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Article

Class-II Molecular Mismatch as an Independent Risk Factor of Chronic Allograft Lung Dysfunction Development in Lung Transplantation

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Abstract

Chronic allograft dysfunction (CLAD) is the main cause of graft loss after lung transplantation (LTR). Within the immunological factors involved in CLAD development, the antibody-mediated rejection (ABMR) has the most impact. However, ABMR diagnosis is difficult due to the limited sensitivity of histopathological, immunohistochemical, and immunological criteria currently used. Growing evidence is demonstrating the impact of molecular mismatch in ABMR; here, we ought to assess the potential role of molecular mismatch in CLAD development. A total of 457 LTR were recruited for the study, with HLA type from donors and recipients to assess molecular mismatch, and with a minimum follow-up of 180 days. The combination of molecular mismatch in class-II (HLA-EMMA and HLA-Matchmaker algorithms) with EMMA DR score >12 and antibody verified eplet mismatch in DRB1345 (AbV DRB1345) > 3 predicts CLAD development independently of ex-smoker, prolonged period of hospitalization (>33 days), acute cellular rejection (ACR), and ABMR. The HR of the prediction model for molecular mismatch in class-II was 1.52 (1.01-2.56, p=0.045). This observation could point to a potential role of poor molecular mismatch in class-II to fill the gap of underdiagnosis of ABMR, previous to CLAD development. Prospective studies should be addressed to confirm the utility of molecular mismatch in the identification of patients at risk of CLAD development.

Keywords: molecular mismatch; immunological risk; CLAD; prediction; lung transplant; ABMR; ACR

1. Introduction

Chronic Lung Allograft Dysfunction (CLAD) is the main cause of graft loss after lung transplantation, directly impacting both patient and graft survival [1]. Immunological and non-immunological factors are involved in CLAD development, but other factors remain to be elucidated [2].

Within the immunological factors, the antibody-mediated rejection (ABMR) is well characterized. However, ABMR diagnosis in lung transplantation remains elusive. The last Banff Lung report criteria classification points to a multidimensional approach for ABMR diagnosis, where

all immunological, histopathological, and immunohistochemical criteria should be fulfilled [3]. There are patients with suspicion of ABMR without donor-specific anti-HLA antibodies (DSA), together with low sensitivity of histopathological and immunohistochemical findings, which make ABMR diagnosis a real challenge [3,4].

Here, we focus on the immunological factors. The classical way to assess immunological compatibility is based on human leukocyte antigen (HLA) match, where the most ABDR HLA mismatch, the worst long-term graft survival in solid organ transplantation, in the kidney [5], liver [6] and heart [7] including lung transplantation [8].

New molecular biology methods in HLA typing have enabled the development of novel approaches to assess donor-recipient compatibility, such as molecular mismatch, based on different algorithms that could better define the immunological risk of donor-recipient pairs. In fact, there is increasing evidence supporting that high molecular mismatch increases the risk of *de novo* anti-HLA antibodies, using a single algorithm as HLA-Matchmaker [9], PIRCHE [10] or combined algorithms [11,12]. Moreover, the combination of algorithms improved the prediction of ABMR in pediatric cardiac allografts [13].

We aim to study the potential role of MM algorithms in CLAD prediction after lung transplantation.

2. Results

2.1. CLAD Development in Lung Transplantation

A total of 592 lung transplant recipients (LTR) were performed in our institution from 2010 to 2024. Ninety-five LTR without HLA type data from donor or recipient were discarded. In addition, 40 of 497 remaining LTR had a follow-up less than 6 months. Finally, a total of 457 lung transplant recipients (LTR) were included in the analysis. The main parameters studied are summarized in Table 1.

Table 1. Description of the parameter included in the study: Recipients, donors, lung transplants, clinical and immunological parameters.

Parameter		Total	Frequency (%)	Mean (SD)	Median (IQR)
Recipient	Recipient sex (% female)	457	180 (39.39)		
	Recipient age (years)	457		56.8 (9.9)	60.0 (53.9-63.5)
	Recipient smoker (never)	454	105 (23.13)		
	Tobacco consumption (cigarettes/day)	341		37.2 (22.1)	35 (20-50)
	Hypertension (yes)	455	91 (20.00)		
	Diabetes (yes)	455	41 (9.01)		
	Dyslipidemia (yes)	453	147 (32.45)		
	Size (meters)	396		1.65 (0.09)	1.66 (1.59-1.72)
	Weight (kilograms)	397		68.0 (12.7)	68 (59-77)
	BMI (kg/m2)	396		24.76 (3.55)	25.0 (22.2-27.7)
	LAS score	347		34.4 (3.56)	33.3 (32.1-35.5)
Donor	Donor sex (% female)	457	236 (51.64)		
	Donor age (years)	457		52.3 (14.2)	55 (44-63)
	Donor smoker (yes)	439	152 (34.62)		
	Hypertension (yes)	436	144 (33.03)		

