The Corneal Transplant Follow-up Study II (CTFS II): a randomized trial to determine whether HLA class II matching reduces the risk of allograft rejection in penetrating keratoplasty

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1 Synopsis

2 The prospective Corneal Transplant Follow-up Study II (CTFS II) found no influence of HLA

3 class II matching in high-risk penetrating keratoplasty (PK). Younger recipient age markedly

4 increased the risk of allograft rejection.

5

6   Abstract

7   Purpose: A randomized trial to test the hypothesis that HLA class II matching reduces the

8   risk of allograft rejection in high-risk penetrating keratoplasty (PK).

9   Methods: All transplants were matched for HLA class I antigens (≤2 mismatches at the A

10   and B loci) and corneas were allocated to patients by cohort minimization to achieve 0, 1, or

11   2 HLA class II antigen mismatches. The corneal transplants (n=1133) were followed for 5

12   years. The primary outcome measure was time to first rejection episode.

13   Results: Cox regression analysis found no influence of HLA class II mismatching on risk of

14   immunological rejection (HR 1.13; 95% CI 0.79, 1.63; p=0.51). The risk of rejection in

15   recipients older than 60 years was halved compared with recipients ≤40 years (HR 0.51;

16   95% CI 0.36, 0.73; p=0.0003). Rejection was also more likely where cataract surgery had

17   been performed after PK (HR 3.68; 95% CI 1.95, 6.93; p<0.0001). In univariate analyses,

18   pre-operative factors including chronic glaucoma (p=0.02), vascularization (p=0.01),

19   inflammation (p=0.03), ocular surface disease (p=0.0007), and regrafts (p<0.001) all

20   increased the risk of rejection. In the Cox model, however, none of these factors was

21   individually significant but rejection was more likely where ≥2 pre-operative risk factors were

22   present (HR 2.11; 95% CI 1.26, 3.47; p<0.003).

23   Conclusions: HLA class II matching, against a background of HLA class I matching, did not

24   reduce the risk of allograft rejection. Younger recipient age, the presence of ≥2 pre-operative

25   risk factors and cataract surgery after PK all markedly increased the risk of allograft

26   rejection.

27

INTRODUCTION

Allograft rejection remains a serious complication after penetrating keratoplasty (PK), accounting for 30-40% of graft failures within 1-2 years.[1-3] Even if treated successfully, just a single rejection episode can jeopardize long-term graft survival.[4] Topical corticosteroid is the treatment of choice for the prevention and management of corneal transplant rejection; however, it is not always successful at reversing rejection episodes and its long-term use can result in raised intraocular pressure and cataract.[5] While promising results have been reported with the use of systemic immunosuppressants such as tacrolimus and mycophenolate mofetil in patients at high risk of rejection,[6] supporting evidence from randomized trials is limited.[7] Lamellar grafts are less likely than PK to suffer allograft rejection but an underlying risk of rejection, albeit very low for Descemet membrane endothelial keratoplasty (DMEK), remains.[8] [9] Moreover, there are still many PKs performed worldwide [10] and alternative approaches for reducing the risk of rejection are needed,[11] [12] which may come through an increased understanding of the immunobiology of corneal transplantation in humans.

Matching for human leucocyte antigens (HLA) between donors and recipients is an effective strategy for reducing the risk of allograft rejection in organ transplantation.[13] However, the results of studies investigating the influence of HLA matching in corneal transplantation remain equivocal, especially for HLA class II matching where there are reports of a beneficial effect, no effect and even a detrimental effect.[14-17] This may in part be due to errors in HLA typing using serological methods [18] since it has been estimated that errors in just 5% of HLA-DR tissue types would be sufficient to reduce any benefit of HLA class II matching.[14] There is also a paucity of large-scale, prospective clinical trials of HLA matching in corneal transplantation and almost all of the available information comes from retrospective analyses of clinical outcome data. Nonetheless, HLA matching is still advocated for corneal transplantation as an approach to reduce the risk of rejection.[19] [20]

55

56 To resolve the uncertainty surrounding the role of HLA class II matching in high risk PK, the

57 corneal transplant follow-up study II (CTFS II) was designed as a randomized clinical trial to

58 determine whether HLA class II matching, against a background of HLA class I matching,

59 reduces the risk of allograft rejection in high-risk PK.[21] To avoid the inevitable errors with

60 serological methods, DNA-based techniques were used for the tissue typing of all donors

61 and recipients.

