Time to occurrence of positivity in C4d+ve patients.

| Pts | Time to occurrence (months) | | | | | |
|---|---|---|---|---|---|---|
| | < 1 year | | >1 year | | Overall | |
| | n (%) | Median (min-max) | n (%) | Median (min-max) | n | Median (min-max) |
| Group 1 | 19 (86) | 2.07 (0.37 - 9.07) | 3 (14) | 96.47 (27.73 - 121.40) | 22 | 2.22 (0.37 - 121.40) |
| Group 2 | 5 (83) | 0.67 (0.43 - 1.40) | 1 (17) | 25.17 | 6 | 0.70 (0.43 - 25.17) |
| Group 3 | 0 (0) | - | 8 (100) | 112.42 (14.10 - 251.87) | 8 | 112.42 (14.10 - 251.87) |
| Total | 24 (67) | 1.58 (0.37 - 9.07) | 12 (33) | 103.48 (14.10 - 251.87) | 36 | 3.00 (0.37 – 251.87) |

Table 3. Mortality of the four study groups

| **Groups of patients** | **Causes of death** | **Time of follow up after C4d positivity and death (median months)** | **Mortality (× 1,000 person-years)** |
|---|---|---|---|
| **Group 0** | 1 Sudden death<br>4 Chronic rejection<br>1 Acute cellular rejection<br>1 Unwitness | - | 15.75 |
| **Group 1** | 1 Chronic rejection<br>1 Unwitness | 12.35 (11.94 - 12.76) | 30.80 |
| **Group 2** | 1 Chronic rejection | 25.32 | 103.73 |
| **Group 3** | 1 Sudden death<br>1 PTLD<br>2 Humoral rejection | 7.64 (3.75 - 11.09) | 50.31 |

### 6.2.1 C4d and survival

Fourteen patients died during the follow-up at a median of 2.7 years following transplantation (range 1-22.5 years). The mortality (see Table 3 ) was higher in group 2 with respect to group 3, but death occur earlier after C4d +ve in
group 3.

The 22 patients in group 1 (61% of total C4d+ve) showed a 18 fold higher risk compared to the C4d negative patients (95% CI 1.960 to 160.022). The 6 patients in group 2 had a 61 fold higher risk (95% CI 3.399 to 1110.360). The 8 patients in group 3 had a 32 fold higher risk (95% CI 5.884 to 179.432), overall $p<0.0001$ (see Table 4). Overall the C4d positive patients showed a statistically significant reduction in survival compared to the C4d negative patients (Figure 2) and this observation was kept in the three different groups (see Figure 3). When the asymptomatic (groups 1 and 2) and symptomatic patients (group 3) were compared with the control group an 18 and 26 fold increase mortality risk was observed, respectively.

Table 4 Results of Cox regression analysis.

| | | Unadjusted | | | | Sex and age adjusted | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Deaths/n | p | HR | 95% CI for HR | | p | HR | 95% CI for HR | |
| Group 0 | 7/71 | | 1 | - | - | | 1 | - | - |
| Asymptomatic | 3/28 | < 0.0001 | 18.8 | 1.6 | 218.3 | 0.0005 | 18.7 | 1.6 | 217.2 |
| Symptomatic | 4/8 | | 26.1 | 5.2 | 129.9 | | 27.8 | 4.97 | 155.9 |

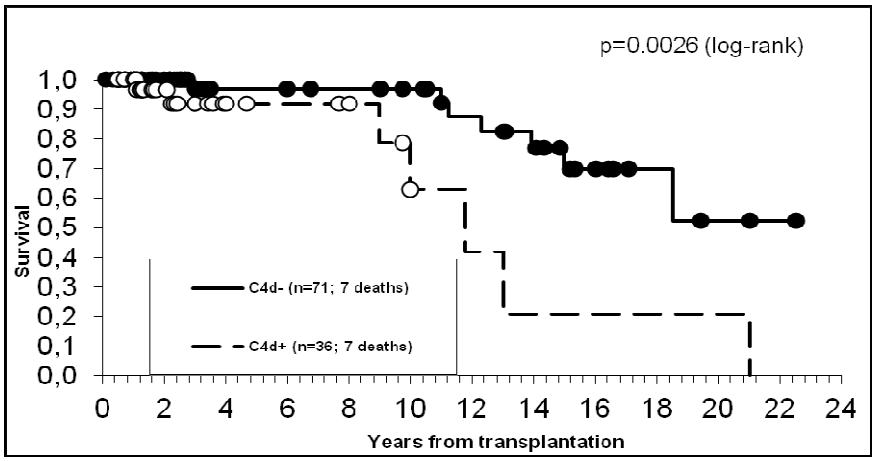



*Figure 2. Kaplan-Meyer survival curve of C4d+ ve patients vs C4d-ve patients.*

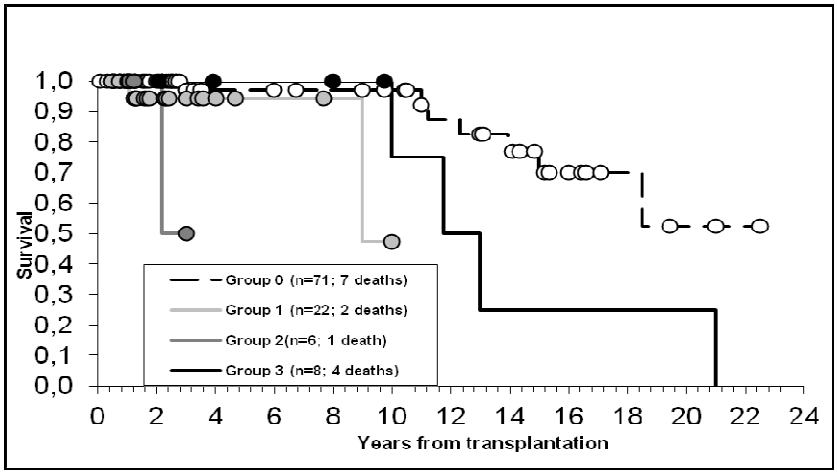



*Figure 3. Kaplan-Meyer survival curve of the four study groups.*

No differences were found between the C4d positive and negative groups in terms of patients' ages, sex, number of transfusions, pregnancy, and time of ischemia during transplantation (Table 1). Of the 107 patients included in this study, 6 received LVAD (6%), 3 were positive and 3 negative to C4d. One case had humoral rejection and recurrence of C4d (2.5%) and 2 patients had Asymptomatic AMR (7.0%)

Pretransplant PRA values were available for all 36 C4d positive patients and for 75% of the control group. There were 3 PRA positive patients in group 3, none in group 2, and 2 in group 1.

Table 5. Univariate analysis of clinical variables.

| **Clinical variables** | C4d+ve DSA-ve Graft Dysf -ve n=22 | C4d+ve DAS+ve Graft Dysf -ve n=6 | C4d+ve DSA+ve Graft Dysf +ve n=8 | C4d+ve n=36 | C4d-ve n=71 | *p-value* |
|---|---|---|---|---|---|---|
| Sex M n (%) | 15 (68.2) | 5 (83.3) | 8 (100.0) | 28 (77.8) | 57 (80.3) | 0.8029* |
| Recipient age yrs median (min-max) | 49.5 (19-69) | 52 (34-60) | 52 (36-59) | 51 (19-69) | 56 (17-73) | 0.1937† |
| Transplant Indications n (%) | | | | | | |
| Cardiomyopathies | 9 (40.9) | 3 (50.0) | 2 (25.0) | 14 (38.9) | 36 (50.7) | 0.5704* |
| Ischemic Cardiomyopathies | 6 (27.3) | 2 (33.3) | 4 (50.0) | 12 (33.3) | 23 (32.4) | |
| Valvular Cardiomypathies | 1 (4.6) | 1 (16.7) | 0 (0.0) | 2 (5.6) | 3 (4.2) | |
| Congenital Heart Disease | 3 (13.6) | 0 (0.0) | 0 (0.0) | 3 (8.3) | 2 (2.8) | |
| Others | 3 (13.6) | 0 (0.0) | 2 (25.0) | 5 (13.9) | 7 (9.9) | |
| Tranfusions n (%) | 6 (28.6) | 2 (33.3) | 1 (12.5) | 9 (25.7) | 7 (12.5) | 0.1563* |
| Mismatch n (%) | 11 (50.0) | 5 (83.3) | 6 (75.0) | 22 (61.1) | 38 (62.3) | 1.0000* |
| Donor age yrs median (min-max) | 35 (15-66) | 46 (15-58) | 32.5 (17-59) | 37 (15-66) | 37 (17-64) | 0.7565† |
| Incompatibility A n (%) | 14 (63.6) | 6 (100.0) | 6 (62.5) | 25 (69.4) | 35 (58.3) | 0.3840* |
| Incompatibility B n (%) | 16 (72.7) | 6 (100.0) | 5 (62.5) | 27 (75.0) | 36 (60.0) | 0.1833* |
| Incompatibility DR n (%) | 15 (68.2) | 6 (100.0) | 4 (50.0) | 25 (69.4) | 34 (56.7) | 0.2797* |
| Donor cause of death n (%) | | | | | | |
| Trauma | 11 (52.4) | 4 (66.7) | 5 (62.5) | 20 (57.1) | 30 (51.7) | 0.2537* |
| Cerebrovascular disease | 10 (47.6) | 2 (33.3) | 3 (37.5) | 15 (42.9) | 23 (39.7) | |
| Other | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.9) | 5 (8.6) | |
| Viruses n (%) | 11 (57.9) | 4 (66.7) | 5 (62.5) | 20 (60.6) | 26 (41.3) | 0.0874* |
| Pregnancies n (%) (only in women) | 6 (100.0) | 1 (100.0) | 0 (0.0) | 7 (100.0) | 10 (83.3) | 0.5088* |
| Time of cold ischemia minutes median (min-max) | 193.5 (0-300) | 160 (110-180) | 120 (120-240) | 180 (0-300) | 162 (0-300) | 0.6709† |
| Rejection score median (min-max) | 0.94 (0.13-1.92) | 0.81 (0.57-1.54) | 1.13 (0.00-1.56) | 0.95 (0.00-1.92) | 0.82 (0.00-2.68) | 0.5012† |
| Rejection score severe median (min-max) | 0.28 (0.00-1.61) | 0.38 (0.29-1.17) | 0.19 (0.00-0.73) | 0.29 (0.00-1.61) | 0.24 (0.00-1.05) | 0.1796† |

*Fisher's exact test; †Wilcoxon rank sum test

### 6.2.2 Recurrence of C4d positivity on EMB

In the positive C4d patients, there were 9 (25%) with recurrent C4d positivity on EMB, 7 of them had multiple rejection episodes (78%) characterized by at least one negative between two positive biopsies; one of these (1/8 of group 3, 12.5%) was diagnosed with AMR seven years after heart transplantation and was treated with plasmapheresis and rituximab. The other 8 became positive within the first year after transplantation (median 1.58 months, range 0.47-8.07). No clinical data were statistically significant this C4d positive group (Table 6).