	Diabetes (yes)	415	34 (8.19)		
	Donor size (meters)	452		1.67 (1.16)	1.69 (1.62-1.75)
	Donor weight (kilograms)	452		73.26 (13.33)	75 (65-80)
Immunological	Anti-HLA class-I antibodies pre transplant (yes)	349	27 (7.74)		
	Anti-HLA class-II antibodies pre transplant (yes)	349	17 (4.87)		
	Anti-MICA antibodies pre transplant (yes)	457	4 (0.88)		
	Antibody verified eplet mismatch All	457		16.91 (6.33)	16 (13-21)
	Antibody verified eplet mismatch ABC	457		8.70 (3.65)	8 (6-11)
	Antibody verified eplet mismatch DQDR	457		8.21 (4.74)	8 (5-11)
	Antibody verified eplet mismatch DQA1DQB1	457		6.08 (3.98)	5 (3-9)
	Antibody verified eplet mismatch DRB1345	457		2.13 (1.86)	2 (1-3)
	PIRCHE II score	457		274.2 (111.9)	270 (195-343)
	PIRCHE II HLA-A	457		54.9 (38.9)	49 (27-76)
	PIRCHE II HLA-B	457		46.8 (28.0)	43 (26-64)
	PIRCHE II HLA-C	457		47.6 (33.8)	41 (23-67)
	PIRCHE II HLA-DQA1	457		47.3 (40.2)	45 (0-74)
	PIRCHE II HLA-DQB1	457		48.9 (32.9)	46 (25-68)
	PIRCHE II HLA-DRB1	457		33.1 (19.8)	31 (19-44)
	EMMA score HLA-All	457		57.4 (22.1)	55 (41-73)
	EMMA score HLA-ABC	457		27.9 (10.5)	27 (21-34)
	EMMA score HLA-A	457		12.8 (7.2)	12 (8-17)
	EMMA score HLA-B	457		8.27 (4.64)	8 (5-11)
	EMMA score HLA-C	457		6.88 (4.14)	7 (4-10)
	EMMA score HLA-DQABDR	457		29.4 (19.0)	26 (14-43)
	EMMA score HLA-DQA1	457		9.81 (8.58)	8 (0-18)
	EMMA score HLA-DQB1	457		11.2 (8.52)	9 (4-18)
	EMMA score HLA-DR	457		8.43 (5.58)	8 (4-12)
Lung transplant	Donation (DBD)	457	350 (76.6)		
	Type of transplant (Unipulmonar)	457	93 (20.4)		
	Indication (Elective)	457	444 (97.2)		
	CMV pairing (High risk)	435	57 (13.1)		
	Donor pO2 (mmHg)	438		382.8 (153.1)	422 (350-484)
	ECMO during surgery (yes)	454	49 (10.8)		
	Ischemic time first (minutes)	455		298.9 (130.6)	275 (235-323)
	Ischemic time second (minutes)	363		419 (155.3)	388 (335-450)
	Surgery time (minutes)	346		291.6 (76.1)	290 (240-330.8)
	Transfusion (yes)	349	112 (32.1)		
	Number of packet RBC (units)	112		2.72 (2.44)	2 (1-4)
	Surgical reintervention (yes)	456	34 (7.46)		
Clinical	Induction (yes)	373	318 (76.6)		
	Calcineurin inhibitor (tacrolimus)	349	334 (95.7)		
	Antimetabolite (mycophenolate)	349	348 (99.7)		
	Tracheostomy (yes)	349	17 (4.87)		
	Hospitalization (days)	453		31.2 (20.0)	25 (21-33)
	Intubation (days)	339		2.28 (3.1)	1 (1-2)

Primary graft dysfunction (yes)	457	125 (27.4)
Primary graft dysfunction grade 3 (yes)	259	37 (14.3)
Acute cellular rejection (yes)	457	229 (50.1)
Antibody mediated rejection (yes)	457	38 (8.32)
Chronic allograft dysfunction (yes)	457	160 (35.0)

* BMI: body mass index; IQR interquartile range; DBD: Donation after Brain Death; RBC: red blood cells; SD: standard deviation.

The median of censored follow-up was 4.7 years, and the proportion of female lung transplants was 39.4%. One hundred and sixty out of 457 (35%) patients developed CLAD. The difference in the demographic, immunological, and clinical variables between groups of LTR developing CLAD or not is summarized in Supplementary Table 1 and Table 2.

Table 2. Frequency comparison and Kaplan Meier analysis of parameters included in the study: Recipients, donors, lung transplants, clinical and immunological parameters.