62

63 MATERIALS AND METHODS

64 *Patients*

65 The design of this randomized clinical trial, the methodology, patient inclusion and exclusion

66 criteria, donor and recipient characteristics, sample size, and allocation to the study groups

67 follow the relevant CONSORT Guidelines (www.consort-statement.org) as described in

68 detail in a previous paper.[21] The study complied with the tenets of the Declaration of

69 Helsinki. Briefly, patients of either sex aged 16 years or older who met the selection criteria

70 for being at increased risk of PK rejection (regrafts, bullous keratopathy, vascularized

71 cornea, active or past inflammatory/infectious disease, glaucoma) and who had given

72 informed consent to participate in the study, were registered with NHS Blood and Transplant

73 and placed on the waiting list for HLA-matched transplants. The donor corneas were all

74 stored by organ culture at 34°C and the minimum endothelial cell density estimated 3 days

75 before surgery was 2200 cells/mm².[22] All tissue typing of donors and recipients used DNA-

76 based methods (PCR-SSP/SSO). For a given cornea, patients were identified on the waiting

77 list with ≤2 HLA class I (HLA-A and -B combined) antigen mismatches with the donor and

78 then the cornea allocated to one of these patients by cohort minimization, which included a

79 weighted randomization, to achieve 0, 1 or 2 HLA class II (HLA-DR) antigen mismatches.[21]

80   Patients were followed for 5 years with data being collected at the time of surgery and

81   postoperatively at 6 months and then on the anniversary of the transplant.

82   *Statistical analysis*

83   The primary outcome measure was time to first rejection episode, regardless of whether it

84   was treated successfully or resulted directly in graft failure. Pre- and per-operative variables

85   of interest, identified from previous studies,[3] were examined univariately by comparing

86   Kaplan-Meier curves with the log-rank test. Subsequently, variables were selected for Cox

87   proportional hazards regression analysis by forward selection using the likelihood ratio test,

88   and all variables significant at the 10% level were included in the final model. The number of

89   HLA class II mismatches and donor sex match were included in the model during variable

90   selection despite lack of significance at the 10% level because of their relevance to the

91   study. Post-operative factors were modelled as time-dependent variables in the Cox model.

92   Some patients received multiple transplants and recipient was therefore modelled as a

93   random effect to allow for this. Survival estimates and hazard ratios (HR) are quoted with

94   95% confidence intervals (95% CI).

95

96   RESULTS

97   Between 3 September 1998 and 2 June 2011, 1133 transplants (all PK) in 980 patients were

98   accrued to the study. Of these 1078 met all the study selection criteria and were randomized

99   to one of the three study groups of 0 (n=182), 1 (n=483) or 2 (n=413) HLA class II (HLA-DR)

100   mismatches.[21] Five-year graft survival for these high-risk grafts was 63% (95% CI 60, 66).

101   The overall rejection-free survival estimate at 5 years was 67% (95% CI 64, 71). Figure 3

102   shows the cumulative numbers of events (i.e., first rejection episodes) and numbers of

103   transplants at risk at each follow-up time point. Of the 298 first rejection episodes reported,

104   79% occurred within the first 2 years after the transplant, with 12%, 6% and 3% of first

105   rejection episodes occurring subsequently in post-operative years 3, 4 and 5, respectively.

106

107 **Univariate analysis**

108 *Donor factors*

109 The only donor factor that influenced risk of rejection was storage time of corneas in organ

110 culture. The Kaplan-Meier rejection free survival estimate at 5 years was higher (i.e., lower

111 risk of rejection) for corneas stored in organ culture >21 days (69%; 95% CI 64, 73) than for

112 corneas stored ≤15 days (55%; 95% CI 42, 67) (p=0.04). Other donor factors, namely age

113 (p=0.8), gender (p=0.83), cornea from male donor into female recipient (p=0.38), donor

114 cause of death (p=0.6), death to enucleation time (p=0.2), and endothelial cell density

115 (p=0.4) had no influence with the risk of rejection.

