

## Coincidence of cellular and antibody mediated rejection in heart transplant recipients – preliminary report

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Kardiologia i Torakochirurgia Polska 2014; 11 (1): 52-55

### Abstract

Antibody mediated rejection (AMR) can significantly influence the results of orthotopic heart transplantation (OHT). However, AMR and cellular rejection (CR) coexistence is poorly described. Therefore we performed a prospective pilot study to assess AMR/CR concomitance in endomyocardial biopsies (EMBs) obtained electively in 27 OHT recipients (21 M/6 F, 45.4 ± 14.4 y/o). Biopsy samples were paraffin embedded and processed typically with hematoxylin/eosin staining to assess CR, and, if a sufficient amount of material remained, treated with immunohistochemical methods to localize particles C3d and C4d as markers of antibody dependent complement activation. With this approach 80 EMBs, including 41 (51%) harvested within the first month after OHT, were qualified for the study. Among them 14 (18%) were C3d+, 37 (46%) were C4d+, and 12 (15%) were both C3d and C4d positive. At least one C3d+, C4d+, and C3d/C4d+ EMB was found in 10 (37%), 17 (63%), and 8 (30%) patients, respectively. Among 37 CR0 EMBs C3d was observed in 4 (11%), C4d in 17 (46%), and both C3d/C4d in 3 (8%) cases. Among 28 CR1 EMBs C3d was observed in 3 (11%), C4d in 11 (39%), and C3d/C4d in 3 (11%) cases. Among 15 CR2 EMBs C3d was observed in 7 (47%), C4d in 9 (60%), and C3d/C4d in 6 (40%) cases. Differences in C3d and C3d/C4d occurrence between grouped CR0-1 EMBs and CR2 EMBs (7/65 – 11% vs. 7/15 – 47%; 6/65 – 9% vs. 6/15 – 40%) were significant ( $p = 0.0035$  and  $p = 0.0091$ , respectively,  $\chi^2$  test). In conclusion, apparently frequent CR and AMR coexistence demonstrated in this preliminary study warrants further investigation in this field.

**Key words:** heart transplantation, cellular rejection, antibody-mediated rejection.

### Streszczenie

Odrzucanie zależne od przeciwciał (AMR) może wpływać na wyniki ortotopowej transplantacji serca (OHT), ale jego współwystępowanie z odrzucaniem komórkowym (CR) nie zostało wyczerpująco opisane. Autorzy przeprowadzili prospektywne badania pilotażowe oceniające współistnienie AMR i CR w biopsjach endomiokardialnych (EMB) pobranych planowo od 27 biorców OHT (21 M/6 K, 45,4 ± 14,4 roku). Biopsje endomiokardialne po zatopieniu w parafinie były przeprowadzane w typowy sposób z użyciem barwienia hematoksyliną/eozyną dla oceny CR, a pozostały materiał był barwiony immunohistochemicznie w celu lokalizacji składników C3d i C4d dopełniacza, jako markerów AMR. Do badania pozyskano 80 EMB, w tym 41 (51%) pobranych w ciągu miesiąca od OHT. Wśród nich 14 (18%) było C3d+, 37 (46%) – C4d+, a 12 (15%) równocześnie C3d i C4d pozytywne. Przynajmniej jedną C3d+, C4d+ i/lub C3d/C4d+ EMB stwierdzono odpowiednio u 10 (37%), 17 (63%) i 8 (30%) pacjentów. Wśród 37 EMB ocenionych jako CR0 C3d obserwowano w 4 (11%), C4d w 17 (46%), a C3d i C4d w 3 (8%) przypadkach. Wśród 28 EMB z CR1 C3d odnotowano w 3 (11%), C4d w 11 (39%), a C3d/C4d w 3 (11%) przypadkach. W grupie 15 EMB wykazujących istotne odrzucanie CR2 C3d było obecne w 7 (47%), C4d w 9 (60%), a C3d/C4d w 6 (40%) przypadkach. Różnice w częstości występowania C3d i C3d/C4d pomiędzy EMB CR0-1 i CR2 (7/65 – 11% vs 7/15 – 47%; 6/65 – 9% vs 6/15 – 40%) były istotne (odpowiednio:  $p = 0,0035$  i  $p = 0,0091$ , test  $\chi^2$ ). Podsumowując – wysoka częstość współwystępowania AMR i CR już na etapie pilotażu uzasadnia kontynuację badań tego zjawiska.

