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BASIC SCIENCE

Matricellular proteins and matrix metalloproteinases mark the inflammatory and fibrotic response in human cardiac allograft rejection

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Aims The cardiac extracellular matrix is highly involved in regulating inflammation, remodelling, and function of the heart. Whether matrix alterations relate to the degree of inflammation, fibrosis, and overall rejection in the human transplanted heart remained, until now, unknown.

Methods and results Expression of matricellular proteins, proteoglycans, and metalloproteinases (MMPs) and their inhibitors (TIMPs) were investigated in serial endomyocardial biopsies ($n = 102$), in a cohort of 39 patients within the first year after cardiac transplantation. Out of 15 matrix-related proteins, intragraft transcript and protein levels of syndecan-1 and MMP-9 showed a strong association with the degree of cardiac allograft rejection (CAR), the expression of pro-inflammatory cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-6 and transforming growth factor (TGF)-β, and with infiltrating $CD3^+$T-cells and $CD68^+$monocytes. In addition, SPARC, CTGF, TSP-2, MMP-14, TIMP-1, Testican-1, TSP-1, Syndecan-1, MMP-2, -9, and -14, as well as IL-6 and TGF-β transcript levels and inflammatory infiltrates all strongly relate to collagen expression in the transplanted heart. More importantly, receiver operating characteristic curve analysis demonstrated that syndecan-1 and MMP-9 transcript levels had the highest area under the curve (0.969 and 0.981, respectively), thereby identifying both as a potential decision-making tool to discriminate rejecting from non-rejecting hearts.

Conclusion Out of 15 matrix-related proteins, we identified synd-1 and MMP-9 intragraft transcript levels of as strong predictors of human CAR. In addition, a multitude of non-structural matrix-related proteins closely associate with collagen expression in the transplanted heart. Therefore, we are convinced that these findings deserve further investigation and are likely to be of clinical value to prevent human CAR.

Keywords Heart transplantation • Rejection • Metalloproteinases • Matricellular proteins • Cardiac matrix • Inflammation • Biomarker

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Introduction

Cardiac allograft rejection (CAR) is characterized by severe cardiac inflammation, cardiomyocyte damage, fibrosis, and progressive ventricular dysfunction of the transplanted heart.[1] Although the 1-year survival rate now approaches 87%, CAR still accounts for most of the early and late deaths after heart transplantation (HTx). The current standard to screen for CAR is the histopathological detection of inflammatory infiltrates and cardiomyocyte necrosis in serial endomyocardial biopsies (EMBs) and grading according to the guidelines of the International Society of Heart and Lung Transplantation (ISHLT).[1] Nevertheless, a more in-depth understanding of the biological and molecular changes associated with CAR is mandatory to develop novel diagnostic and therapeutic strategies.

During the last decade, it has become clear that dynamic changes in the cardiac ECM, including altered expression and activity of matricellular proteins, proteoglycans, and the matrix metalloproteinase (MMP) system, do not only determine the mechanical properties of the heart, but also modulate key inflammatory and reparative pathways in animal models of cardiac stress and disease.[2–4] As such, reports from several groups advocate a pivotal role for matricellular proteins and proteoglycans, including, thrombospondin (TSP)-1 and -2, connective tissue growth factor (CTGF), secreted protein, acidic and rich in cysteine (SPARC), syndecan (synd)-1 and -4, and testican-1 in the inflammatory process.[4–8] Metalloproteinases and their specific tissue inhibitors (TIMPs), on the other hand, are strong regulators of the cardiac ECM and have the capability to directly or indirectly modulate the inflammatory response.[2,9] As a result, they determine the structural and functional outcome of diseased hearts. Studies in MMP-2- and -9-deficient mice revealed that both gelatinases influence graft survival by modulating T-cell alloreactivity in a model of acute CAR,[10] whereas the use of broad-spectrum MMP-inhibitors significantly enhanced graft survival by suppressing the inflammatory response.[11] Besides inflammation, cardiac allograft fibrosis has been identified as an important limiting factor towards long-term graft survival.[5] Interestingly, the majority of these ECM-related macromolecules have been reported to influence expression, deposition, assembly, and/or maturation of newly deposited extracellular collagen matrix in various cardiac pathologies.[2,4,6]

Despite their implication in the inflammatory and reparative pathways of the heart, evaluation of matricellular proteins, proteoglycans, MMPs and TIMPs in relation to inflammation, fibrosis, and the degree of human CAR are scarce and fragmentary. In present study, we focused on the intragraft transcript and protein levels of matricellular proteins (TSP-1 and -2, CTGF and SPARC), proteoglycans (synd-1 and -4, Testican-1), MMPs (MMP-1, -2, -9, MMP-14), and TIMPs (TIMP-1 to -4) in relation to (i) the degree of CAR as measured by ISHLT grading; (ii) the expression of pro-inflammatory cytokines interleukin (IL)-6, tumour necrosis factor (TNF)-α and transforming growth factor (TGF)-β; (iii) the influx of $CD3^+$ T-cells and $CD68^+$ monocytes; and (iv) collagen expression within the first year after HTx. Finally, we also evaluate whether particular EMB-derived transcriptional profiles could potentially provide a valuable diagnostic tool to identify those patients with CAR.