Table 6 Mutivriate analysis for repeated C4d on EMBs

| | | Unadjusted | | | | Sex and age adjusted | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Deaths/n | p | HR | 95% CI for HR | | p | HR | 95% CI for HR | |
| C4d –ve | 7/71 | < 0.0001 | 1 | - | - | 0.0002 | 1 | - | - |
| C4d+ve | 7/36 | | 24.959 | 5.114 | 121.800 | | 26.112 | 4.819 | 141.493 |

Six out of the nine (67%) patients were positive for anti HLA antibodies and among these, 3 had DSA and 3 no DSA. Survival was worse in the C4d positive patients with recurrence with respect to controls (p=0.0662)

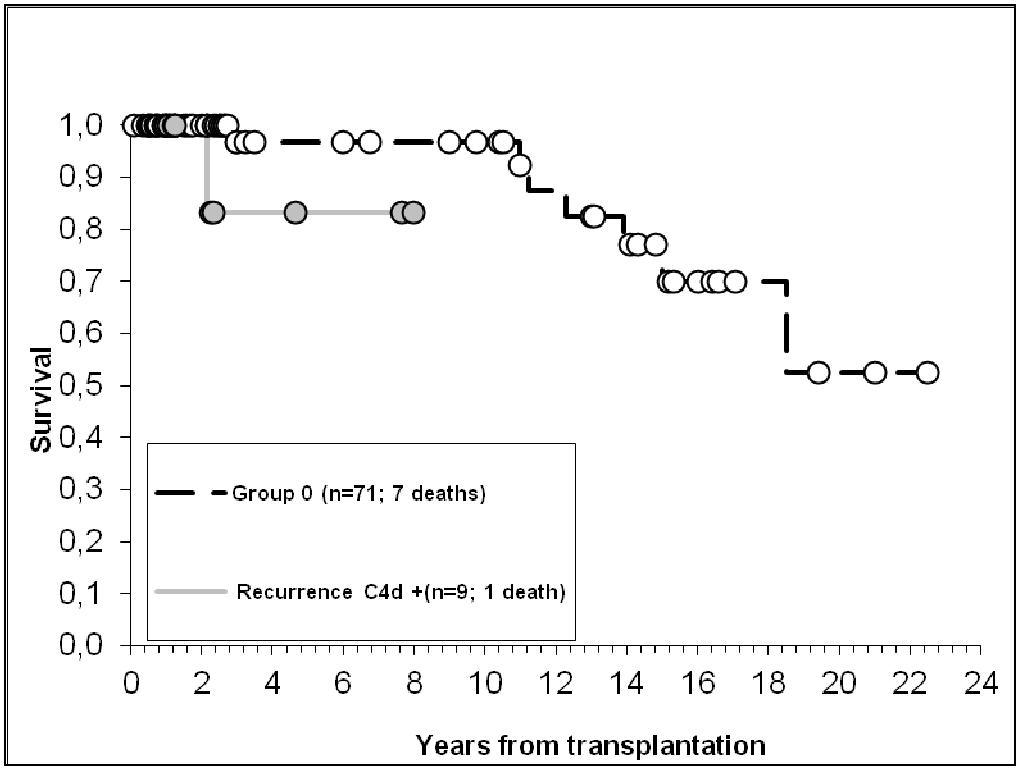



*Figure 4. Kaplan-Meyer survival curve for patients with recurrence of C4d +ve on EMBs*

### 6.2.3 Outcome of patients with AMR

AMR diagnosis was confirmed in eight patients on the basis of the ISHLT criteria. AMR occurred at a median of 9 years (range 1 month-21 years) after heart transplantation. Two patients had recurrence of C4d positivity and DSA detection after antibody mediated rejection therapy. Two of the 8 patients had received a left ventricle assistance device before heart transplantation, and 4 had a previous diagnosis of Epstain-Barr virus-related post transplant lymphoproliferative disease (PTLD). All of them were on a chronic immunosuppressive regimen with cyclosporine, cyclosporine plus everolimus (5/8 62%), cyclosporine plus mycophenolic acid (3 patients) and 3 patients were on steroids.

Six (66%) of the AMR patients were treated with Rituximab and plasmapheresis, 1 patient received only plasmapheresis and 2 patients did not received a specific treatment because of the contraindications

Of the four (33%) patients who died as a result of AMR, 1 had never received rituximab or plasmapheresis due to their contraindications. Another patient , transplanted 21 years earlier, had been treated with 3 cycles of plasmapheresis. Ejection fraction ameliorated after the second cycle, but the patient died suddenly. The other two patients showed improvement after plasmapheresis and rituximab, but they died some time later, one due to post-transplant lymphoproliferative disorder (PTLD) and the other due to AMR.

### 6.2.4 C4d and CAV

Coronary angiography results were available for 59 out of 71 (83%) C4d negative patients and 30 out of 36 C4d (83%) positive patients. CAV developed at a median of 1.37 years (range 0.04-20.7) in the C4d positive patients. No differences in terms of angiographically detectable CAV were found in the C4d positive (7/30) with respect to the C4d negative (16/59) patients. No statistically significance was found with respect to CAV when comparing the four groups (p=0.5697). Two patients (25%) in group 3 (AMR) developed CAV, none did in group 2, and 5 (31%) did in group 1.

### 6.2.5 C4d and acute cellular rejection (ACR)

Absence of acute cellular rejection was observed in 24 (24/56, 43%) of the (56/985) C4d positive EMBs. Grade 1R (focal acute cellular rejection) was found in 26 (26/56, 46%),

grade 2R (moderate cellular rejection) was detected in 5 (9%) and 3R (severe cellular rejection) was found in 1 (2%). There were no differences in terms of rejection score and severe rejection score in the four groups (Table 1). Nonetheless, patients with recurrent C4d positivity had a more severe rejection score with respect to the C4d negative patients (median 0.63 range=0.00-1.17 vs median 0.24 range=0.00-1.05 respectively p=0.034).

## *6.3 Clinico-pathological profile of AMR in Pediatric population.*

Seven patients (33%) showed a C4d +ve intra-graft capillary deposition. Of these 4 were positive for circulating donor specific anti HLA antibodies; none were positive in the C4d negative group. One patient presented also graft dysfunction (14% of C4d+ve) within 3 months after transplantation. One patient with C4d positivity died for heart failure after 2 years since the C4d positivity detection. The other five patients with C4d positivity are still alive at 2, 3, 4 , 14 years respectively (Table 7,8). In the C4d positive group the rejection score was high compare to C4d negative patients. (2.2 vs 0.2)

Table 7. Clinical variables in pediatric C4d+ve pts and C4d-ve pts.

| **Clinical variables** | **Negative C4d Group n=14** | **PositiveC4d Group n=7** |
|---|---|---|
| **Sex M n (%)** | 55 | 60 |
| **Recipient age yrs media (standard deviation)** | 8,3±6,1 | 6,8±6,8 |
| **Transplant Indications n (%)** | | |
| **Cardiomyopathies** | 93 | 86 |
| **Ischemic Cardiomyopathies** | - | - |
| **Valvular Cardiomypathies** | - | - |
| **Congenital Heart Disease** | 7 | 14 |
| **Others** | - | - |
| **Tranfusions n (%)** | 7% | 25 |
| **LVAd** | 6% | 0 |
| **Mismatch n (%)** | 78 | 0 |
| **Donor age yrs media (standard deviation)** | 9±6,8 | 12,2±15,5 |
| **Incompatibility A n (%)** | 89 | 40 |
| **Incompatibility B n (%)** | 89 | 40 |
| **Incompatibility DR n (%)** | 78 | 40 |
| **Donor cause of death n (%)** | | |
| **Trauma** | 89 | 60 |
| **Cerebrovascular disease** | - | 20 |
| **Other** | 11 | 20 |
| **Viruses n (%)** | 67 | 100 |
| **Time of cold ischemia minutes media (standard deviation)** | 180,9±63,2 | 242±78,2 |
| **Rejection score median (min-max)** | 0,2 (0-1,1) | 2,2 (0,6-4) |
| **Angiografically CAV (%)** | 11 | 0 |
| **Follow up (media, SD) years** | 9.6±6.2 | 9.5±5.1 |

Table 8. Clinical characteristics of C4d+ve patients.

| **Pts ID** | **Age at H Tx** | **Sex** | **Transplant Indications** | **CDC PreHtx** | **Luminex Pre THx** | **time of C4d +ve post-TX** | **Luminex PostHTx** | **Follow up** |
|---|---|---|---|---|---|---|---|---|
| **1** | 12 | M | Cardiomyopathy post myocardities | negative | DSA+ | 1,5 months | DSA + | Alive at 3 y post HTx |
| **2** | 16 | F | Arithmogenic cardiomyopathy | negative | - | 1 year | - | Alive at 3 years |
| **3** | 1 | M | Restrictive cardiomyopathy | negative | - | 11 years | - | Survival at 4 years |
| **4** | 1 | F | Dilated cardiomyopathy | negative | - | 5 years | - | Death at 7 years |
| **5** | 4 | M | Restrictive cardiomyopathy | negative | negative | 14 years | DSA+ | alive |
| **6** | 18 | M | Dilated cardiomyopathy | negative | DSA+ | 2weeks | DSA+ | Alive at 2 years |
| **7** | 18 | M | Congenital heart disease | negative | DSA+ | 2 weeks | DSA+ | Alive 3 years |

All the EMBs positive to C4d were evaluated for the histological parameters of AMR. The results (Table 9) showed that morphological features were present in C4d positive group and also in C4d negative group. Prominent intracapillary accumulation of macrophages were presented in 63% of C4d positive pts. The single case of symptomatic AMR of our study showed intracapillary accumulation of macrophages and all the morphological feature indicative of AMR.

Table 9. Histological characteristics in C4d+ve EMBs compared to C4d-ve EMBs.

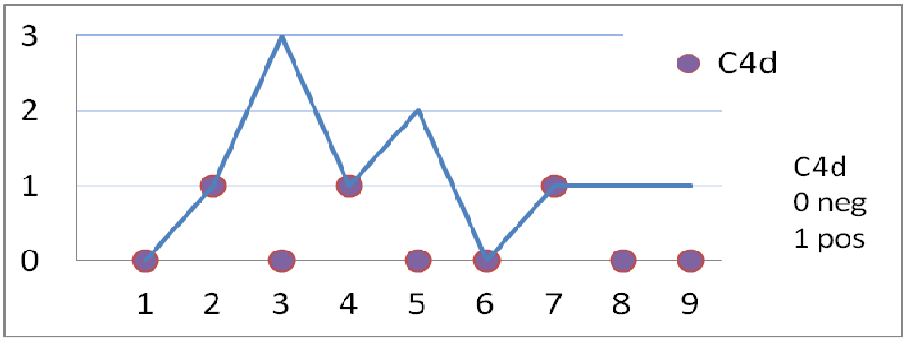
| **Histological Characteristics** | **C4d negative (Grading 0-1) (n=14) %** | **C4d positive (Grading 2-3) (n=7) %** |
|---|---|---|
| Endothelial swelling | 78 | 75 |
| Mononuclear cells in capillaries | 22 | 63 |
| Myocyte injury | 55 | 25 |
| Myocyte necrosis | 22 | 12 |
| Neutrophils in capillaries | - | 25 |
| Edema | 78 | 88 |
| Hemorrhage | - | - |
| Microthrombi | 11 | 12 |

### 6.3.1 Pediatric index case of AMR.

A 12 years old boy with cardiomyopathy as evolution of a myocarditis underwent heart transplant . He had mismatches donor HLA: HLA-A1; B8;DR3,11. This antibody was always detected, when assessed, at follow up.The first episode of C4d positivity, on the second monitoring EMB (Figure 5), was characterized by the detection of DSA class I, not present at the time of transplantation and the patients was symptomatic (Figure5). He was

treated with immunoglobulin, chorticosteroids and after 10 days treatment C4d and DSA class I were negative . After three months since the first episode of AMR EMBs showed C4d positivity again without symptoms (Figure 6).