Parameter		No-CLAD group Frequency (total valid cases)	CLAD group Frequency (total valid cases)	Chi- square (p-value)	Kaplan- Meier Log-rank test (p-value)
Recipient	Recipient sex (% female)	43.43 (297)	31.88 (160)	0.0208	0.0082
	Recipient age bin	26.60 (297)	21.88 (160)	0.3173	0.4561
	Recipient smoker (never)	24.24 (297)	21.02 (157)	0.5107	0.0198
	Tobacco consumption bin	22.52 (222)	21.01 (119)	0.8535	0.9966
	Hypertension (yes)	21.55 (297)	17.09 (158)	0.3128	0.7294
	Diabetes (yes)	7.74 (297)	11.39 (158)	0.2619	0.9251
	Dyslipidemia (yes)	36.70 (297)	24.36 (156)	0.0105	0.7057
	Size bin	21.01 (276)	25.00 (120)	0.4561	0.2142
	Weight bin	24.19 (277)	26.67 (120)	0.6906	0.7257
	BMI bin	23.55 (276)	27.50 (120)	0.4776	0.5464
	LAS score bin	25.10 (255)	25.00 (92)	1.0000	0.9220
Donor	Donor sex (% female)	50.51 (297)	44.38 (160)	0.2490	0.1152
	Hypertension (yes)	33.68 (285)	31.79 (151)	0.7691	0.8568
	Diabetes (yes)	10.47 (277)	3.62 (138)	0.0274	0.1229
	Donor smoker (yes)	32.06 (287)	39.47 (152)	0.1474	0.4799
	Donor age bin	26.94 (297)	17.50 (160)	0.0316	0.2675
	Donor size bin	17.69 (294)	19.62 (158)	0.7049	0.2864
	Donor weight bin	24.91 (293)	20.13 (159)	0.3008	0.5310
Immunological	Anti-HLA class-I antibodies pre transplant (yes)	9.02 (255)	4.26 (94)	0.2106	0.3894
	Anti-HLA class-II antibodies pre transplant (yes)	5.10 (255)	4.26 (94)	0.9648	0.7539
	Anti-MICA antibodies pre transplant (yes)	1.01 (255)	0.63 (94)	1.0000	0.9468
	Antibody verified eplet mismatch All bin	20.20 (297)	25.00 (160)	0.2870	0.4267
	Antibody verified eplet mismatch ABC bin	20.20 (297)	23.75 (160)	0.4460	0.1478
	Antibody verified eplet mismatch DQDR bin	20.88 (297)	27.50 (160)	0.1377	0.7146

	Antibody verified eplet mismatch DQA1DQB1 bin	18.86 (297)	23.13 (160)	0.3372	0.8879
	Antibody verified eplet mismatch DRB1345 bin	20.88 (297)	25.00 (160)	0.3722	0.0864
	PIRCHE II score bin	26.60 (297)	21.25 (160)	0.2498	0.1915
	PIRCHE II HLA-A bin	24.58 (297)	22.50 (160)	0.7021	0.9443
	PIRCHE II HLA-B bin	26.26 (297)	21.25 (160)	0.2827	0.1881
	PIRCHE II HLA-C bin	23.91 (297)	26.25 (160)	0.6596	0.5086
	PIRCHE II HLA-DQA1 bin	24.58 (297)	25.63 (160)	0.8941	0.4268
	PIRCHE II HLA-DQB1 bin	26.26 (297)	22.50 (160)	0.4393	0.2650
	PIRCHE II HLA-DRB1 bin	24.58 (297)	24.38 (160)	1.0000	0.6668
	EMMA score HLA-All bin	22.90 (297)	26.88 (160)	0.4055	0.3461
	EMMA score HLA-ABC bin	25.59 (297)	23.75 (160)	0.7489	0.9439
	EMMA score HLA-A bin	22.90 (297)	24.38 (160)	0.8100	0.2867
	EMMA score HLA-B bin	23.91 (297)	21.25 (160)	0.5981	0.5953
	EMMA score HLA-C bin	20.54 (297)	20.00 (160)	0.9883	0.7256
	EMMA score HLA-DQABDR bin	22.22 (297)	26.88 (160)	0.3182	0.4498
	EMMA score HLA-DQA1 bin	14.48 (297)	21.88 (160)	0.0609	0.0936
	EMMA score HLA-DQB1 bin	22.90 (297)	21.88 (160)	0.8952	0.6383
	EMMA score HLA-DR bin	20.20 (297)	25.63 (160)	0.2245	0.0391
Lung transplant	Donation (DBD)	74.07 (297)	81.25 (160)	0.1069	0.7096
	Type of transplant (Unipulmonar)	14.48 (297)	31.25 (160)	<0.0001	<0.0001
	Indication (Elective)	98.32 (297)	95.00 (160)	0.0820	0.1855
	CMV pairing (High risk)	14.74 (285)	10.00 (150)	0.2142	0.4139
	Donor pO2 (mmHg)	23.84 (281)	25.48 (157)	0.7904	0.3530
	ECMO during surgery (yes)	11.49 (296)	9.49 (158)	0.6220	0.2436
	Ischemic time first bin	23.57 (297)	27.22 (158)	0.4574	0.1018
	Ischemic time second bin	22.92 (253)	26.36 (110)	0.5676	0.4334
	Surgery time bin	25.30 (253)	24.73 (93)	1	0.1341
	Transfusion (yes)	30.20 (255)	37.23 (94)	0.2626	0.9374
	Number of packed RBC bin	22.08 (77)	17.14 (35)	0.7286	0.3023
	Surgical reintervention (yes)	6.40 (297)	9.43 (159)	0.3225	0.0835
Clinical	Induction (yes)	90.64 (267)	71.70 (106)	<0.0001	0.0327
	Calcineurin inhibitor (tacrolimus)	97.25 (255)	91.49 (94)	0.0395	0.0197
	Antimetabolite (mycophenolate)	99.61 (255)	100.00 (94)	1	0.5640
	Tracheostomy (yes)	4.31 (255)	6.38 (94)	0.6056	0.2502
	Hospitalization bin	20.27 (296)	29.94 (157)	0.0286	0.0245
	Intubation bin	18.15 (248)	23.08 (91)	0.3890	0.4635
	Primary graft dysfunction (yes)	27.95 (297)	26.25 (160)	0.7810	0.9693
	Primary graft dysfunction grade 3 (yes)	14.29 (126)	14.29 (133)	1	0.3167
	Acute cellular rejection (yes)	41.08 (297)	66.88 (160)	<0.0001	0.0002
	Antibody mediated rejection (yes)	3.37 (297)	17.50 (160)	<0.0001	<0.0001

* BMI: body mass index; IQR interquartile range; DBD: Donation after Brain Death; RBC: Red Blood Cells; SD: standard deviation. All quantitative parameters were binned (bin) as described in section 4.5.3. p values <0.05 (bold) and <0.01 (italic).