Methods

Study design, patient characteristics, experimental materials and methods, and statistical analysis are described extensively in the Supplementary material online. Briefly, protocol EMBs and plasma samples were simultaneously collected and properly stored during the routine follow-up of 39 patients within the first year after cardiac transplantation (Supplementary material online, *Table S1*). A first subset of the obtained EMBs were graded for cellular rejection by a pathologist blinded to other clinical or scientific data according to the criteria of the ISHLT, whereas a second subset of EMBs and plasma specimens were processed for further molecular and histological analysis, including, RNA isolation and real-time polymerase chain reaction (RT–PCR), immunohistochemical analysis, *in situ* gelatin zymography, and enzyme-linked immunosorbent assay. The study was approved by the medical ethical commission of the KULeuven and all patients gave written informed consent, consistent with the Declaration of Helsinki.

Results

Intragraft synd-1, MMP-9, and TIMP-1 transcript levels relate to the degree of cardiac allograft rejection

A total of 102 EMBs from our cohort of 39 HTx patients were examined by real-time quantitative PCR to determine the differential mRNA expression of 15 matrix-related proteins in relation to the different ISHLT rejection grades (*Table 1* and Supplementary material online, *Table S1*). Normalized intragraft transcript levels of synd-1, MMP-9, and TIMP-1 showed the strongest significant and positive correlation with the degree of CAR (*Table 1*). Whereas no major differences were seen between Grade 0 and Grade 1A EMBs, synd-1, MMP-9, and TIMP-1, respectively, showed a 1.8-, 3.1-, and 1.8-fold increase in Grade 1B ($P < 0.001$) and a 37.2-, 9.4-, and 7.4-fold increase in Grade 3 ($P < 0.001$), when compared with Grade 0 EMBs (*Table 1*). In contrast, only a moderate or no correlations were observed for the other matricellular proteins, proteoglycans, MMPs, and TIMPs included in this study (*Table 1*).

Transcript levels of synd-1, MMP-9, CTGF, and TIMP-1 relate to IL-6, TNF-α, and/or TGF-β

Within our cohort of EMBs, intragraft transcript levels of pro-inflammatory cytokines IL-6, TNF-α, and TGF-β significantly increased in Grade 1B and 3 rejecting EMBs and significantly relate to the degree of CAR (*Figure 1A*). Interleukin-6, TNF-α, and TGF-β function as potent chemoattractants and provide essential signals for the dynamic trafficking of T-cells and monocytes into allografts.[5,12] In addition, both IL-6 and TGF-β negatively regulate cardiac allograft survival through their pro-fibrotic effects.[5] Interestingly, normalized synd-1 and MMP-9 transcript levels revealed the strongest association with the expression levels of IL-6, TNF-α, and TGF-β (*Figure 1B* and *D*). Whereas TIMP-1 and CTGF, both implicated in stimulating cardiac fibrosis,[4,9] showed a strong correlation with IL-6 and TGF-β, but not with TNF-α (*Figure 1C* and *E*). Markedly, synd-1, CTGF, SPARC, MMP-1, -9, and TIMP-1 correlated stronger

Table 1 Intragraft transcript levels in relation to rejection grades on endomyocardial biopsy