Figure 1



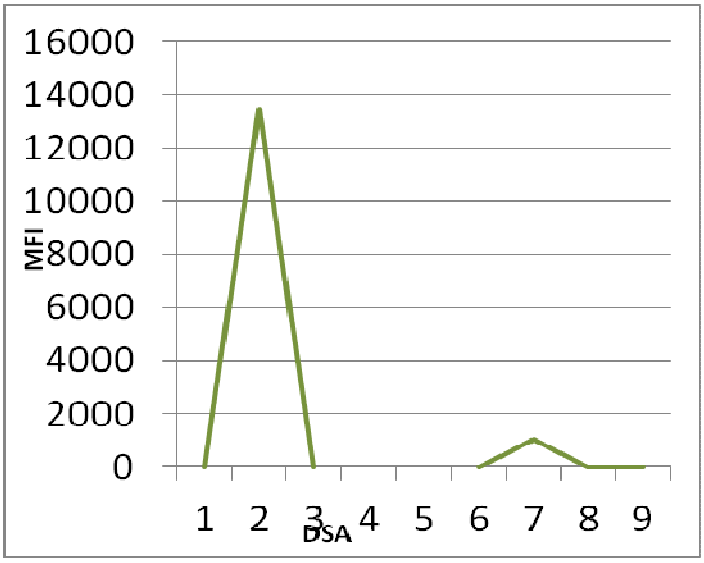




*Figure 5.C4d+ve on EMBs compared to antiHLA antibodiesexpressed in MFI*

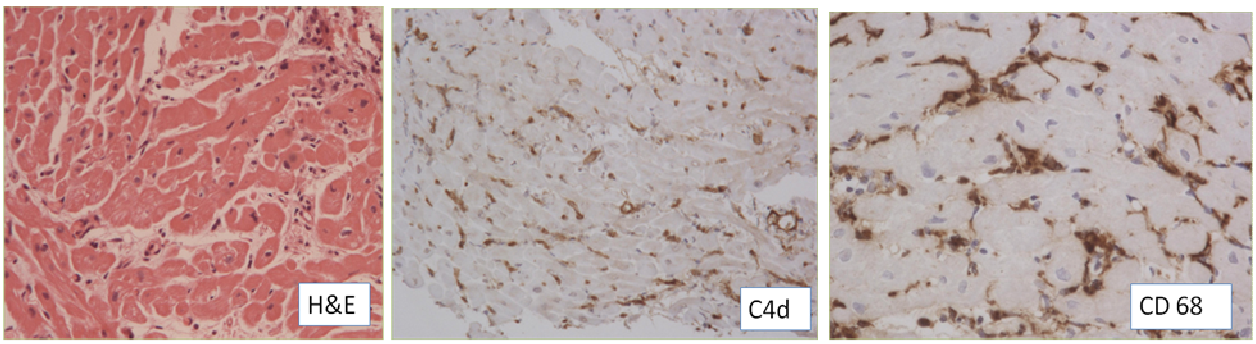



*Figure 6. Histological characteristic of subsequent episode of C4d+ on EMBs after therapy. No signs of AMR on H&E, diffuse capillary immunostain for C4d and presence of intracapillary macrophages.*

## *6.4 Pilot study on reproducibility of ISHLT2004 classification system*

As for the two cases of AMR, 35% of pathologists identified correctly the suspicion of AMR on HE slides, while in 17 out of the other 18 cases negative for AMR, few pathologists , with a percentage ranging between 5- to 33%, gave a misdiagnosis of " suggestive for AMR" (see table 10).

Table 10 Distribution of agreement between referent pathologists (ACR/AMR Index Diagnosis) and 18 pathologists for diagnosis of AMR on HE staining for each of the 20 cases

| Case Code | Referent pathologists ACR/AMR Diagnosis | Pathologists diagnosis for AMR | | Total |
|---|---|---|---|---|
| | | Negative for AMR | Suggestive for AMR | |
| **p001** | 0 (AMR) | 11(61) | 7(39) | 18 |
| **p002** | 0/neg | 17(94) | 1(6) | 18 |
| **p003** | 0/neg | 15(83) | 3(17) | 18 |
| **p004** | 1A/neg | 17(94) | 1(6) | 18 |
| **p005** | 3A/neg | 16(89) | 2(11) | 18 |
| **p006** | 0/neg | 15(83) | 3(17) | 18 |
| **p007** | 1A/neg | 15(83) | 3(17) | 18 |
| **p008** | 1A/neg | 14(78) | 4(22) | 18 |
| **p009** | 1B(AMR) | 12(67) | 6(33) | 18 |
| **p010** | 4/neg | 13(72) | 5(28) | 18 |
| **p011** | 1A/neg | 13(72) | 5(28) | 18 |
| **p012** | 0/neg | 17(94) | 1(6) | 18 |
| **p013** | 1A/neg | 12(67) | 6(33) | 18 |
| **p014** | 1A/neg | 14(78) | 4(22) | 18 |
| **p015** | 3B/neg | 15(83) | 3(17) | 18 |
| **p016** | 3B/neg | 11(61) | 7(39) | 18 |
| **p017** | 1A/neg | 16(89) | 2(11) | 18 |
| **p018** | 2/neg | 17(94) | 1(6) | 18 |
| **p019** | 1A/neg | 16(89) | 2(11) | 18 |
| **p020** | 0/neg | 18(100) | 0 | 18 |
| **Total** | | 294(82) | 66(18) | 360(100) |

## *6.5 Morphological parameters on EMBs to detect AsAMR and AMR*

Aim: to evaluate if morphological parameters detect signs of early subclinical or latent stages of AMR and their correlation with C4d staining in cardiac transplants recipients.

Of the 8 histological characteristic evaluated (Table 11), only two could be considered fair predictors of C4d capillary positivity in the light of their sensitivity: endothelial swelling with a 78.7 % sensitivity (its specificity was very poor 28.8%) and interstitial edema with a 77.1% sensitivity (its specificity was 31.8%). Intracapillary macrophages had a sensitivity of 39.3% only and a specificity of 68.2%. The sensitivity and specificity of the histological parameters in EMBs of patients with C4d positivity (Table 11) were similar to those observed in EMBs of patients affected by AMR (Table 12).

Table 11. Sensitivity, Specificity, positive and negative Likelihood Ratio of histological characteristics in predicting C4D positivity

| **Histological Characteristics** | **C4d negative (Grading 0-1) (n=66)** | **C4d positive (Grading 2-3) (n=61)** | **Sensitivity (95% CI)** | **Specificity (95% CI)** | **Positive LR (95% CI)** | **Negative LR (95% CI)** |
|---|---|---|---|---|---|---|
| Endothelial swelling | 47 | 48 | 78.7 (66.3-88.1) | 28.8 (18.3-41.3) | 1.10 (0.90-1.35) | 0.74 (0.40-1.37) |
| Mononuclear cells capillaries | 21 | 24 | 39.3 (27.1-52.7) | 68.2 (55.6-79.1) | 1.24 (0.77-1.98) | 0.89 (0.69-1.16) |
| Myocyte injury | 16 | 21 | 34.4 (22.7-47.7) | 75.8 (63.6-85.5) | 1.42 (0.82- 2.46) | 0.87 (0.69-1.09) |
| Myocyte necrosis | 3 | 9 | 14.8 (7.0-26.2) | 95.5 (87.3-99.1) | 3.25 (0.92-11.44) | 0.89 (0.80-1.00) |
| Neutrophils in capillaries | 5 | 20 | 32.8 (21.3-46.0) | 92.4 (83.2-97.5) | 4.33 (1.73-10.82) | 0.73 (0.60-0.88) |
| Edema | 45 | 47 | 77.1 (64.5-86.9) | 31.8 (20.9-44.4) | 1.13 (0.91-1.40) | 0.72 (0.40-1.29) |
| Hemorrhage | 7 | 10 | 16.4 (8.2-28.1) | 89.4 (79.4-95.6) | 1.55 (0.63-3.81) | 0.94 (0.81-1.07) |
| Microthrombi | 1 | 6 | 9.8 (3.7-20.2) | 98.5 (91.8-100.0) | 6.49 (0.80-52.39) | 0.92 (0.84-1.00) |

CI=Confidence Interval; LR=Likelihood Ratio

Table.12 . Sensitivity, Specificity, positive and negative Likelihood Ratio of histological characteristics in predicting AMR (C4d positivity, DSA positivity, presence of graft dysfunction).

| **Histological Characteristics** | **Control group (n=66)** | **AMR group (n=9)** | **Sensitivity (95% CI)** | **Specificity (95% CI)** | **Positive LR (95% CI)** | **Negative LR (95% CI)** |
|---|---|---|---|---|---|---|
| Endothelial swelling | 47 | 9 | 100.0 (66.4-100.0) | 28.8 (18.3-41.3) | 1.40 (1.21-1.64) | - |
| Mononuclear cells in capillaries | 21 | 1 | 11.1 (0.3-48.3) | 68.2 (55.6-79.1) | 0.35 (0.05-2.29) | 1.30 (0.98-1.73) |
| Myocyte injury | 16 | 2 | 22.2 (2.8-60.0) | 75.8 (63.6-85.5) | 0.92 (0.25-3.35) | 1.03 (0.71-1.49) |
| Myocyte necrosis | 3 | 1 | 11.1 (0.3-48.3) | 95.5 (87.3-99.1) | 2.44 (0.28-21.06 | 0.93 (0.74-1.18) |
| Neutrophils in capillaries | 5 | 3 | 33.3 (7.5-70.1) | 92.4 (83.2-97.5) | 4.40 (1.26-15.37) | 0.72 (0.45-1.15) |
| Edema | 45 | 5 | 55.6 (21.2-86.3) | 31.8 (20.9-44.4) | 0.81 (0.44-1.50) | 1.40 (0.62-3.14) |
| Hemorrhage | 7 | 1 | 11.1 (0.3-48.3) | 89.4 (79.4-95.6) | 1.05 (0.15-7.56) | 0.99 (0.78-1.27) |
| Microthrombi | 1 | 3 | 33.3 (7.5-70.1) | 98.5 (91.8-100.0) | 22.00 (2.55-189.50) | 0.68 (0.43-1.08) |