**Słowa kluczowe:** transplantacja serca, odrzucanie komórkowe, odrzucanie zależne od przeciwciał.

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## Introduction

There has been vigorously growing interest recently in the field of transplanted heart damage caused by antibodies against donor HLA and complement activation, evolving from the early concept of hyperacute humoral rejection [1] to the relatively well-established modern definition of antibody-mediated rejection (AMR) [2-4]. This progress, assessed due to the easy availability of tools to depict complement involvement in endomyocardial biopsies (EMBs) [5], has changed the idea of AMR, which is now thought to be a phenomenon typical not only for the early phase but also for the late phase after orthotopic heart transplantation (OHT) [6], and it is often linked to cardiac allograft vasculopathy (CAV) [7]. Moreover, it seems possible to treat AMR more and more effectively with the use of modern drugs aimed directly at antibody production [8-10]. Despite these advances, there are still some serious doubts about the role of the complement fragments' deposition in EMBs [11], and a proper definition of AMR [12].

Surprisingly, relatively little is known about the coincidence between AMR and cellular rejection (CR), which remains the most frequent immunologic-based complication of OHT [13]. Therefore we aimed to assess the incidence of concomitant AMR and CR occurrence in a prospective study involving OHT recipients.

## Material and methods

A group of 27 patients after OHT performed in our institution, characterized in Table I, was enrolled in the study. Twenty-four of them were consecutive OHT recipients included during the 1<sup>st</sup> year after the surgery, undergoing elective EMBs according to the local protocol (4 EMBs every week starting on the 7<sup>th</sup> day after OHT, followed by the EMBs obtained at the end of the 6<sup>th</sup> and 8<sup>th</sup> week, and the 3<sup>rd</sup>, 6<sup>th</sup>, 9<sup>th</sup>, 12<sup>th</sup>, 18<sup>th</sup>, 24<sup>th</sup> and 36<sup>th</sup> month after OHT). All these patients were without any clinical or echocardiographic signs of transplanted heart malfunction. The decision to perform EMB in the remaining 3 patients over 1 year after OHT was undertaken during the elective outpatient visit due to the drop of left ventricle contractility assessed by ejection fraction (LVEF) using echocardiography. One of these patients was the only one to present mild symptoms of heart failure, including mild deterioration of exercise tolerance (NYHA II).

All patients were treated with triple drug immunosuppression until the end of the 1<sup>st</sup> year after OHT, with a calcineurin inhibitor (tacrolimus in 26 patients and cyclosporine in 1 patient), lymphocyte proliferation inhibitor (mycophenolate mofetil in 26 patients and azathioprine in 1 patient), and prednisone (which was discontinued at the end of the 12<sup>th</sup> month after OHT). Additionally, all 24 patients enrolled within the 1<sup>st</sup> year after OHT received two typical doses of basiliximab perioperatively.

EMBs were obtained using typical access (mostly the jugular vein) from the interventricular septum, embedded in paraffin, and after routine pathologic processing including hematoxylin/eosin staining CR was graded using the

ISHLT scale [14]. According to the essence of the agreement issued by the bioethic committee of the local medical school, none of the specimens was harvested to assess AMR, but only the material left after CR evaluation was used for further studies if sufficient. With this approach 80 paraffin embedded biopsy samples were qualified for immunohistochemical analysis.

Tissue slices 5  $\mu$ m thick were placed on poly-L-lysine coated slides, then slides were dried in a paraffin oven at 58°C for two hours. After deparaffinization in xylene, the slides were rehydrated in decreasing concentrations of ethyl alcohol and the antigen retrieval procedure in a pressure cooker in TRILOGY solution took place (100°C, 20 min). The slides were washed with Tris solution, and endogenous peroxidase was blocked using Hydrogen Peroxide Block (Thermo Scientific) solution for 10 min. Ready to use antibodies against C3d and C4d (Thermo Scientific) were used in the incubation procedure (humid chamber, room temperature). The coupled antibodies were detected using the UltraVision Quanto Detection System (Thermo Scientific) with subsequent diaminobenzidine developing system. Finally, nuclei were counterstained with hematoxylin and slides were dehydrated and mounted in synthetic resin.