116

117 *Pre- and per- operative recipient factors*

118 Recipient age had a marked influence on risk of rejection (Figure 1) with younger patients

119 having a rather lower rejection-free survival at 5 years (i.e., increased risk of rejection) than

120 older patients (p<0.0001). Indication for transplantation (p<0.0001) and preoperative risk

121 factors, including inflammation (p=0.03), chronic glaucoma (p=0.02), ocular surface disease

122 (p=0.007), deep vascularization (p=0.01), and whether the PK was a regraft (p<0.0001) all

123 increased the risk of rejection (Table 1). Moreover, Figure 2 shows the more risk factors

124 present, the lower the rejection-free survival at 5 years (p<0.0001). Factors not reaching the

125 10% level of significance included: number of HLA class I mismatches (p=0.7), total number

126 of HLA class I + HLA class II mismatches (p=0.8), reason for graft to improve vision only

127 (p=0.26), recipient trephine diameter (p=0.19), donor-recipient trephine diameter difference

128 (p=0.39), and cataract extraction (p=0.22) or vitrectomy (p=0.26) at the time of the transplant

129 operation. Finally, the Kaplan-Meier plot in Figure 3 shows that the number of HLA class II

130 mismatches had no influence on rejection-free survival at 5 years (p=0.6).

131

8

Table 1. Kaplan-Meier rejection-free survival estimates at 5 years for significant donor, recipient and transplant factors (p<0.05). Rejection-free survival data for the influence of recipient age, numbers of risk factors, and level of HLA class II mismatch are shown, respectively, in Figures 1-3.

Factor	n	Survival (%)	95% CI	p*
DONOR				
Corneal storage time in organ culture (days)				0.04
<15 d	80	55	42, 67	
15-21 d	461	67	62, 71	
22-35 d	521	69	64, 73	
PRE- AND PER-OPERATIVE				0.03
Inflammation				
No	843	68	65, 72	
Yes	234	62	54, 68	
Chronic glaucoma				0.02
No	816	69	65, 73	
Yes	261	60	53, 67	
Ocular surface disease				0.007
No	908	68	65, 72	
Yes	169	59	50, 67	
Deep vascularization				0.01
No	450	70	65, 73	
Yes	627	63	57, 68	
Regraft¥				<0.0001
No	497	73	68, 77	
Yes	580	62	57, 66	
Indication (original indication if regraft)§				<0.0001
Ectasias	74	60	47, 71	
Dystrophies	227	72	65, 78	
Previous ocular surgery	310	69	62, 74	
Infection	197	69	61, 75	
Injury	75	40	27, 54	
Opacification	61	69	53, 81	
Other	133	67	57, 75	

*Comparing rejection-free survival up to 5 years after transplantation.

¥Ipsilateral regraft was considered a risk factor rather than a specific indication for transplantation. Regrafts were therefore included in the list of indications under their original indication for transplantation.

§Ectasias (70 keratoconus, 2 keratoglobus, 2 'other'); Dystrophies (166 Fuchs endothelial dystrophy, 61 'other'); Previous ocular surgery (227 pseudophakic bullous keratopathy, 53 aphakic corneal oedema, 30 'other').

128 **Cox proportional hazards regression**

129 The final Cox model is shown in Table 2. For recipients aged 40 years or younger, the risk of

130 rejection was approximately double that for patients aged between 61 and 80 years (HR

131 0.51; 95% CI 0.36, 0.73; p=0.0003) and for patients over 80 years old (HR 0.49; 95% CI

132 0.30, 0.81; p=0.005). The presence of 2 or more preoperative risk factors more than doubled

133 the risk of rejection (HR 2.11; 95% CI 1.28, 3.47; p=0.003). Cataract surgery after the

134 corneal transplant was associated with a more than threefold increased risk of rejection (HR

135 3.68; 95% CI 1.95, 6.93; p<0.0001): it was not known whether patients were on topical

136 steroid at the time of cataract surgery. When controlling for other factors in the final Cox

137 model, HLA class II matching had no influence on risk of rejection (e.g., for 2 vs 0

138 mismatches: HR 1.13; 95% CI 0.79, 1.63; p=0.51) (Table 2 and Figure 3).