CI=Confidence Interval
LR=Likelihood Ratio

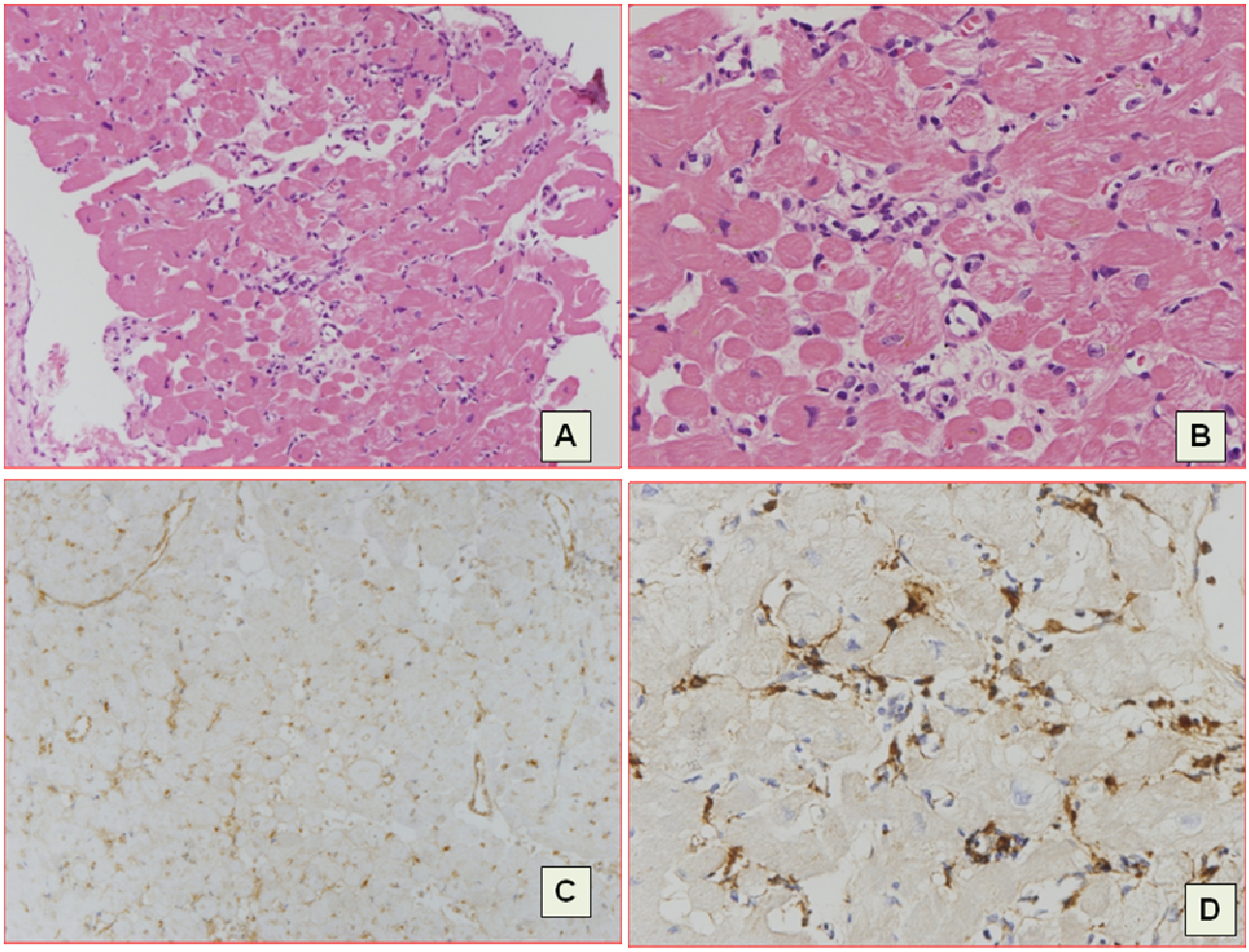



**Figure 7.** *74 years old man was heart transplanted 4 months ago. DSA were presented before transplantation and at the time of biopsy, but he was asymptomatic. A) H&E staining (original magnification125x) showed the interstitial edema and some perivascular inflammatory cells. B) H&E staining high magnification (250x) evidence the intravascular inflammatory cells. C) C4d staining (125x of magnification) showed diffuse strong staining. D) CD68 staining (original magnification250x), showed the presence of single intravascular macrophages.*

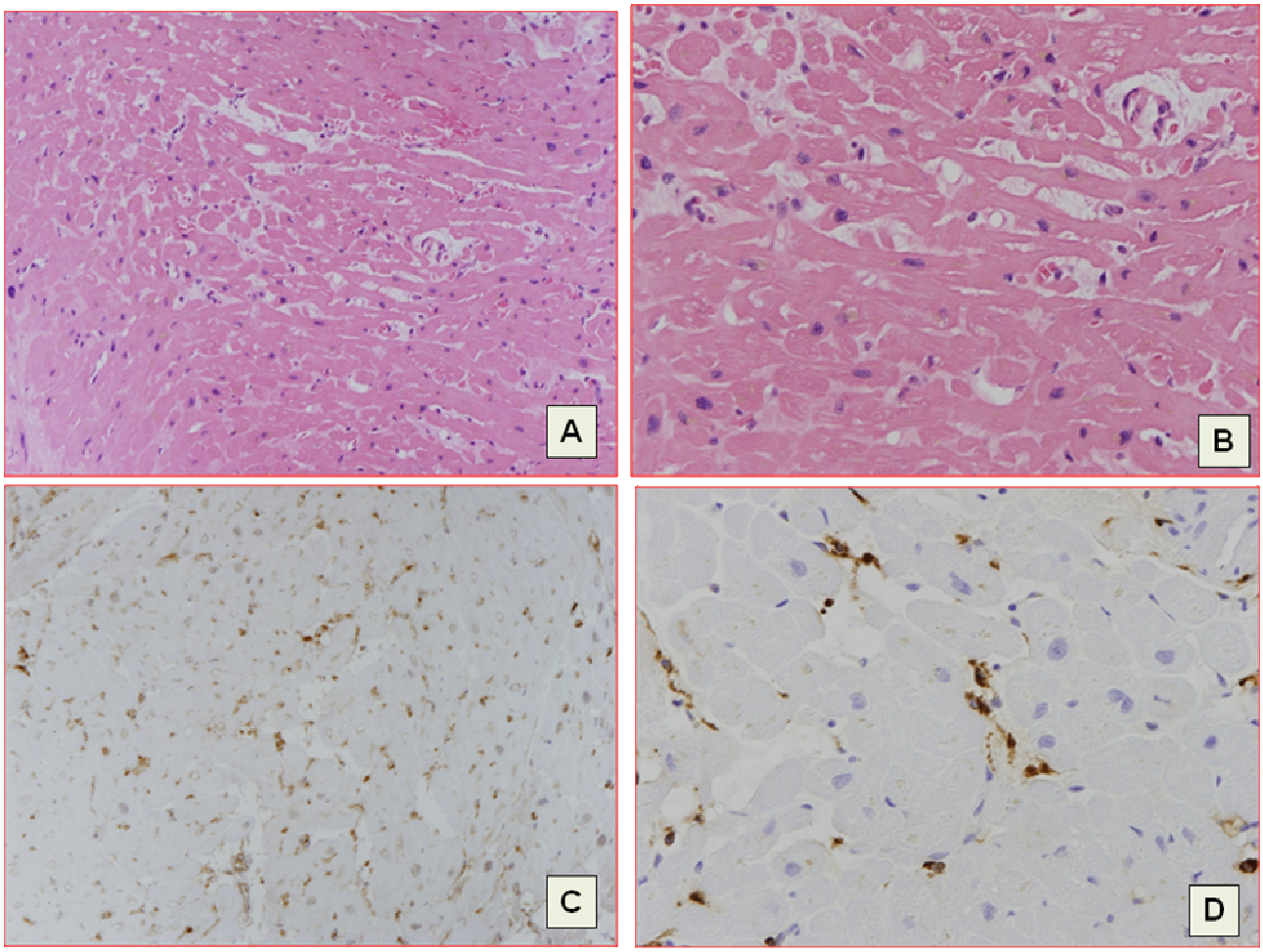



**Figure 8.** *65 years old man was heart transplanted 5 years ago and we was symptomatic (reduction of ejection fraction). DSA were presented at the time of biopsy. A) and B) H&E staining showed only interstitial edema (original magnification 125x and 250x respectively) . C) C4d staining showed multifocal strong staining on capillaries (original magnification 125x). D) CD68 showed the presence of single intravascular macrophages (original magnification 250x).*

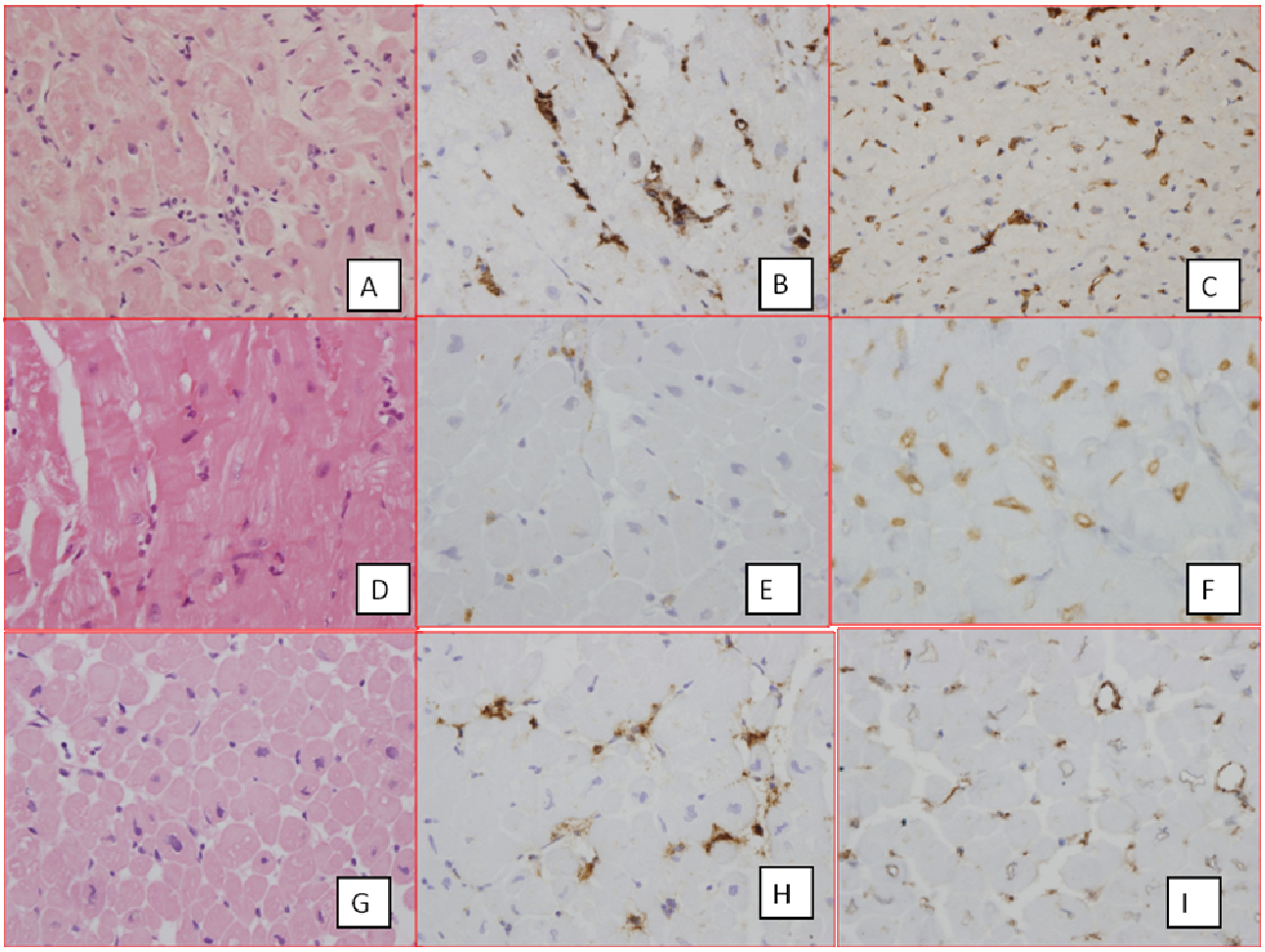



Figure 9. *A,B a 48 years old man with AMR: A, Ematoxilin-Eosin (E&E) stain (original magnification 320x) showed the vascular infiltrate (ACR grade 1A,1R), interstitial oedema and endothelial swelling, B CD 68 IHC staining with intracapillary macrophages, C, intense linear C4d staining of capillary endothelium with a diffuse pattern and strong intensity; D, E, F a 60 years old man of group 2 D, scanty perivascular inflammatory infiltrate (E&E, original magnification 320x, ACR grade 0); E: some macrophages in capillaries (CD68 original magnification 320x) and F:C4d staining of capillary endothelium with diffuse pattern and strong intensity (original magnification 320x). G,H,I a 49 years old man of group 1; G: negative for ACR and no signs suggestive for AMR (E&E, original magnification 320x), H: some intracapillaries macrophages (CD68 original magnification 320x), I: C4d staining with a diffuse pattern and moderate intensity (original magnification 320x).*

Analysis of the histological characteristics in relation to the time after transplantation (Table 14) revealed that myocyte necrosis and neutrophils within capillaries were more frequent during the first 3 months after transplantation and decreasing over time. The presence of intracapillary macrophages was not found to be time related.