Patients who developed CLAD were significantly younger, had higher body weight at listing, lower donor pO2, and experienced longer hospitalization times after transplantation. With increased frequency of single transplant, acute cellular rejection (ACR), antibody-mediated rejection (ABMR), and decreased frequency of recipient dyslipidemia, donor diabetes, induction treatment, less tacrolimus as calcineurin inhibitor treatment, and a low rate of females. Of note, since 2016, all lung

transplant recipients have received basiliximab induction, and cyclosporine has no longer been used as a calcineurin inhibitor treatment. The last single-lung transplant was performed in 2019. For these reasons, the last variables were no longer selected for further analysis of CLAD prediction models.

2.2. Immunological Variables Associated with CLAD-Free Survival

Within the immunological variables, only a high EMMA-DQA score was significantly increased in the CLAD development group, median [interquartile range (IQR)] of 8 [0-16] vs 10 [0-18] in LTR without and with CLAD development, $p=0.04$. The results of the remaining molecular mismatch variables are summarized in Supplementary Table 1. To perform survival analysis, all molecular mismatch variables were transformed into binary based on 75 percentile [14], and combined each molecular mismatch algorithm as described in the Materials and Methods section to focus on the higher value of each variable with the risk of CLAD development, and the Kaplan Meier test was performed. The results of the log-rank test, area under the curve, sensitivity, and specificity are described in Supp Table 2. Within the new variables, calculated only molecular mismatch in DR (combination of HLA-Matchmaker and HLA-EMMA) was significantly associated with shorter time to CLAD development in our cohort, $p=0.0055$. The combination of antibody-verified eplet mismatch in DRB1345 (AbV DRB1345) > 3 and EMMA DR score >12 was associated with CLAD, with very low sensitivity (19.4%) but high specificity (87.5%). This variable (identified as MM-DR2) was further used as a high immunological parameter.

2.3. Prediction Model for CLAD

The next step was to assess the time free of CLAD survival using all demographic, clinical, and immunological parameters in a univariate Cox regression analysis. The results of each parameter are summarized in Supp Table 3. To perform a multivariate regression model to predict CLAD development, the significant parameters in the univariate Cox analysis were selected (recipient sex, never smoker, EMMA score DR bin, Snow score HLA-C bin, days of hospitalization bin, ACR, ABMR, and high immunological risk variable DR2), and parameters previously described [15,16] as being involved in CLAD were also included (recipient and donor age, donor smoker, donor pO2, ischemic time of the first lung, type of donation, higher risk of CMV pairing and Grade 3 of primary graft dysfunction).

In order to test the robustness of variable selection for the Cox model and to address collinearity among variables, we conducted a penalized Cox model using Lasso selection, and a further stepwise selection based on the Akaike Information Criterion (AIC) approach was performed. The proportional hazards assumption was tested to evaluate a constant effect over time. The final variables included in the model were: ACR, ABMR, high immunological risk variable DR2, Recipient never smoker, and hospitalization bin. The DR2 variable was independently associated with CLAD development of humoral and cellular rejection, recipient smoker, and days at Hospital post transplant_bin (>33 days), with a hazard ratio (HR) of 1.499 ($p=0.05$) (Table 3). The hazard ratio and p-values of the variables included in the model are depicted in Figure 1.

Table 3. Multivariate Cox model for CLAD development prediction.

Parameter	Variable	Univariate			Multivariate (Lasso+AIC) (n=215)			Multivariate Final Model (n=457)		
		HR	95CI	p-value	HR	95CI	P-value	HR	95CI	p-value
Recipient	Recipient sex (male)	1.56	1.12-2.18	0.0087						
	Recipient smoker (never)	0.63	0.43-0.93	0.0209	0.59	0.37-0.93	0.0225	0.58	0.39-0.86	0.0072

Donor	Donor sex (male)	1.28	0.94-1.76	0.1163						
	Donor age_bin	0.79	0.53-1.20	0.2690						
	Donor smoker	1.13	0.81-1.56	0.4798						
Immunological	EMMA score	1.45	1.02-2.07	0.0403						
	HLA-DR_bin									
	MM-DR2	1.74	1.17-2.57	0.0061	1.62	1.02-2.58	0.0426	1.50	1.01-2.26	0.0452
Lung transplant	Donation (DBD)	1.08	0.72-1.62	0.7091						
	CMV pairing (High risk)	0.80	0.47-1.37	0.4147						
	Donor pO2_bin	1.19	0.83-1.70	0.3532						
	Ischemic time first_bin	1.34	0.94-1.90	0.1027						
Clinical	Hospitalization days_bin	1.48	1.05-2.08	0.0254	1.42	0.94-2.15	0.0983	1.55	1.09-2.20	0.0141
	PGD 3 (yes)	1.28	0.79-2.09	0.3186						
	ACR	1.86	1.34-2.58	<0.0001	1.69	1.10-2.60	0.0173	1.51	1.07-2.13	0.0180
	ABMR	3.84	2.54-5.80	<0.0001	2.70	1.48-4.93	0.0013	2.91	1.88-4.51	<0.0001

* ABMR antibody antibody-mediated rejection, AIC: Akaike Information Criteria, ACR: acute cellular rejection, DBD: Donation after Brain Death, PGD: primary graft dysfunction.