139

Table 2. Final Cox model for risk of allograft rejection over a 5-year period adjusting for recipient as a random effect to account for patients with multiple grafts* (n=1078).

Factor§	n	HR	95% CI	p
HLA class II mismatches (p=0.4)				
0	182	1.00	-	-
1	483	1.02	0.71, 1.47	0.91
2	413	1.13	0.79, 1.63	0.51
Number of pre-operative risk factors (p=0.0005)				
0	136	1.00	-	-
1	293	1.49	0.89, 2.50	0.13
2	347	2.11	1.28, 3.47	0.003
3+	301	2.79	1.69, 4.60	<0.0001
Recipient age (p=0.0015)				
≤40	161	1.00	-	-
41-60	295	0.71	0.49, 1.02	0.064
61-80	480	0.51	0.36, 0.73	0.0003
≥81	141	0.49	0.30, 0.81	0.0053
Donor-recipient sex match				
All other matches	801	1.00	-	-
Male donor to female recipient	276	0.96	0.72, 1.30	0.81
Selective adjustment/removal of sutures¥				
No	562	1.00	-	-
Yes	516	0.77	0.56, 1.06	0.11
Cataract surgery after transplant				
No	1005	1.00	-	-
Yes	73	3.68	1.95, 6.93	<0.0001
Random effect for transplant recipient accounting for multiple grafts in the same recipient (p=0.06)				

*Multiple grafts include both ipsilateral and contralateral grafts in the same recipient.

§Factors considered but not included in the final Cox model as p>0.1:

Donor factors: donor age (p=0.13), corneal storage time (p=0.18), endothelial cell density (p=0.21)

Pre- and per-operative factors: indication (p=0.19), deep vascularization (p=0.65), regraft (p=0.24), inflammation (p=0.53), glaucoma (p>0.99), ocular surface disease (p=0.53), recipient trephine diameter (p=0.16), donor-recipient trephine difference (p=0.80), suturing method (p=0.71)

Postoperative factors: wound leak (p=0.44), glaucoma medication (p=0.65), other immunosuppresants (p=0.18), elective removal of all sutures (0.53), loose or broken stitch (p=0.12)

¥Removal of interrupted or a continuous suture where a double-running suture technique was used, or adjustment of a continuous suture to even out tension.

139   DISCUSSION

140   We found no influence of HLA class II matching on risk of rejection in this randomized study

141   of a large cohort of high-risk, full-thickness corneal transplants. Previous studies that

142   observed a benefit or detrimental effect of class II matching are therefore not supported by

143   the present findings. In marked contrast to renal transplantation,[13] the lack of influence of

144   HLA class II matching suggests that the direct pathway of allorecognition is not activated in

145   corneal transplantation,[23] perhaps owing in part to a lack of professional antigen presenting

146   cells (APC) in the corneal graft. The cornea is not devoid of APCs: Langerhans cells are

147   present in the corneal epithelium but are confined to the periphery of normal corneas and

148   therefore not transplanted in significant numbers in a corneal graft. Despite this, storage time

149   of donor corneas in organ culture has been postulated to reduce corneal graft

150   immunogenicity through loss of APCs during prolonged storage. In the univariate analyses,

151   longer storage time in organ culture was indeed associated with reduced risk of rejection

152   (p=0.04), but this was not the case in the Cox model when other factors were controlled for.

153   The presence of immature dendritic cells (DC) in the central cornea has been reported in

154   mice.[24][25] Under certain conditions, such as transplantation of a cornea into an inflamed graft

155   bed, these cells mature to express HLA class II antigens and activate host T-cells by the

156   direct pathway. In the present study, the lack of influence of HLA class II matching would

157   suggest that either a similarly immature population of DCs is not present in human cornea or

158   there was a failure to stimulate maturation even though CTFS II included only high-risk

159   grafts.