Table 14. of histological characteristics of EMBs related to C4d positivity and to time interval after heart transplantation

| **Histological Characteristics** | **0-3 months post-Htx** | | **3<-12 months post-HTx** | | **>12 months post-Htx** | |
|---|---|---|---|---|---|---|
| | **C4d neg (n=10)** | **C4d pos (n= 27)** | **C4d neg (n=25)** | **C4d pos (n=10)** | **C4d neg (n=31)** | **C4d pos (n= 24)** |
| | n( %) | n( %) | n( %) | n( %) | n( %) | n( %) |
| Endothelial swelling | 8 (80) | 19 (70) | 13 (52) | 9 (90) | 26 (84) | 20 (83) |
| Mononuclear cells in capillaries | 5 (50) | 12 (44) | 4 (16) | 4 (40) | 12 (39) | 8 (33) |
| Myocyte injury | 2 (20) | 7 (26) | 5 (20) | 4 (40) | 9(29) | 10 (42) |
| Myocyte Necrosis | 0 (0) | 4 (15) | 2 (8) | 2 (20) | 1 (3) | 3 (13) |
| Neutrophils in capillaries | 0 (0) | 8 (30) | 2 (8) | 3 (30) | 3 (10) | 9 (38) |
| Edema | 9 (90) | 20 (74) | 12 (48) | 9 (90) | 24 (77) | 18 (75) |
| Hemorrhage | 1 (10) | 6 (22) | 2 (8) | 2 (20) | 4 (13) | 2 (8) |
| Microthrombi | 0 (0) | 2 (7) | 0 (0) | 1 (10) | 1 (3) | 3 (13) |

CI=Confidence Interval
LR=Likelihood Ratio

### 6.5.1 Circulating antiHLA antibodies and morphological features

The sensitivity and specificity and positive and negative likelihood ratio of histological characteristics in predicting DSA were similar to those reported for C4d positive staining and for AMR (Table 15).

Table 15 . Sensitivity, Specificity, positive and negative Likelihood Ratio of histological characteristics in predicting DSA

| **Histological Characteristics** | **DSA Positive (n=26)** | **DSA Negative (n=9)** | **Sensitivity (95% CI)** | **Specificity (95% CI)** | **Positive LR (95% CI)** | **Negative LR (95% CI)** |
|---|---|---|---|---|---|---|
| Endothelial swelling | 22 | 6 | 84.6 (65.1-95.6) | 33.3 (7.5-70.1) | 1.27 (0.78-2.07) | 0.46 (0.13-1.68) |
| Mononuclear cells in capillaries | 7 | 4 | 26.9 (11.6-47.8) | 55.6 (21.2-86.3) | 1.32 (0.70-2.47) | 0.46 (0.10-2.22) |
| Myocyte injury | 12 | 4 | 46.2 (26.6-66.6) | 55.6 (21.2-86.3) | 1.04 (0.45-2.41) | 0.97 (0.49-1.92) |
| Myocyte necrosis | 4 | 1 | 15.4 (4.4-34.9) | 88.9 (51.8-99.7) | 1.39 (0.18-10.82) | 0.95 (0.72-1.26) |
| Neutrophils in capillaries | 7 | 4 | 26.9 (11.6-47.8) | 55.6 (21.2-86.3) | 0.61 (0.23-1.59) | 1.32 (0.70-2.47) |
| Edema | 18 | 6 | 69.2 (48.2-85.7) | 33.3 (7.5-70.1) | 1.04 (0.61-1.76) | 0.92 (0.31-2.74) |
| Hemorrhage | 4 | 2 | 15.4 (4.4-34.9) | 77.8 (40.0-97.2) | 0.69 (0.15-3.16) | 1.09 (0.74-1.60) |
| Microtrombi | 4 | 0 | 15.4 (4.4-34.9) | 100.0 (66.4-100.0) | - | - |

CI=Confidence Interval
LR=Likelihood Ratio

### 6.5.2 Repeated positivity of C4d staining

Morphological features with regard to patients with more than one episode of C4d positivity were analyzed separately. Endothelial swelling and intracapillary macrophages were more frequently detected in the 2nd EMB (Table 6).

**Table 6.** . Histological characteristics of antibody mediated rejection assessed on the first C4d positive EMB and on the recurrent C4d positive EMB of the same patients

| **Histological Characteristics** | **C4d first C4d pos (n=13)** | **More than 1 C4d pos (n=13)** |
|---|---|---|
| | n (%) | n (%) |
| Endothelial_swelling | 12 (92.3) | 12 (92.3) |
| Mononuclear cells in capillaries | 7 (53.8) | 8 (61.5) |
| Myocyte_injury | 8 (61.5) | 6 (46.1) |
| Myocyte_Necrosis | 5 (38.5) | 4 (30.8) |
| Neutrophils in capillaries | 6 (46.15) | 5(38.5) |
| Edema | 11 (84.62) | 13 (100.0) |
| Hemorrhage | 3 (23.1) | 0 (0.0) |
| Microthrombi | 4 (30.8) | 1 (7.7) |

### 6.5.3 ROC curves

We tested the possibility that the use of two histological parameters together (such as endothelial swelling and intracapillary macrophages) considered as markers of AMR damage could improve their sensitivity and/or specificity. As can be seen from the figure

(area under curve=0.544) combining the two parameters did not cause a significant improvement (Figure 3), sensitivity was 31.1% (CI 19.9-44.3) and specificity was 71.2% (CI 58.8-81.7), positive likelihood ratio 1.08 (CI 0.68-1.84)and negative likelihood ratio 0.97 (CI 0.77-1.22) . In the ROC curve constructed for those patients who had circulating DSA in the presence of C4d positivity the AUC was 0.656 (Figure 4), sensitivity was 23.1% (CI 9.0-43.7) and specificity was 66.7% (CI 29.9-92.5), positive likelihood ratio 0.69 (CI 0.22-2.21) and negative likelihood ratio 1.15 (CI 0.69-1.92) .

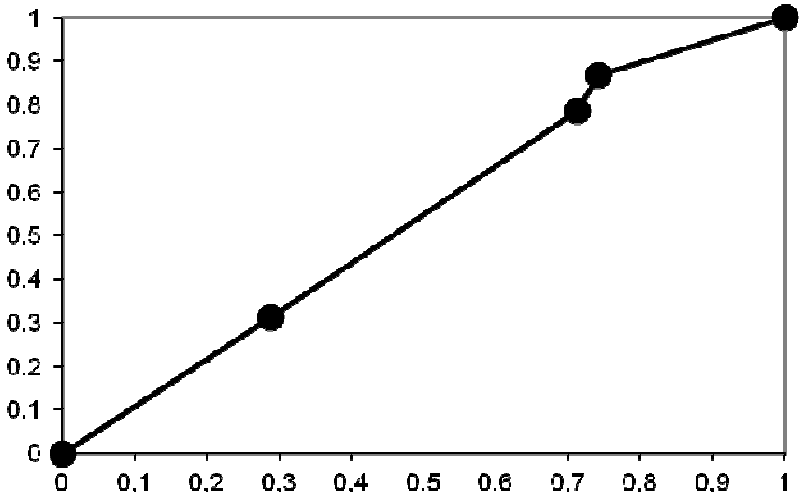



*Figure 10. ROC curve of combination of Endothelial swelling and aggregates of mononuclear cells in intracapillaries to detect C4d depositions on capillaries by IP in biopsies without cellular rejection. Combining the two parameters do not improve the capacity to predict C4d positivity*.

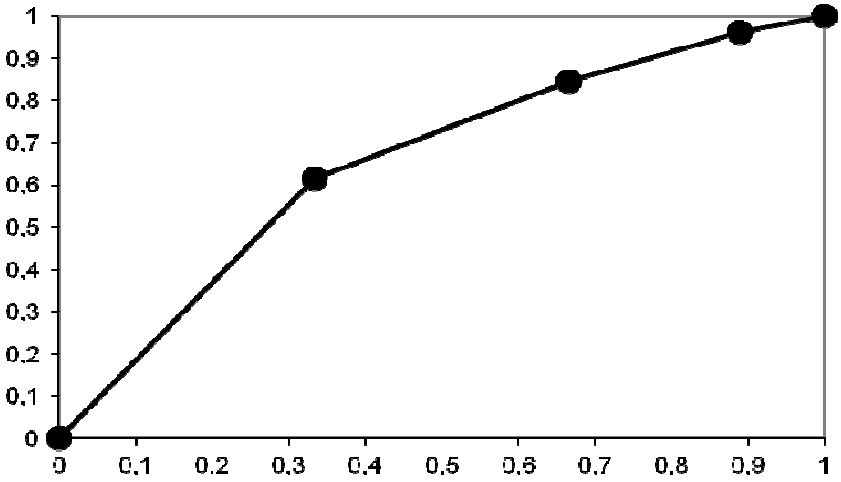



*Figure 11. ROC curve of combination of Endothelial swelling and aggregates of macrophages intracapillaires to detect circulating antibodies anti HLA on serum. Combining the two characteristic do not improve the capacity to predict presence of antibodies anti HLA.*

## *6.6 Immunostaining for C3d in the diagnosis of AMR.*

| Aim: to evaluate utility of C3d, another biomarker of complement activation, in identifying the different stages of Antibody Mediated Rejection |
|---|

Of the 61 EMBs positive for C4d, 36 were also positive for C3d (59.0%, 36/61). In the control group, 66 EMBs negative for C4d, 10 were positive for C3d (15.1% 10/66). In the asymptomatic AMR group (52 EMBs of 52 patients) 31 were also positive for C3d (59,6%, 31/52). In the symptomatic AMR group (9 EMBs of 9 patients) 5 were also positive for C3d (55% 5/9). We calculated the sensitivity and specificity of C4d, C3d and combined C4d and C3d to predicting circulating DSA (Table 16 ).