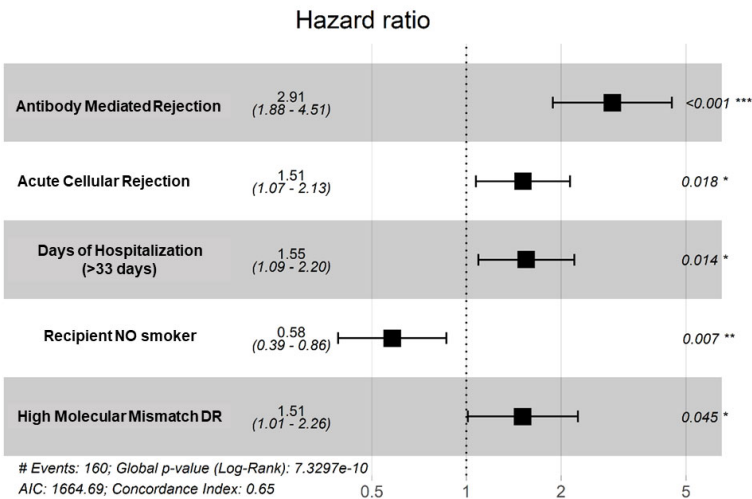


Figure 1. Forest plot of parameters associated with CLAD development: final multivariate Cox analysis. Multivariable Cox result. The parameters included in the final multivariate model were selected using Lasso penalization and stepwise based on Akaike Information Criteria. The forest plot depicts hazard ratios (black squares) and 95% CI (horizontal black lines). The exact p-values are shown. (Total number of Lung transplant recipients 457, 160 with CLAD development).

2.4. Dynamic Model for CLAD Prediction

In order to evaluate the dynamic impact of the CLAD prediction model, which incorporates five variables at different stages of lung transplantation, we analyzed the sequential effect of each risk factor, from baseline through the occurrence of humoral rejection, on the risk of developing CLAD. We further assessed the cumulative risk of CLAD associated with each additional parameter relative to the optimal scenario. The mean CLAD-free survival decreased to 3,714 days among smokers. For smokers with high immunological risk, mean CLAD-free survival decreased to 2,761 days. If these lung transplant recipients also remained hospitalized for over 33 days post-transplant, CLAD-free survival declined further to 1,966 days; with the onset of ACR, this dropped to 1,461 days, and with confirmed ABMR, the mean CLAD-free survival was 928 days. The comparative risks under each scenario are illustrated in Figure 2.

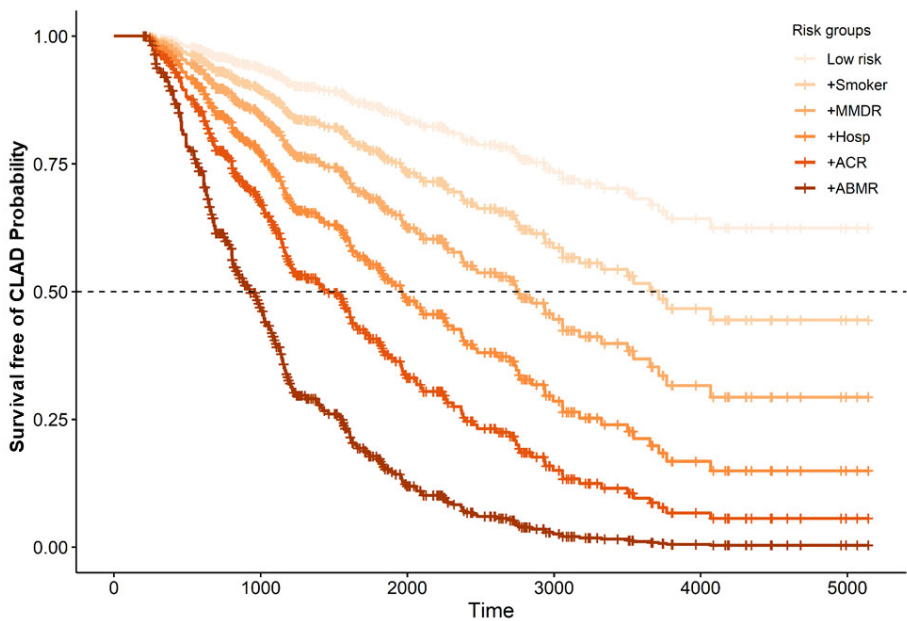


Figure 2. Survival free of CLAD probability. Using the CLAD prediction model with the data of 457 lung transplant recipients, the mean CLAD-free survival decrease when adding each risk factor from inclusion in waiting list to ABMR diagnosis. Hosp (>33 days of hospitalization after lung transplantation), MMDR (class-II mismatch molecular in DR), ACR (acute cellular rejection), ABMR (antibody mediated rejection). .