160

161   While the potential benefit or otherwise of HLA class I matching could not be evaluated in

162   our study, further studies in mice have suggested little influence of major histocompatibility

163   (MHC) mismatches in corneal transplant rejection, despite evidence to the contrary from

164   some human studies.[14] Instead, non-MHC antigen mismatches have been shown to play a

165   major role in rejection in mice.[26] The role of non-MHC mismatches is more difficult to

166   elucidate in humans. Nonetheless, reports of increased risk of rejection when corneas from

167   male donors, expressing the HLA class I restricted H-Y antigen, are transplanted into female

168   recipients suggest that non-MHC antigens may have a role in stimulating allograft rejection

169   of human corneal transplants.[27] However, inclusion of donor-recipient sex match in our

170   CTFS II Cox model failed to show an increased risk of rejection when female recipients

171   received corneas from male donors (p>0.99). Other factors such as cytokine gene

172   polymorphisms may influence transplant outcome. Tumour necrosis alpha (TNF-α) and

173   interleukin-10 (IL-10) polymorphisms have been shown to be associated with transplant-

174   related death in stem cell transplantation.[28] Using a subset of CTFS II recipients we found

175   two TNF-α haplotypes one of which was associated with increased and the other with

176   decreased risk of rejection in these high-risk corneal graft recipients.[29]

177

178   The use of systemic immunosuppressants in addition to topical corticosteroids was not

179   significant in the Cox model (p=0.18) (Table 2). A Cochrane Review highlighted the lack of

180   strong evidence for systemic immunosuppression in corneal transplantation,[7] although there

181   are reports of its value in high-risk cases.[6]

182

183   The presence of blood vessels in the cornea before transplantation has typically been

184   considered to be a risk factor for rejection in PK.[2] However, even though we found

185   vascularization in the cornea before PK to increase the risk of rejection in the univariate

186   analyses, this observation was not confirmed in the Cox model. Since the detection of lymph

187   vessels in the cornea,[30] the relative importance of haem- *vs.* lymphangiogensis in corneal

188   transplant rejection has been discussed and may help to explain, at least in part, our failure

189   to see a clear influence of vascularization in our study of high risk PK.[31][32] Individual

190   preoperative risk factors were not significant in the multivariate Cox regression analysis but

191   the risk of rejection did increase when two or more of these risk factors were present (Fig. 2

13

192 and Table 2). The most frequent combinations of risk factors were regraft and

193 vascularization (n=197), vascularization, glaucoma and regraft (n=78), and vascularization

194 and infection (n=76).

195

196 We found a marked reduction in risk of rejection with increasing recipient age (Figure 1 and

197 Table 2), which supports an earlier retrospective study in the UK that reported a similar

198 finding.[3] It is well-established that both the innate and acquired immune systems change

199 with age, a process termed immunosenescence. As a result, the elderly have an increased

200 susceptibility to infection and inflammatory disease, and poorer response to vaccination,

201 which is where most of the research into this phenomenon has been directed.[33] In particular,

202 there are alterations in DC subsets, a reduction in peripheral naïve T-cells, an increase in

203 memory T-cells and alterations in cytokine expression, which overall leads to a reduced

204 ability to respond adequately to novel antigens. There have been some studies of the effects

205 of immunosenescence in transplantation, which show reduced risk of acute rejection in older

206 kidney recipients.[34 35] These changes to the immune system with age may help to explain

207 our findings of reduced risk of rejection in recipients over 60 years old.

208

209 In summary, unlike in renal transplantation, HLA class II matching did not reduce the risk of

210 rejection in high-risk, full-thickness corneal transplantation. Other factors are therefore likely

211 to play an important role in corneal transplant immunology and a project (VISICORT) is

212 currently underway to find adverse immune signatures in corneal transplant patients.[36] The

213 reduced risk of rejection with increasing recipient age certainly warrants further investigation.