Table 16. Sensitivity, Specificity, positive and negative Likelihood Ratio of C4D and C3D in predicting DSA positivity

| | **DSA negative (n=9)** | **DSA positive (n=26)** | **Sensitivity (95% CI)** | **Specificity (95% CI)** | **Positive LR (95% CI)** | **Negative LR (95% CI)** |
|---|---|---|---|---|---|---|
| C4D (grade 2-3) | 4 | 22 | 84.6 (65.1 – 95.6) | 55.6 (21.2 – 86.3) | 1.9 (0.9 – 4.0) | 0.3 (0.1 – 0.8) |
| C3D (grade 2-3) | 1 | 11 | 42.3 (23.4 – 63.1) | 88.9 (51.8 – 99.7) | 3.8 (0.6 – 25.5) | 0.6 (0.4 – 1.0) |
| C4D and C3D (at least one grade 2-3)* | 4 | 22 | 84.6 (65.1 – 95.6) | 55.6 (21.2 – 86.3) | 1.9 (0.9 – 4.0) | 0.3 (0.1 – 0.8) |

More in detail, the sensitivity of C3d to predicted DSA and NDSA was 42.3% and 43% respectively, the specificity of C3d was 56% for DSA and 70% for NDSA.

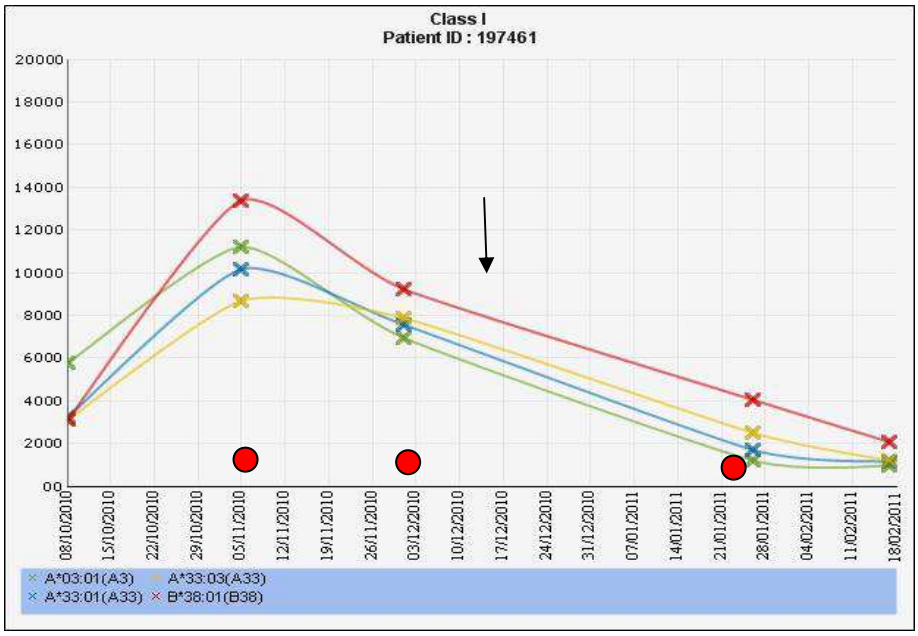
## *6.7 Alloantibody profile in the Symptomatic AMR*

Aim: to investigate whether the C4d positive staining on intramyocardial capillaries predict the levels of circulating anti HLA antibodies in symptomatic AMR

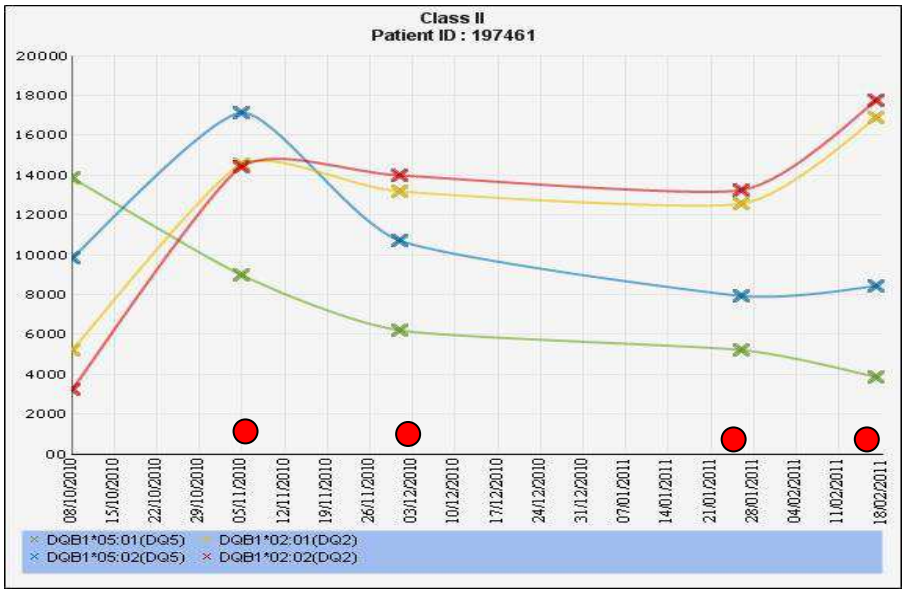
Prevalence of alloantibodies in C4d positive patients was 85%. (22/37). Of the 9 patients with symptomatic AMR , four died early after diagnosis of AMR. Only 5 symptomatic AMR pts we can follow over time. Three of them showed pre-transplant anti HLA in two patients class II and one patient class I. Three developed de novo anti HLA class II and one patient switched class II in class I in the early post-transplant and then causes AMR. C4d was positive in all symptomatic pts. At follow up all symptomatic patients underwent C4d staining on EMBs and at the same time circulating DSA were detected and quantify (see table 17 and figure 13) . 63% of circulating DSA showed C4d on EMBs. In all cases with MFI>10000 C4d was always positive, in the range between 1000-9000 MFI C4d were variable detect, with MFI<1000 C4d was always negative.

Table 17. Characteristics of symptomatic AMR group.

| Pts ID | Pre-transplant CDC or Luminex | Onset of AMR after transplant (years) | Type DSA | MFI | C4d | Symptoms | Therapy | Outcome |
|---|---|---|---|---|---|---|---|---|
| **1** | DQ7 | 0.4 | A1 | 13450 | + | + | chorsticosteroids | Alive after 3 years |
| **1** | | | | 1030 | - | | | |
| **2** | - | 9.4 | DQ1 | 1300 | + | | | Alive after 3 years |
| **2** | - | 9.6 | | 13898 | + | + | Plasmapheresis retuximab | |
| **2** | - | 11.4 | | <1000 | - | | | |
| **3** | A23 | 3.0 | A23 | 7882 | + | + | Plasmapheresis retuximab | Alive after 3.6 years |
| **3** | | 3.1 | | 8212 | - | | | |
| **3** | | 5.4 | | 1295 | - | | | |
| **3** | | 5.9 | | 3146 | + | + | | |
| **4** | HLA II + | 7.2 | DQ4 | 6800 | + | + | | Alive after 3.7 years |
| **4** | | 7.2 | | 6000 | - | | Plasmapheresis, retuximab | |
| **4** | | 7.3 | | 3200 | + | | | |
| **4** | | 7.5 | | 7200 | - | | | |
| **4** | | 8.2 | | 2400 | - | | | |
| **5** | - | 0.5 | A | 6000 | + | | | |
| **5** | | | DRb | 17000 | + | | | |
| **5** | | | DRb | 19000 | + | | | |
| **5** | | | DQ | 14000 | + | + | plasmapheresis | |
| **5** | | | DQ | 18000 | + | | | Alive after 6 months |




AntiHLA class I antibodies




Anti HLAclass II

*Figure 13. Symptomatic AMR patients: anti HLA antibodies class I and class II profile in the follow up . After therapy (black arrow)we observed decreasing of alloantibodies class I and increaseing of alloantibodies class II. C4d (red point) was costantly positive.*

The relation between MFI and the morphological features detected in the endomyocardial biopsies are shown in the table 18. Intracapillary neutrophils and microthrombi are signs of severe antibody mediated rejection and are associated with high MFI compared to other histological characteristics.

Table 18. The relation between histological characteristic and Anti HLA profile Expressed in MFI (Mean Fluorescence Intensity).

| **Histological characteristics** | **Anti HLA antibodies profile (MFI) (14 cases)** |
|---|---|
| Endothelial swelling | 7746.85 ± 4478.97 |
| Monocites in capillaries | 7584.43 ± 5410.20 |
| Myocyte_injury | 6056.83 ± 4506.05 |
| Myocyte_Necrosis | 6910.25 ± 5481.41 |
| Neutrophils in capillaries | **10012.50 ± 4470.71** |
| Edema | 7540.00 ± 4612.82 |
| Hemorrhage | - |
| Microthrombi | **10687.50 ± 3341.75** |

## *6.8 Microvasculopathy and C4d*

Aim:to identify the role of AMR in the development of allograft vasculopathy

We matched pathological and clinical archives to identify and select patients that performed monitoring endomyocardial biopsy and coronary flow reserve through an invasive echocardiogram at the same time. We selected nineteen patients , of these 7 had immunostaining C4d positive and 12 were negative (average age 50.1±8.7 years). In the group of patients with C4d positivity 4 were late symptomatic AMR (mean time after Htx 13.9±4.9 years) and three of them died after few months since the diagnosis of AMR. Three patients had asymptomatic AMR (mean time after HTx 5.3±3.4 years).

Coronary flow reserve (CFR) of patients with C4d+ was statistically significant reduced compared to C4d- group 1.28±0.48 vs 3.28±1.03 respectively, p=0.0297 (see figure 15)

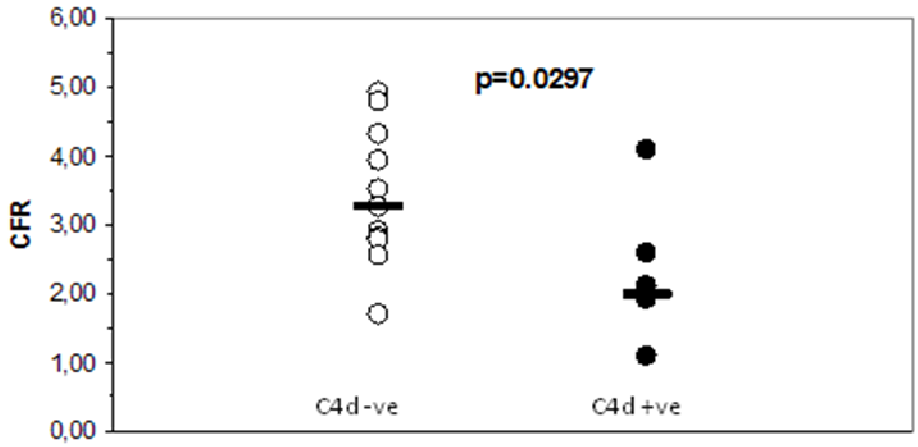



*Figure 14 . CFR in C4d+pts group compare to C4d-ve pts group*

CFR rate of patients without CAV at angiography in f both groups highlighted the difference between the two groups with a low value of CFR in the C4d+ group. (see figure 16)