3. Discussion

CLAD development is the main clinical event affecting lung allograft survival. However, its multifactorial nature makes its approach very complicated. One of the main factors is the development of DSA and ABMR. However, the diagnosis of ABMR in lung transplantation is currently a challenge due to the lack of immunohistochemical and immunopathological markers. Furthermore, in suspected humoral rejection after lung transplantation, anti-HLA antibodies are often not detected in the circulation. It has been proposed that these DSA may infiltrate lung tissue [17]. In fact, recently, the detection of graft DSA has demonstrated better sensitivity for ABMR diagnosis than peripheral DSA [17]. Furthermore, the development of non-HLA antibodies has also been implicated in the humoral component of CLAD in a multicentre study [18]. In our lung transplant cohort, the non-HLA antibodies were also associated with ABMR[19].

The main reason eliciting an alloimmune response is the antigen HLA mismatch between donor and recipient, as demonstrated several decades ago. The greater the HLA mismatch, the lower the graft survival in solid organ transplantation [20,21]. However, this observation was not confirmed in the collaborative transplant study in LTR, where the well-matched HLA pairs did not show superior

graft survival in the early stages after lung transplantation, probably motivated by the impact of infections and surgical issues [8]. Some studies confirm that a greater number of HLA disparities is related to the development of CLAD [22]. It would be necessary to have tools to minimize the risk of developing CLAD in LTRs to increase graft and patient survival. These studies have been conducted using HLA typing tools that determine disparity at the antigenic level. However, improvements in molecular biology techniques have allowed for studying HLA typing at the allelic level, facilitating the identification of disparities between donor and recipient through molecular disparities, which has been proposed as a new tool to define immunological risk between donor and recipient. However, the lack of large multicenter studies is delaying the implementation of these methods in the workflow of histocompatibility laboratories. Greater molecular disparity measured by class II eplet load has been shown to be useful in identifying patients at risk for developing *de novo* DSA in renal [23,24] and lung transplantation [25]. The risk epitope mismatch in DQA1*05 has been previously identified as a major cause of the development of *de novo* anti-HLA antibodies in cardiothoracic transplantation [26]. We did not include this analysis in our study; however, given the evidence presented, it will be a factor to consider in future CLAD predictive models.

A large multicentre cohort in kidney transplantation confirmed the utility of molecular mismatch in class-II, which was independently associated with ABMR [27]. In some studies, it has been observed that the combination of different molecular mismatch algorithms in class-II increased the predictive capacity of ABMR [25].

The majority of the studies on molecular mismatch were focused on *de novo* anti-HLA antibody or ABMR, but not on chronic rejection. Here, we ought to study the combined molecular mismatch algorithm and assess the ability to predict the risk of CLAD development. Despite the limitation of a single-centre study, we studied a large cohort of LTR where the main clinical factors involved in CLAD, as humoral rejection and ACR [28], are confirmed in this study.

Although several variables classically associated with CLAD (such as CMV mismatch [29], donor age [30], or prolonged ischemic time [31]) were not significant in our cohort, this may be related to sample size, centre-specific practices (ie. different CMV prophylaxis) [32], or collinearity with other included parameters.

On the contrary, recipient dyslipidemia or diabetic donor frequency was decreased in our cohort of CLAD. Although these findings may appear counterintuitive within this clinical context, we observed that patients who subsequently developed CLAD had a higher body weight at the time of inclusion on the transplant waiting list. Post-transplant weight gain has been previously associated with distinct CLAD phenotypes [33]. In this study, CLAD development was analyzed regardless of restrictive or bronchiolitis obliterans syndrome phenotypes.

In our cohort, we observe the impact of molecular mismatch in class-II as an independent factor for CLAD development adjusted by the smoking history of the patients, prolonged inpatient after transplantation, ACR, and ABMR. This observation points to a potential usefulness of immunological risk assessment for predicting chronic rejection at early stages. These findings suggest that incorporating molecular mismatch assessment, particularly in class II loci, could improve donor-recipient risk stratification and guide individualized post-transplant monitoring strategies. However, the model obtained in this study takes parameters along the lung transplantation, so the cumulative risk for the patient increases over time, remaining the presence of ABMR the most important factor in CLAD development. The fact that molecular mismatch in DR class-II predicts CLAD independently of ABMR could be explained in terms that the current criteria for ABMR diagnosis have low sensitivity. Thus, the disparity in class II could fill the gap in underdiagnosed ABMR. Future multicentre prospective studies integrating molecular mismatch algorithms with non-HLA antibody detection and dynamic clinical models will be essential to refine CLAD risk prediction.

4. Materials and Methods

4.1. Study Design

All consecutive lung transplant recipients were followed in the Pneumology Department at Marqués de Valdecilla Hospital since 2010. All the patients signed informed consent, and the study was approved by the Regional Committee with the Code number (2022.202). This retrospective observational study consisted of a total of 497 LTR with donor HLA typing studies. To assess the CLAD involvement and to avoid bias, we selected those LTR with a follow-up more than 180 days after lung transplantation. A total of 457 LTR were finally selected for the study.

4.2. Clinical Data

The clinical data were gathered by clinicians at the Pneumology Department prospectively. The details of clinical data are described in 4.5.1, including demographic data from both donor and recipients, transplant, and finally, the immunological parameters were collected by the histocompatibility laboratory staff.