214

215

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FIGURE LEGENDS

Figure 1. Kaplan-Meier plot for rejection-free survival stratified by recipient age and 5-year rejection-free survival estimates.
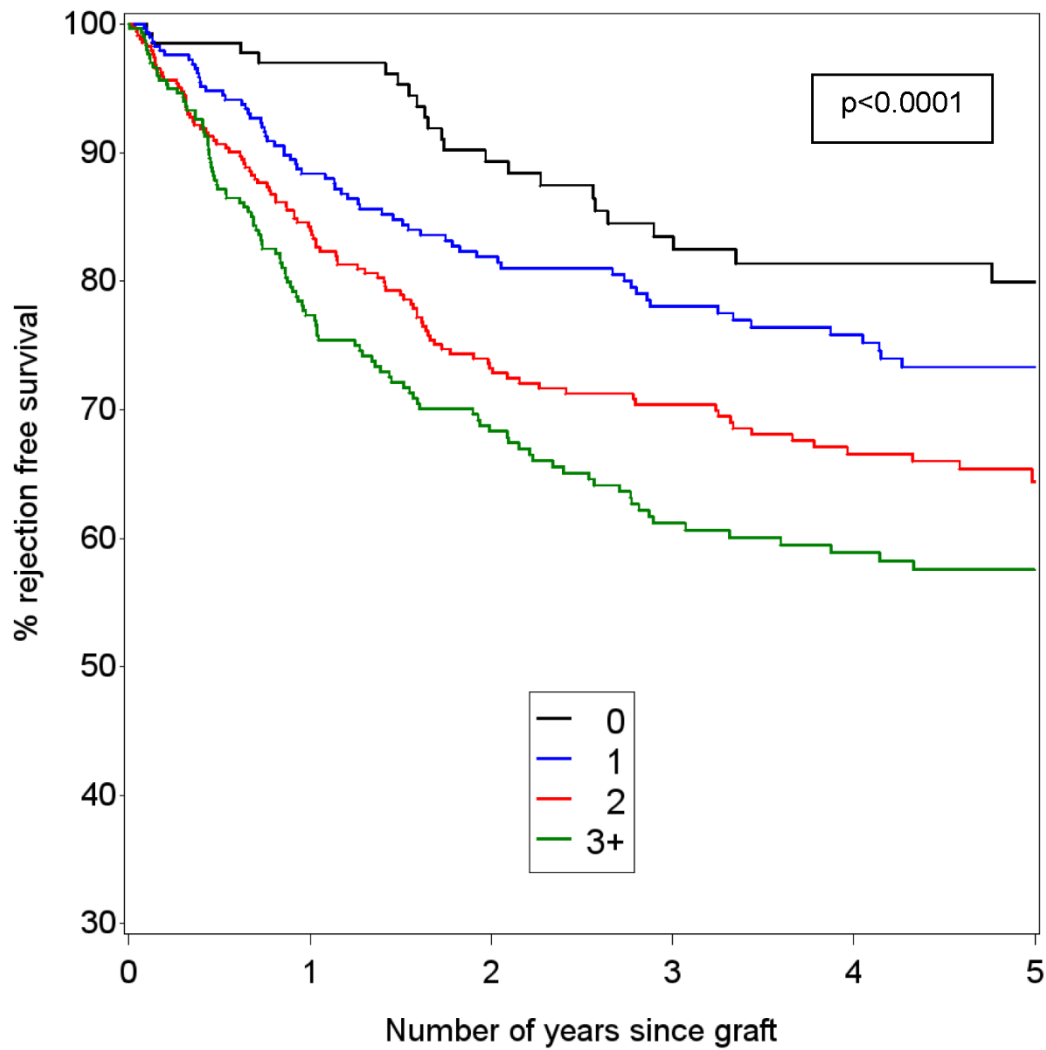
Figure 2. Kaplan-Meier plot for rejection-free survival stratified by number of pre-operative risk factors (regraft, vascularization, glaucoma, inflammation, ocular surface disease and 'other') and 5-year rejection-free survival estimates.