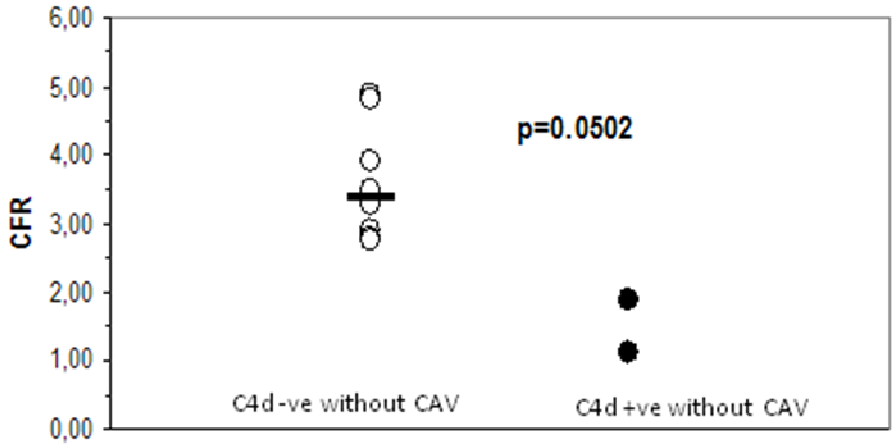



*Figure 15. CFR in C4d+ve pts and C4d-ve pts without CAV*

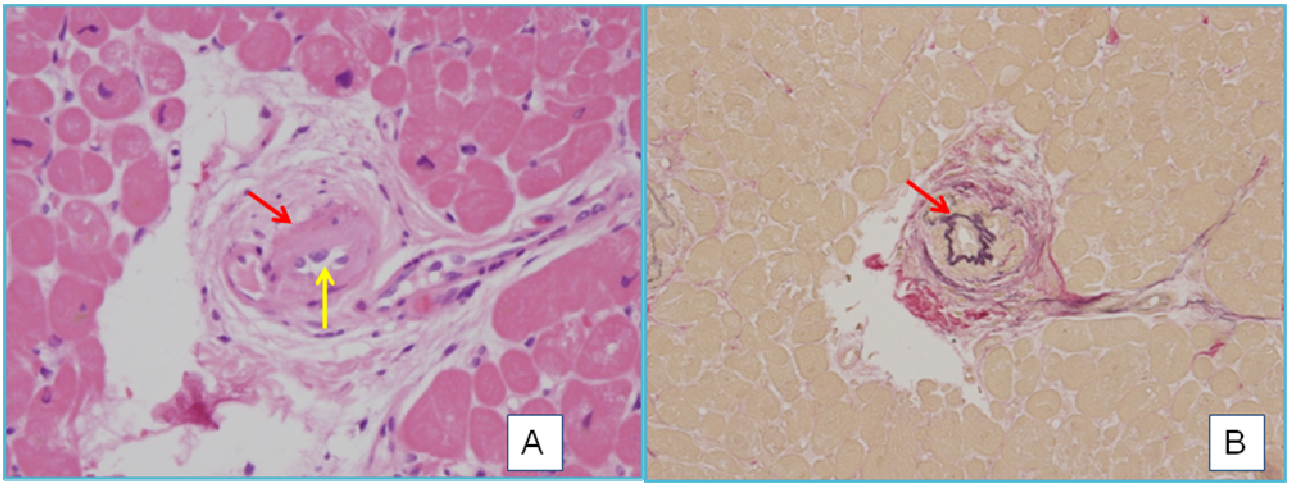



*Figure 16. A H&E staining showed intramyocardial arteriola with a narrowing lumen caused by thickening of arteriola's wall(red arrow) and endothelial layer thickening with prominent endothelial nuclei (yellow arrow) (200x of magnification). In figure B elastic fiber van Gison special staining was performed to evidence the internal elastic lamina (red arrow) and evaluated the thickening of arteriola's wall and to confirm the diagnosis of stenotic microvasculopathy.*

# 7 DISCUSSION

The most important findings of this PhD thesis indicate that C4d predicts worse prognosis and the presence of DSA and graft dysfunction further improve risk stratification. C4d+ve and DSA can be used as early mortality predictors in patients without signs of graft dysfunction. Moreover AMR is a complex and ongoing phenomenon with different phenotypic features. C4d positivity detected on EMBs is an important marker for diagnosis of AMR identifying also the asymptomatic AMR.

Morphological parameters alone, that are considered the fist screening tool for AMR, are not adequately sensitive and with low positive likelihood ratio to be used as screening tools for early asymptomatic AMR detection. Screening recommendations should, therefore, be modified to include more sensitive tests such as C4d staining in the routine protocol to improve patient risk stratification.

Our results recognized : 1) the importance of C4d screening to identify subclinical humoral rejection; 2) the presence of late AMR entity that could presented in the long time period after transplantation; 3) the relation between anti HLA antibodies and chronic rejection.

### *7.1 Standardization of the immunohistochemistry method of C4d.*

Due to the evidence of difficult approach in the routinely management of frozen tissue and IF stain for complement activation diagnosis, many heart transplant centres were looking for standardization of immunoperoxisade stain on endomyocardial biopsy . the introduction on the market of a new antibody for the identification of C4d fragment of the complement cascade required assessment of the feasibility and reproducibility of the method of C4d immunoperoxidase staining on paraffin embedded tissue. We have chosen to adopt the Chantranuwat et al [86] grading since it represents the most frequently adopted scheme for heart in the literature for paraffin-embedded samples and it turned out to be an easy and practical tool. [111](personal communication on ISHLT 2011 and BANFF 2011)

The results from the multicenter study on the standardization of the protocol for C4d staining confirmed the consistency of our protocol and adopted grading scheme .

### *7.2 Clinico-pathological profile of AMR of adult population*

Our findings indicated that C4d staining performed on a routine basis after heart transplantation on paraffin embedded tissue sections can predict outcome in heart transplant recipients and supporting the utility of C4d stain on routine base. Moreover categorizing patients into 4 groups on the basis of their C4d, DSA and graft function profile stratify the mortality risk [61], the higher the group the worse the outcome.. Group 3 ( C4d, DSA and graf dysfunction ) had half the mortality risk of group 2 (32 vs 61 fold increase) is only apparently contradictory. Group 3 had the longest time interval between heart transplant and EMB C4d positivity but the shortest survival time between C4d +ve

and death. Early acute versus late acute of chronic rejection episods, humoral or cellular-humoral, might represent different pathogenic situation with different prognosis, virus infections, particularly cardiotrophic virus, iimunosopression, lymphoproliferative disorders, solid neoplasia, might producing "injury" with antigen modifications and consequently antibody formation. Injury produced by activation of the complement cascade on the endothelium requires time to act and to dissolve the equilibrium produced by the regulatory mechanism (CD55 and CD59 proteins) for blockage of complement cascade [84], but when dysfunction appears the patients' outcome worsens.

Group 1 could represent those patients in whom DSA, although present, are undetected for two possible reasons: 1) the insensitivity of antibody detection methods; 2) sequestration of low levels of DSA in the graft [112].

Intragraft deposition of C4d complement split fragments could, then, be regarded as 1) a subclinical form of AMR incapable of producing clinically relevant graft dysfunction but acting over a long period of time as an immunological *noxa* contributing to allograft vasculopathy or 2) accommodation as acquired resistance to pathological effects of graft-specific antibodies and complement fixation.[45, 96, 113, 114]. In a recent paper, Rodriguez et al. hypothesized that the complement cascade could be inhibited by the presence of regulatory proteins CD 55 and CD59 capable of halting the activation of complement cascade[82,115, 116] . Our findings indicate that over time the graft's ability to inhibit/neutralize the complement cascade activation on endothelial cells could decrease leading to graft dysfunction.

Our results are at difference with those of Wu et al.[82], the first to assess asymptomatic AMR patients and to compare them to treated AMR, who reported no differences in fact between asymptomatic (our group 1 + 2), treated AMR (our group 3) and controls (our

group 0) in terms of their 5 year survival rate. They did not, assess the presence of DSA in their population of asymptomatic patients. Our study, in which DSA was assessed and the asymptomatic patients were divided into DSA positive and negative groups, showed that detection of DSA is a negative prognostic marker indicative of worse outcome [117].

Clinical data considered were unable to differentiate between the symptomatic and asymptomatic patients and between both of them and the controls . No factors, like positive PRA with impact of sensitization, were found not significant. We cannot exclude that this could be ascribe to low percentage of PRA before transplant which is low compared to majority of other studies of HTx (10-20%) reasonable explanation the high proportions of male (80%) included in this study. Patients with more than one EMB specimen positivity for C4d (25% of the C4d positive patients) had a worse survival rate and more severe rejection scores. These results are compatible with the hypothesis that serial occurrences of C4d positivity are indicative of repetitive episodes of endothelial injury [118]. Our data seem to parallel those of Hammond et al. who found that repetitive episodes of AMR increase the risk of cardiovascular mortality with an incremental risk of 8% for each episode[77].

Controversy continues to grow with regard to the significance of C4d positivity and consequently to patient management both in the early and late post-transplantation stages as little data are available especially with regard to paraffin embedded tissue [98, 119, 120]. Our findings indicate that C4d positivity is associated with a poor outcome. If and how these patients should be treated is another controversial issue, but plasmapheresis, intravenous immunoglobulins and rituximab could be an initial therapeutic approach. Close surveillance is mandatory to detect early signs of graft dysfunction.

In contrast with previous studies[60, 121] showing a correlation between AMR and onset of CAV and between Asymptomatic AMR and CAV, we were unable to find any correlation between C4d positivity and development of CAV . However we detected only DSA no other type of antibody which has been recognize as potential triggers of AMR. We can not exclude that lack of correlation could be ascribed to the presence of no-complement fixed antibodies or to the numerosity of the groups, to the recognized low sensitivity of angiography to detecting CAV or even to the fact that C4d at immunoperoxidase is less sensitive than at immunofluorescence [76]. The relatively small number of patients evaluated and the fact that circulating DSA were not routinely assessed in the C4d negative patients are all limitations of this study. Notwithstanding these constraints, the implications of the use of C4d immunostaining in the surveillance of heart transplant recipients warrant consideration and further studies.

### *7.3 Clinico-pathological profile of Pediatric population*

This study reported our experience on a small population of pediatric HTx from a single institution who were screened for AMR pathological criteria [13]. We found 14% of our pediatric population with C4d positivity on EMBs and 57% of them had elevated PRA. Elevated PRA pretransplantation has been described as risk factors for AMR.[122]. However PRA monitored by CDC method underestimated the prevalence of anti HLA antibodies.[123, 124]. In our study all positive cases with antiHLA antibodies were detected by Luminex methodology. On the contrary they were false negative with CDC method. Most of pediatric pts with positive C4d were without graft dysfunction at the time of EMB. Only one patient had clinical symptoms.[55].

We recommended C4d immunostaining routinely also in the pediatric populationThe evaluation of PRA pre-transplant should be performed with a more sensitive method such as Luminex to identify patients at risk of developing AMR: [125]

### *7.4 Pilot study on reproducibility of ISHLT2004 classification system*

The main objective of this European network is to address diagnosis and research to evaluate the reproducibility of Acute Cellular Rejection grading system. We have select only two cases of AMR asking to pathologists to recognize AMR with only on Haematoxilyn & Eosin stain.