Primary graft dysfunction (PGD) was defined and graded according to the criteria of the International Society for Heart and Lung Transplantation (ISHLT) [34]. Acute cellular rejection after transplantation was defined and graded according to the ISHLT Working Formulation [35]. CLAD diagnosis and phenotyping were performed using ISHLT criteria [36]. Finally, the ISHLT consensus was used for the diagnosis of antibody-mediated rejection [37].

4.3. HLA Typing

The HLA typing results from donors and recipients were available at the serological level or with a 2-digit resolution for Class-I (A, B, C loci) and Class-II (DRB1 and DQB1 loci). The HLA typing was performed using Luminex technology with sequence-specific oligonucleotide probes from both vendors (One Lambda, Inc, Canoga Park, CA, USA) and Werfen, LifeCodes). The National Marrow Donor Program (NMDP) haplotype reference database (<https://haplostats.org/haplostats>) was used to infer a second field (4-digit) resolution HLA typing. The most likely high-resolution HLA genotypes are listed among ambiguous typing results according to the highest haplotype frequency in Caucasians.

4.4. Molecular Mismatch Algorithms

4.4.1. Eplet Mismatch

The eplet mismatch scores were calculated from second field resolution HLA typing. The HLA-Matchmaker tool (HLA-ABCEpletMatching v4.0 and DRDQDPEpletMatching Program v3.1 from (<http://www.epitopes.net/downloads.html>) was used; the number of antibody-verified (AbV) eplets was considered for HLA eplet mismatch scores.

4.4.2. HLA-EMMA Mismatch

The solvent amino acid (SAA) mismatch between donor and recipient was assessed by HLA-EMMA software (<https://hla-emma.com>) [38]. The SAA mismatch in each locus was gathered from the software report.

4.4.3. PIRCHE-II Scores

The PIRCHE-II algorithm predicts mismatched HLA-derived peptides that can be presented by class-II molecules from recipients, which is related to the indirect CD4+ T cell alloreactivity. We record both the total HLA-locus specific mismatched peptides (sum of HLA-A, -B, -C, DRB1, -DQB1 derived peptide count) and the locus-specific mismatched peptides (HLA-A, -B, -C, -DR and -DQ) presented by recipient HLA-DRB1 molecules (<https://www.pirche.com>).

4.5. Statistical Analysis

4.5.1. List of Variables and Description, and Calculation

Recipient parameters:

- 1. Recipient’s sex: dichotomous variable (male or female)
- 2. Recipient’s age: numeric variable (years)
- 3. Recipient smoker (history of smoking at inclusion): dichotomous variable (never or Yes)
- 4. Tobacco consumption: numeric variable (cigarette packs/year)
- 5. Hypertension: dichotomous variable (yes or no)
- 6. Diabetes: dichotomous (yes or no)
- 7. Dyslipidaemia: dichotomous (yes or no)
- 8. Recipient’s size: numeric variable (meters)
- 9. Recipient’s weight: numeric variable (kilograms)
- 10. Recipient’s body mass index (BMI): numeric variable (kg/m2)
- 11. Lung allocation score: numeric variable

Donor parameters:

- 12. Donor’s sex dichotomous variable (male or female)
- 13. Donor’s age: numeric variable (years)
- 14. Donor smoker: dichotomous variable (yes or no)
- 15. Hypertension: dichotomous variable (yes or no)
- 16. Diabetes: dichotomous (yes or no)
- 17. Donor’s size: numeric variable (meters)
- 18. Donor’s weight: numeric variable (kilograms)

Immunological parameters:

- 19. Anti-HLA class-I antibodies pre-transplant: dichotomous (yes or no)
- 20. Anti-HLA class-II antibodies pre-transplant: dichotomous (yes or no)
- 21. Anti-MICA antibodies pre-transplant: dichotomous (yes or no)
- 22. Antibody verified eplet mismatch All: numeric variable (eplet mismatched)
- 23. Antibody verified eplet mismatch ABC: numeric variable (eplet mismatched)
- 24. Antibody verified eplet mismatch DQDR: numeric variable (eplet mismatched)
- 25. Antibody verified eplet mismatch DQA1DQB1: numeric variable (eplet mismatched)
- 26. Antibody verified eplet mismatch DRB1345: numeric variable (eplet mismatched)
- 27. PIRCHE II score: numeric variable (no unit)
- 28. PIRCHE II HLA-A: numeric variable (no unit)
- 29. PIRCHE II HLA-B: numeric variable (no unit)
- 30. PIRCHE II HLA-C: numeric variable (no unit)
- 31. PIRCHE II HLA-DQA1: numeric variable (no unit)
- 32. PIRCHE II HLA-DQB1: numeric variable (no unit)
- 33. PIRCHE II HLA-DRB1: numeric variable (no unit)
- 34. EMMA score All: numeric variable (solvent amino acids mismatched)
- 35. EMMA score HLA-ABC: numeric variable (solvent amino acids mismatched)
- 36. EMMA score HLA-A: numeric variable (solvent amino acids mismatched)
- 37. EMMA score HLA-B: numeric variable (solvent amino acids mismatched)
- 38. EMMA score HLA-C: numeric variable (solvent amino acids mismatched)

- 39. EMMA score HLA-DQABDR: numeric variable (solvent amino acids mismatched)
- 40. EMMA score HLA-DQA1: numeric variable (solvent amino acids mismatched)
- 41. EMMA score HLA-DQB1: numeric variable (solvent amino acids mismatched)
- 42. EMMA score HLA-DR: numeric variable (solvent amino acids mismatched)