Figure 3. Kaplan-Meier plot for rejection-free survival stratified by number of HLA class II mismatches, 5-year rejection-free survival estimates, numbers of events (i.e., first rejection episode) and numbers of transplants at risk at each postoperative follow-up time.
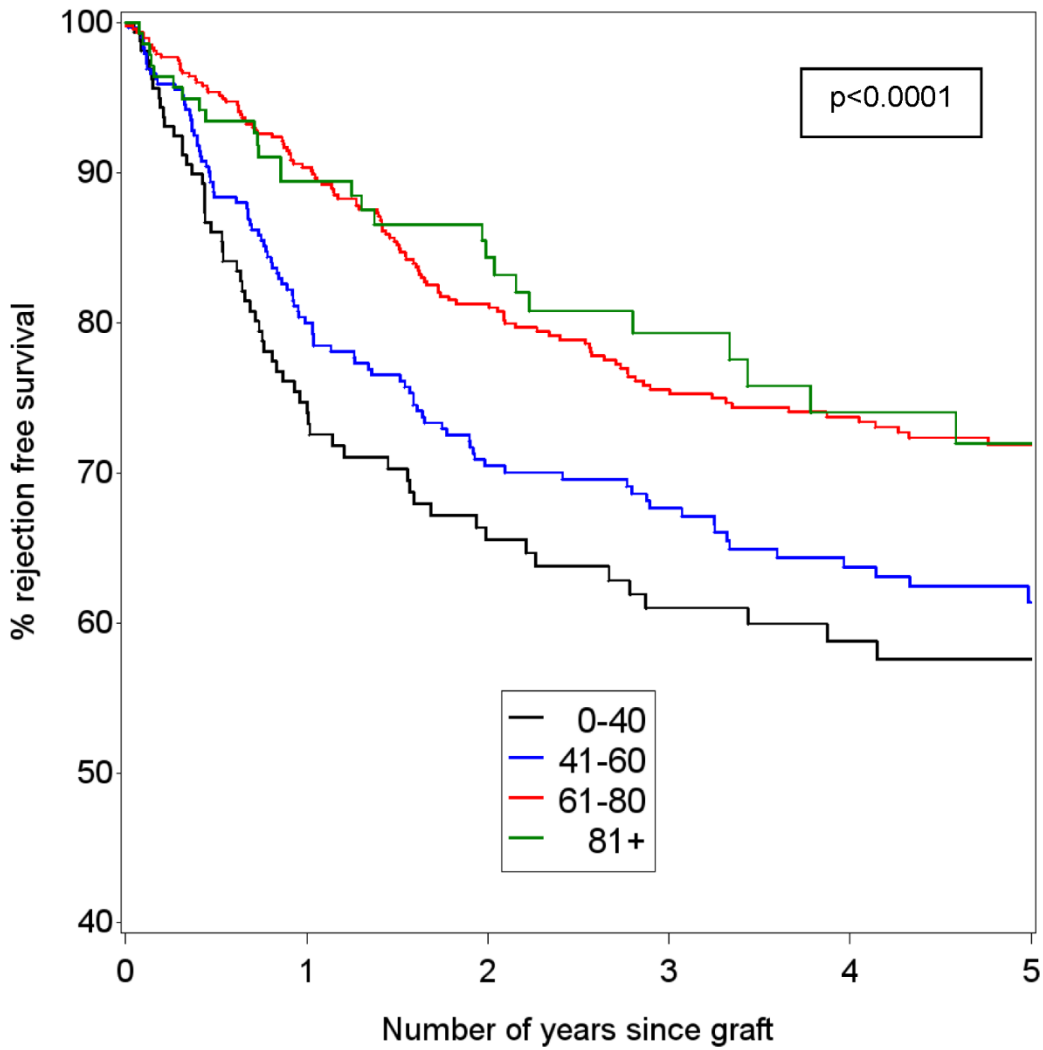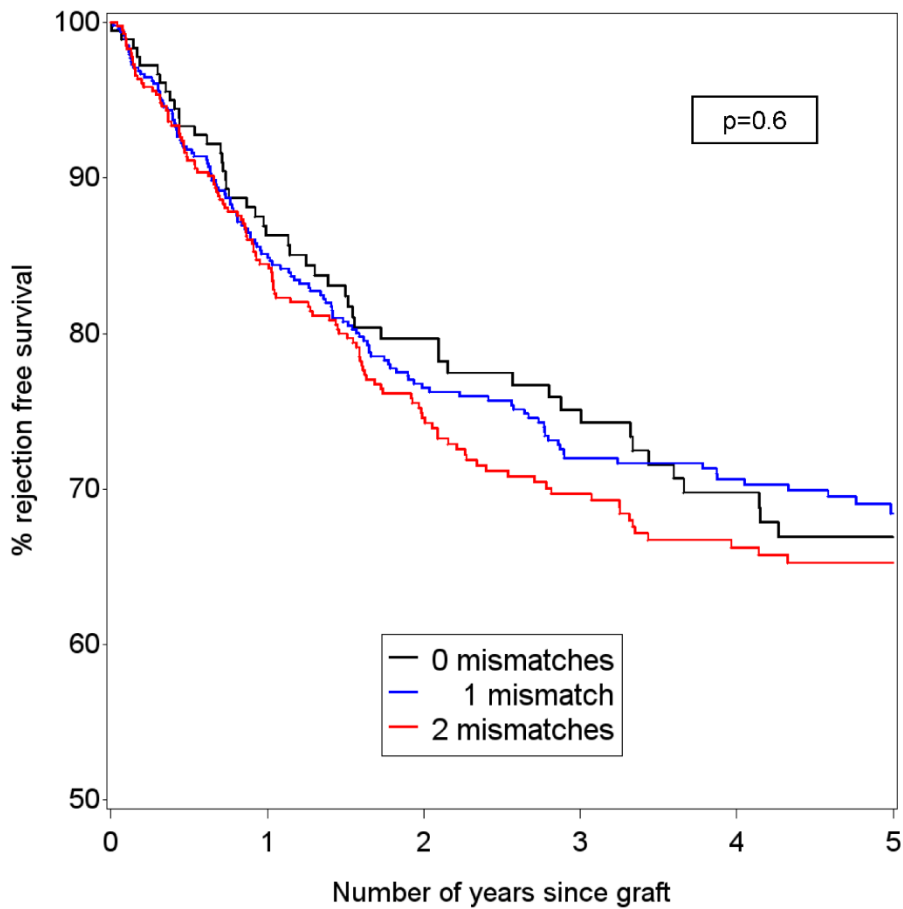
Recipient age	n	5-year rejection-free survival (%)	
		Estimate	95% CI
0-40 years	161	58	49, 66
41-60 years	295	61	55, 67
61-80 years	480	72	67, 76
81+ years	141	72	61, 81