The most important result is AMR [82, 101, 117] that morphology on Haematoxylin Eosin is not specific and cannot be taken as a guiding tool to identify or to raise the suspicion of AMR, thus requiring further histochemical and immunological evaluation to reach the diagnosis of antibody mediated rejection [84]. It was questioning the criteria adopted by ISHLT 2004 classification and forced us to further evaluation with a more extensive study of morphologic parameters to better definition of the morphological and immunopathological criteria for both symptomatic and asymptomatic AMR.

In the definitive study , already planned with more cases representative of the distribution of the grading scale in every day experience, it will be also important to evaluate an increase number of cases to identify eventually the area of major difficulties in interpretation by pathologists.[126]

### *7.5 Morphological parameters on EMBs to detect AsAMR and AMR.*

In the light of this study's findings, the eight histological characteristics of endothelial, myocyte and vascular damage, previously identified as markers of AMR[13, 102], do not appear to be efficacious tools to detect allograft antibody-mediated rejection in heart

transplant recipients. The recent demonstration that asymptomatic AMR can be present in patients with circulating DSA and intracapillary deposition of C4d, but without clinical dysfunction [6,10,82], have encouraged pathologists to continue the search for markers detecting early antibody mediated damage to the graft even before there are any clinical manifestations. To our knowledge few data can be found in the literature regarding the efficacy of histological features in detecting AMR (106), and for the most part referring to immunofluorescence markers on frozen tissue as main criteria of AMR and no data in relation to C4d IHC on formalin-fixed paraffin embedded tissues.

The histological features evaluated in our study were those selected taking into account data in the literature and included in the ISHLT Working formulation.

According to the 2005 ISHLT consensus recommendations, only when there are positive histological features indicative of AMR is further immunohistochemical testing required. In fact, the most recent diagnostic criteria of AMR included: endothelial-cell swelling and intravascular macrophage accumulation with immunophenotypic evidence of immunoglobulin and complement deposition in capillaries by immunofluorescence on frozen sections or by immunohistochemistry on formalin -fixed, paraffin embedded material and CD68 staining of intravascular macrophages in capillaries. Other criteria such as myocyte damage, intracapillary leucocytes, interstitial edema, haemorrhage, and microthrombi were considered of low diagnostic value in the light of their low sensitivity for AMR since they are shared by other conditions such as acute cellular rejection (ACR) and ischemia-reperfusion injury (IRI) and, if present , are associated to later stages and more severe AMR.

The sensitivity and specificity of pathological markers are, moreover, strongly influenced by interobserver variability in the pathologic interpretation of endomyocardial biopsy

results and by different criteria used in the formulation of a histological diagnosis[127]. Through its task force, the ISHLT continues to strive to address the inconsistencies in the use of their grading system and technical considerations in biopsy processing and evaluation.

At the moment criteria for the diagnosis of AMR are based on the histological identification of antibody mediated myocardial damage and the detection of complement activation fragments deposition, evidenced by intracapillary staining for C4d. In our study morphology on H&E staining, C4d positivity, circulating DSA, and graft dysfunction were evaluated separately and compared with histological features in the attempt to identify a more complete, consistent guideline for diagnosis. Considering C4d positivity alone at IHC (the immunopathological parameter) the sensitivity and specificity were poor for all eight morphological features evaluated. The same was true if EMBs in patients with C4d positivity and DSA and/or graft dysfunction were considered. While endothelial swelling had an high sensitivity, its specificity was low, meaning that high number of true negative patients could not be ruled out. Endothelial swelling and edema could be present in many cases without both asymptomatic and symptomatic antibody mediated rejection as in the setting of ischemic-reperfusion injury, inflammatory cell infiltration, late ischemic damage, infection. There was no statistically significant difference between DSA positive and negative patients and between AMR and negative controls. While these results are in keeping with those reported by Michaels [55], the fact that macrophages had a relatively high specificity but low sensitivity and were present in only one third of patients with C4d and DSA positivity makes this parameters inadequate for screening purposes [128].

As for the myocyte injury feature C4d positive EMBs and C4d negative EMBs did not show any statistically significant difference. The presence of myocyte injury in C4d negative

EMBs could be unexpected. However this could be the result of different noxae as the early ischemic-reperfusion damage (within the first 3 months) or late after 3 months as the result of earlier development of CAV or microvasculopathy in marginal donors. In fact in the last years in our institution an increasing number of marginal donors has been reported due to the decrease in donations. Infections also could account for a cytopathic effects at any time.

Even markers considered indicative of more severe forms of AMR (presence of neutrophils, hemorrhage and microthrombi) were found to be highly specific but with low sensitivity meaning that they are absent in many positive cases and as such cannot be considered diagnostic markers . They are not always present in positive patients but when present they are highly suggestive of AMR. When C4d-positive repeat biopsies were evaluated the results were similar to those for single episodes. The ROC curve for the two parameters considered together did not detect enhanced sensitivity and specificity in indentifying DSA or C4d positivity.

In conclusion these results suggest that morphological parameters alone are not adequately sensitive and with low positive likelihood ratio to be used as screening tools for early asymptomatic AMR detection. Screening recommendations should, therefore, be modified to include more sensitive tests such as C4d staining in the routine protocol to improve patient risk stratification.[129-131].Further multicenter study collecting more C4d positive cases are needed to validate our results

### *7.6 immunostaining for C3d in the diagnosis of AMR.*

Our results showed that C3d on formalin fixed paraffin embedded tissue did not contribute to increase the sensitivity and specificity in identifying pts affected by

symptomatic or asymptomatic AMR carrying circulating DSA, which are considered our gold standard for AMR. Although C3d was more specific in detecting DSA vs NDSA ( 55% vs 70% specificity, respectively) , it did not increase the specificity of C4d to recognize DSA.

The significance of C3d in the diagnosis of AMR is still controversial. Our result s are in keeping with those reported in kidney transplantation, which showed 50% of C3d positivity in pts with C4d positivity and AMR [48, 49]. Rodriguez ER et al already reported the presence of both positivity for C4d and C3d in all AMR subgroups ,using immunofluorence . . C4d and C3d were presented also in asymptomatic AMR group [61, 84].

In our study C3d did not improve our ability to identify circulating DSA and this is in keeping with Rodriguez et al; different combination of C4d and C3d positivity in symptomatic and asymptomatic AMR could be justify by the "accommodation"phenomenon and/or by the presence of complement regulators on the endothelium..

Similar to Rodriguez experience with immunofluorence we also found multiple combination of C4d+ and C3d+, C4d+ C3d-, C4d- C3d+, C4d- and C3d-. The cases in which C4d + but not C3d was detected may represent a subgroup of patients in whom no or limited C3 activation occurred after the deposition of C4d because of the presence of effective complement regulatory factors Rodriguez ER[59] and Tan CD[82] showed a higher sensitivity and specificity of C3d to detect circulating DSA and humoral rejection.

In our experience C3d did not improve our ability to detecte symptomatic and asymptomatic AMR, C4d and C3d seems to carry the same sensitivity and specificity in detecting DSA as C4d alone. Combined C3d and C4d did not increase our ability to

detected DSA in HTx patients. Differences in sensitivity and specificity of immunofluorence and Immunoperozidase could explain these diffent results.

Since C3d is not standardized yet and recognized as valuable antibodies to be applied in the diagnosis of AMR both for immunofluorence and immunoperozidase we concluded that nowadays C4d is the best marker to detected circulating antibodies in HTX recipients also those asymptomatic. [132]

### *7.7 Alloantibodies profile in the symptomatic AMR*

This preliminary study indicated that de novo DSA are frequent in patients presenting with indications for late endomyocardial biopsies and they are associated to 57% of C4d positivity. De novo DSA were mostly class II HLA type. Previously studies on kidney transplantation [133] indicated association between de novo DSA class II and late microvascular injury on renal biopsy and graft loss. Also in heart transplant patients a similar predominance of DSA class II in association with CAV and decrease in graft survival is observed as well as the association of high grade cellular rejection and increase risk of accelerated CAV [134]. In our previously study[92] we showed that AMR group has worse outcome compared to C4d negative patients, all cases developed AMR in the late period.

The capability to HLA-DSA to activate the complement system is likely a key element for their clinical relevance. In our data C4d positivity did not correlate with MFI. Honger et al [135] showed that in vitro complement activation do not predict the occurrence of AMR. Burns et al [136] demonstrated in patients with pretransplant HLA-DSA that only those with increasing HLA-DSA titer post-transplant developed AMR. This suggested that the occurrence of humoral memory response is the key factor for the development of AMR and could explain our apparently contradictory results. Complement activation by HLA

antibodies is influenced by several factors: 1) antibody concentration 2) specificity for the target epitope 3) the proximity of the targeted molecules/epitopes allowing for synergistic action of several antibodies [137, 138].

Our preliminary data showed : 1) the difference of type of anti HLA-DSA in the early and late period post-transplant, 2) low levels of MFI do not correlate with AMR or C4d deposition on EMBs, 3) humoral memory response and specificity for the target epitope can explain the absence of correlation between complement activation and development of AMR.

### *7.8 Microvaculopathy and C4d*

To investigate the hypothesis that chronic complement activation and/or presence of anti HLA-antibodies could develop microvasculopathy with consequence reduction of coronary flow reserve we compared C4d positive patients with CFR measurements. Our results showed the important correlation between positive staining for C4d on EMBs (complement activation) and reduction of CFR, establishing a strict relation between complement activation and microvascular damage. Hiemann et al [39] showed a prevalence of microvasculopathy within the first year after HTx. This findings may represent microvascular remodeling characterized by an increase of vascular smooth muscle alfa actin over time [139]. Endothelial swelling has been linked to poor survival, and it was significantly associated with stenotic microvasculopathy[39]. The same risk factors for developed of CAV such as age of recipient, or donor, CMV were identified for stenotic microvasculopathy [140, 141]. For the first time we identified the association between humoral rejection and immune-mediated mechanism for development of microvasculopathy.

Microvasculopathy may be a sensitive marker of development of epicardial vasculopathy and it could be found even months before manifestation of epicardial vasculopathy. The relation between complement deposition C4d on EMBs and reduction of coronary flow reserve showed the strict relation of antibody mediate damage and chronic rejection, the "key" of the interpretation of pathophysiology of AMR and cardiac allograft vasculopathy, the major cause of heart graft loss.

# 8 CONCLUSIONS

Our findings indicate that AMR is a complex and ongoing phenomenon with different phenotypic features. C4d+ve is an important marker for diagnosis of AMR , thus identifying asymptomatic AMR .

C4d predicts worse prognosis and DSA and graft dysfunction further improve risk stratification. C4d+ve and DSA can be used as early mortality predictors in patients without signs of graft dysfunction.

Morphological parameters alone, , are not adequately sensitive and carry low positive likelihood ratio as screening tools for early asymptomatic AMR detection. Screening recommendations has been recently modified to include more sensitive tests such as C4d staining in the routine protocol to improve patient risk stratification.

The assessment of anti HLA antibodies, class I and II and their relation with C4d (complement activation)showed : 1) a difference in type of anti HLA-DSA in the early and late period post-transplant,with class I anti HLA early appearance and class II anti HLA late after TX 2) low levels of MFI do not correlate with AMR or C4d deposition on EMBs, 3) humoral memory response and specificity for the target epitope can explain the absence of correlation between complement activation and onset of AMR.

Endothelial damage and complement deposition produce microvascular remodeling and microvasculopathy with increased graft loss.

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