Lung transplant parameters

- 43. Donation type was categorized as DBD vs non-heart-beating donors)
- 44. Type of transplant: dichotomous variable (Unipulmonar or Bipulmonar)
- 45. Indication: dichotomous variable (Elective or Urgent)
- 46. CMV pairing: dichotomous variable (high risk or another pairing)
- 47. Donor pO2: numeric variable (mmHg)
- 48. ECMO during surgery: dichotomous variable (yes or no)
- 49. Ischemic time first: numeric variable (minutes)
- 50. Ischemic time second: numeric variable (minutes)
- 51. Surgery time: numeric variable (minutes)
- 52. Transfusions: dichotomous variable (yes or no)
- 53. Number of packet RBC: numeric variable (units)
- 54. Surgical reintervention: dichotomous variable (yes or no)

Clinical parameters

- 55. Induction: dichotomous variable (yes or no)
- 56. Calcineurin inhibitor: dichotomous variable (tacrolimus vs cyclosporine)
- 57. Anti-metabolite: dichotomous variable (MMF vs Aza)
- 58. Tracheostomy: dichotomous variable (yes or no)
- 59. Hospitalization: numeric variable (days)
- 60. Intubation: numeric variable (days)
- 61. Primary graft dysfunction: dichotomous variable (yes or no)
- 62. Primary graft dysfunction grade 3: dichotomous variable (yes or no)
- 63. Acute cellular rejection: dichotomous variable (yes or no)
- 64. Antibody-mediated rejection: dichotomous variable (yes or no)
- 65. Chronic allograft dysfunction: dichotomous variable (yes or no)

4.5.2. Calculation of New Immunological Parameters

To focus on the importance of high immunological risk in the CLAD development, we calculated new immunological variables using the 75th percentile (p75) as cut-off for each molecular mismatch algorithm to convert to a dichotomous variable. Furthermore, to perform combinations of molecular mismatch algorithms, firstly, the combination of 2 molecular mismatch algorithms (HLA-EMMA and HLA-Matchmaker) was performed, when the 2 parameters were over p75 in both algorithms, immunological high risk was assigned, and the other combinations were defined as low risk. Further combination with PIRCHE molecular mismatch was performed using the same approach.

4.5.3. Survival Analysis

Time to CLAD development was calculated from the transplant date in all LTR. In order to perform survival analysis, all numeric variables were binned into dichotomous using the 75th percentile as a cut-off to perform Kaplan Meier analysis, the log rank test p-value, area under the curve, sensitivity, and specificity for each parameter were calculated (Supp Table 2).

4.5.4. Cox Regression Approach

Survival analysis was performed using a Cox proportional hazards regression model to identify risk factors associated with the development of CLAD after lung transplantation. Variables included in the modelling process were selected based on clinical relevance and statistical significance ($p < 0.05$) in the univariate analyses (Suppl Table 3).

Variable selection for the multivariable Cox model followed a two-step strategy. First, the Least Absolute Shrinkage and Selection Operator (LASSO) penalization technique was applied using 10-fold cross-validation to identify a parsimonious set of predictive covariates and prevent model overfitting. Second, stepwise selection based on the Akaike Information Criterion (stepwise AIC, bi-directional) was conducted among variables shortlisted by LASSO to further optimize model fit and interpretability.

The final model was fit by maximizing the partial likelihood, and the proportional hazards assumption was rigorously assessed with Schoenfeld residual-based testing. Model outputs are expressed as hazard ratios (HRs) with their 95% confidence intervals (Table 3). Model performance was evaluated by measuring concordance, Wald statistics, and likelihood ratio tests.

The model was calculated with data from 215 patients. In order to increase the robustness of the prediction model, all the LTR data were assessed. The final multivariate Cox regression model was calculated with the variables selected by LASSO and using the method of inferring missing data in 6 LTR. In detail, multiple imputation was performed using the predictive mean matching method with five imputations (m=5) and a fixed random seed to ensure reproducibility. One imputed dataset was extracted and used for subsequent analysis.

The adjusted survival functions were estimated for different combinations of risk factors, enabling the visualization of CLAD-free survival probability as a function of key clinical profiles throughout the peri-transplant period.

4.5.5. Packages Used

R version 4.5.1,.

Packages:

library(survival 3.8-3), library(MASS v7.3-65), library(survminer v0.5.0), library(ggplot2 v3.5.2), library(dplyr v1.1.4), library(tidyr v1.3.1), library(writexl v1.5.4), library(gt v1.0.0), library(pROC v1.18.5), library(mice v.3.18.0).

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: Comparison of Parameters included in the study: Recipients, donors, lung transplants, clinical and immunological parameters in Lung transplant recipients based on CLAD development; Table S2: Data of Area Under de Curve (AUC), sensitivity and specificity, and Kaplan Meier p-value of the parameters included in the study: Recipients, donors, lung transplants, clinical and immunological parameters; Table S3: Univariate Cox-regression model with the parameters included in the study: Recipients, donors, lung transplants, clinical and immunological parameters.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Cantabria Ethics and Research Committee (CEIm) under study code 2022.202.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data generated or analyzed in this study are included in this article. Further enquiries can be directed to the corresponding author.

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