Figure 1. Kaplan-Meier plot for rejection-free survival stratified by recipient age and 5-year rejection-free survival estimates.

Number of risk factors	n	5-year rejection-free survival (%)	
		Estimate	95% CI
0	136	80	71, 86
1	293	73	67, 79
2	347	64	58, 70
3+	301	58	51, 64

Figure 2. Kaplan-Meier plot for rejection-free survival stratified by number of pre-operative risk factors (regraft, vascularization, glaucoma, inflammation, ocular surface disease and 'other') and 5-year rejection-free survival estimates.

HLA Class II match grade	n	5-year rejection-free survival (%)	
		Estimate	95% CI
0 mismatches	182	67	58-74
1 mismatch	482	68	63-73
2 mismatches	413	65	60-70

Number of years since graft	0	0.5	1	2	3	4	5
HLA class II match grade							
0 mismatches							
Number at risk	182	164	142	110	89	73	33
Cumulative events	0	12	24	34	40	46	49
1 mismatch							
Number at risk	482	429	364	291	240	198	101
Cumulative events	0	39	69	104	120	124	129
2 mismatches							
Number at risk	413	363	314	228	174	138	65
Cumulative events	0	36	62	96	110	118	120

Figure 3. Kaplan-Meier plot for rejection-free survival stratified by number of HLA class II mismatches, 5-year rejection-free survival estimates, numbers of events (i.e., first rejection episode) and numbers of transplants at risk at each postoperative follow-up time.