Results are presented as the absolute and proportional values. Due to the low numbers, the statistical analysis was limited to the  $\chi^2$  test.

## Results

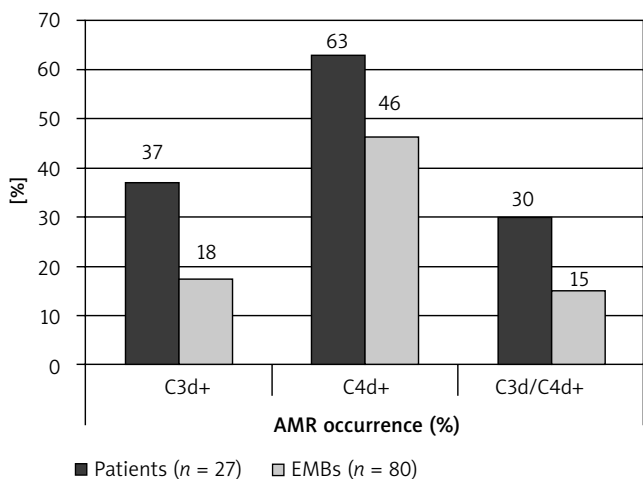
The overall number of patients with at least one EMB positive for C3d and/or C4d, as well as the number of EMBs positive for C3d, C4d and both of them occurring concomitantly, is presented in Figure 1.

Coincidence of CR and AMR is illustrated in Figure 2. Grouping EMBs with CR grades 0 and 1 ( $n = 65$ ) to compare their results with biopsies showing significant rejection (CR2,  $n = 15$ ), we were able to demonstrate that C3d occurrence (7 out of 65 – 11% vs. 7 out of 15 – 47%) and concomitant C3d and C4d occurrence (6 out of 65 – 9% vs. 6 out of 15 – 40%) were significantly more frequent in patients with the presence of acute cellular rejection ( $p = 0.0035$  and  $p = 0.0091$ , respectively).

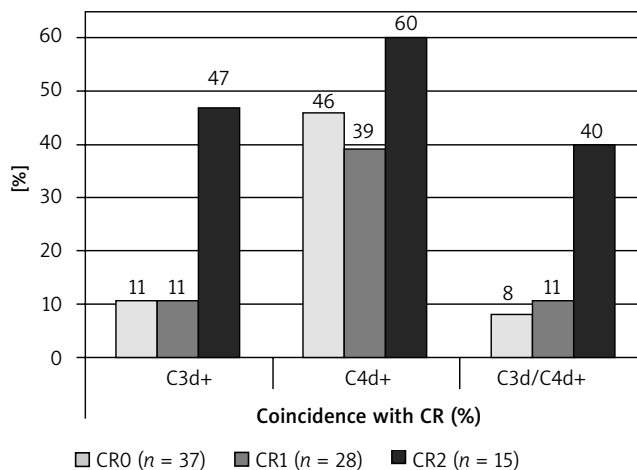
Tab. I. Description of the study group

No. of patients	27
Age [years]	45.4 $\pm$ 14.4
Gender (M/F)	21 (78%)/6 (22%)
Indication for OHT (CAD/nCAD)	9 (33%)/18 (67%)
Donor age [years]	31.4 $\pm$ 9.1
Donor/recipient gender match (Y/N)	20 (74%)/7 (26%)
Ischemic time [minutes]	167 $\pm$ 54

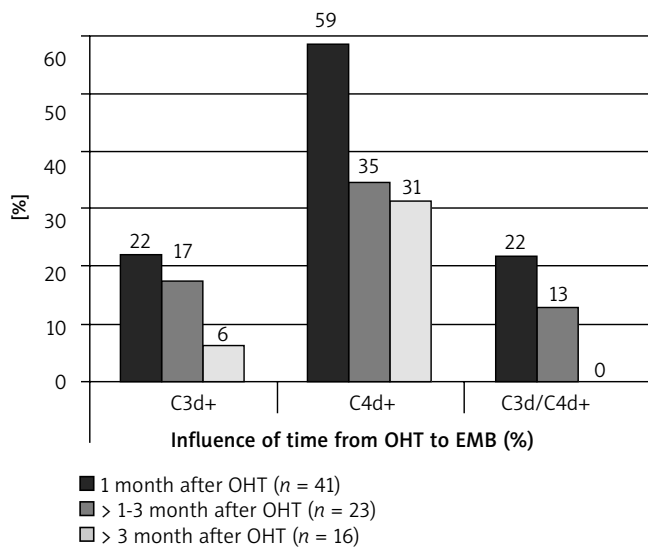
M – male, F – female, OHT – orthotopic heart transplantation, CAD – coronary artery disease, nCAD – no coronary artery disease, Y – yes, N – no



**Fig. 1.** Percentage of patients with at least one positive endomyocardial biopsy (EMB), and percentage of all EMBs positive for C3d, C4d, and concomitant occurrence of both C3d and C4d in the same sample



**Fig. 2.** Percentage of EMBs positive for C3d, C4d, and concomitant occurrence of both C3d and C4d in the same sample according to the presence of cellular rejection (CR) grades 0-2



**Fig. 3.** Percentage of EMBs positive for C3d, C4d, and concomitant occurrence of both C3d and C4d in the same sample according to the time between orthotopic heart transplantation (OHT) and biopsy collection

The influence of the time between OHT and EMB procedures on AMR occurrence is presented in Figure 3. The only statistical significance was demonstrated when the number of C4d positive EMBs was compared between samples obtained within the 1<sup>st</sup> month after OHT and later (24 out of 41 vs. 13 out of 39,  $p = 0.042$ ).

During 12 months of follow-up, between December 2012 when the 1<sup>st</sup> EMB included in the study was obtained, and December 2013 when the database was completed, none of the patients experienced deterioration of transplanted heart function. The only death was due to complications after non-cardiac surgery, and occurred in a C3d/C4d negative patient.

### Discussion

Despite the low number of enrolled OHT recipients and analyzed EMBs, the observation that occurrence of complement activation features, particles C3d and/or C4d deposition, is correlated with CR presence and the early phase after OHT, when CR is the most frequent consequence of immunologic mismatch between donor and recipient, seems to be the apparent result of this study.

A high level of CR and vascular rejection (in 33% of AMR cases) was revealed in relatively old papers by Hammond *et al.* [15, 16]. There is also a far more recent report by Loupy *et al.* [17] showing frequent CR/AMR concomitance (55%), but it was observed over 7 years after OHT. However, current guidelines claim that it is infrequent and more characteristic for lower grades of cellular rejection (currently described as CR1), but without announcement of the basic source of this information [4]. Additionally, the authors of this statement acknowledge that CR and AMR coexistence is not surprising, considering current understanding of transplant immunology mechanisms, but it is poorly explored due to the common practice of excluding mixed CR/AMR cases from further analysis to “purify” the newborn concept of antibody-caused rejection. This approach seems to be a little bit reckless in a field limited by a huge number of black holes, heavily experienced also by the authors of this paper.

First of all, it is not certain if the presence of C3d and/or C4d justifies the nomenclature of rejection. It should be easier in the presence of myocardial damage (observed in CR2 cases), but still it can be questionable in the absence of transplanted heart failure features. The presence of C4d deposits in stable long-term survivors, described by many authors [18, 19], also puts in doubt its role as a marker of treatment-requiring rejection. The choice of C3d and C4d as markers of AMR seems to be the best anchored in the literature [5, 20]; however, current guidelines underline the role of macrophage antigen staining to establish the AMR

diagnosis when using paraffin section immunohistochemistry [4, 21], which was not performed as part of this study. We also lack an investigation of the presence of donor-specific antibodies in our patients, while there is a growing body of evidence for their crucial role in the development of the clinical consequences of AMR [22-25].

All these important limitations, which should be considered in the context of the preliminary nature of the current study, do not challenge our conclusion that further studies to establish the frequency and role of AMR and CR coexistence early after OHT are warranted.

## Acknowledgments

This study was supported by Medical University of Silesia grant no. KNW-1-156/P/2/0.

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