	Grade 0 ($n = 38$)	Grade 1A ($n = 31$)	Grade 1B ($n = 25$)	Grade 3 ($n = 8$)	ANOVA post-test for linearity	
					R^2-value	*P*-value
Matrix glycoproteins						
Synd-1	0.4 ± 0.2	0.6 ± 0.2	0.7 ± 0.4	14.9 ± 21.1*,**,***	0.286	<0.0001
Synd-4	48 ± 22,4	42 ± 21,0	49 ± 22,1	43 ± 17,7	0.001	0.803
TSP-1	210 ± 159	181 ± 117	234 ± 107	297 ± 170	0.034	0.073
TSP-2	812 ± 574	960 ± 850	1194 ± 1081	1894 ± 1369*	0.107	<0.01
CTGF	0.6 ± 0.6	0.4 ± 0.2	0.6 ± 0.6	1.3 ± 1.1**	0.069	<0.05
SPARC	10.4 ± 16.1	11.0 ± 14.3	12.4 ± 8.0	17.5 ± 12.1	0.018	0.197
Testican-1	19.0 ± 14.0	16.4 ± 15.2	17.9 ± 12.9	22.4 ± 9.6	0.006	0.516
MMPs and TIMPs						
MMP1	2.2 ± 1.5	3.0 ± 2.2	5.4 ± 3.0*	6.2 ± 3.3*,**	0.118	<0.01
MMP2	19.8 ± 11.5	22.4 ± 13.2	22.2 ± 9.6	24.4 ± 7.6	0.001	0.748
MMP9	2.7 ± 3.6	3.0 ± 3.7	8.4 ± 9.2*,**	25.5 ± 8.2*,**,***	0.520	<0.0001
MMP-14	1.3 ± 1.0	1.4 ± 1.1	1.9 ± 3.4	2.3 ± 2.0	0.022	0.147
TIMP1	58 ± 41.8	58 ± 32.4	104 ± 72*,**	429 ± 317*,**,***	0.479	<0.0001
TIMP2	112 ± 30.07	105 ± 22.5	116 ± 30.17	136 ± 31.6	0.046	<0.05
TIMP3	0.7 ± 0.3	0.5 ± 0.3	0.9 ± 0.6	1.8 ± 0.4*,**	0.267	<0.01
TIMP4	0.10 ± 0.04	0.06 ± 0.03	0.11 ± 0.20	0.31 ± 0.09*,**	0.167	<0.05

ISHLT grade indicates the histopathological grade of cardiac allograft rejection as set by the International Society of Heart and Lung Transplantation. Transcript levels are given in arbitrary units relative to GAPDH expression and presented as mean ± SD. Total $n = 102$, including $n = 38$ for Grade 0 collected from 27 patients, $n = 31$ for Grade 1A from 22 patients, $n = 25$ for Grade 1B form 15 patients and $n = 8$ for Grade 3 from 5 patients.
EMB, endomyocardial biopsy; Synd-1, syndecan-1; TSP, thrombospondin; CTGF, connective tissue growth factor; SPARC, secreted protein acidic and rich in cysteine; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinases.
*$P < 0.05$ vs. Grade 0.
**$P < 0.05$ vs. Grade 1A.
***$P < 0.05$ vs. Grade 1B.

with the expression of one or more pro-inflammatory cytokines when compared with their relation with the degree of CAR (*Figure 1*, Supplementary material online, *Figure S1* and *Table S1*). Only moderate or no correlations were noted for the other proteins included in this study (Supplementary material online, *Figure S1*).

Together, these data suggest that intragraft expression synd-1, CTGF, MMP-9, and TIMP-1 strongly mirror the expression of IL-6, TNF-α, and/or TGF-β during human CAR.

Transcription of synd-1, MMP-9, and CTGF, but not TIMP-1 coincide with the influx of T-cells and monocytes in the transplanted heart

Inflammatory infiltrates associated with CAR, largely consist out of T-cells and monocytes.[1] Therefore, a total of 71—randomly chosen—EMBs were examined by immunohistochemistry to evaluate the influx of $CD3^+$ T-cells and $CD68^+$ monocytes (*Figure 2A–C*). The number of infiltrating $CD3^+$ T-cells and $CD68^+$ monocytes strongly increased with increasing Grade of CAR and these were especially present in EMBs displaying a severe rejection episode accompanied by myocyte necrosis (ISHLT Grade 3; *Figure 2A–C*). Furthermore, transcript levels of the pro-inflammatory cytokines IL-6, TNF-α, and TGF-β were highly associated with the influx of $CD3^+$ and $CD68^+$ cells (Supplementary material online, *Figure S1A–C*), indicating a pivotal role for these cytokines in the inflammatory pathways underlying human CAR. More importantly, the influx of $CD3^+$ T-cells and $CD68^+$ monocytes strongly related to the transcription of synd-1, MMP-9, CTGF (from highest to lowest; *Figure 2D–F*), but not to that of TIMP-1 (*Figure 2G*). Weak or absence of significant correlations was noted for the remaining proteins included in this study (Supplementary material online, *Figure S1D–N*).

Together, these data reveal a close relationship between inflammation and the induction of synd-1, MMP-9, CTGF in the transplanted heart.

Synd-1 and MMP-9 protein expression mark the influx of T-cells and monocytes during cardiac allograft rejection

Encouraged by our consistent findings on synd-1 and MMP-9, we next investigated whether synd-1- and MMP-9-protein expression marked the degree of inflammation in the transplanted hearts.

In agreement with the mRNA expression profiles, synd-1 and MMP-9 protein levels significantly increased with increasing

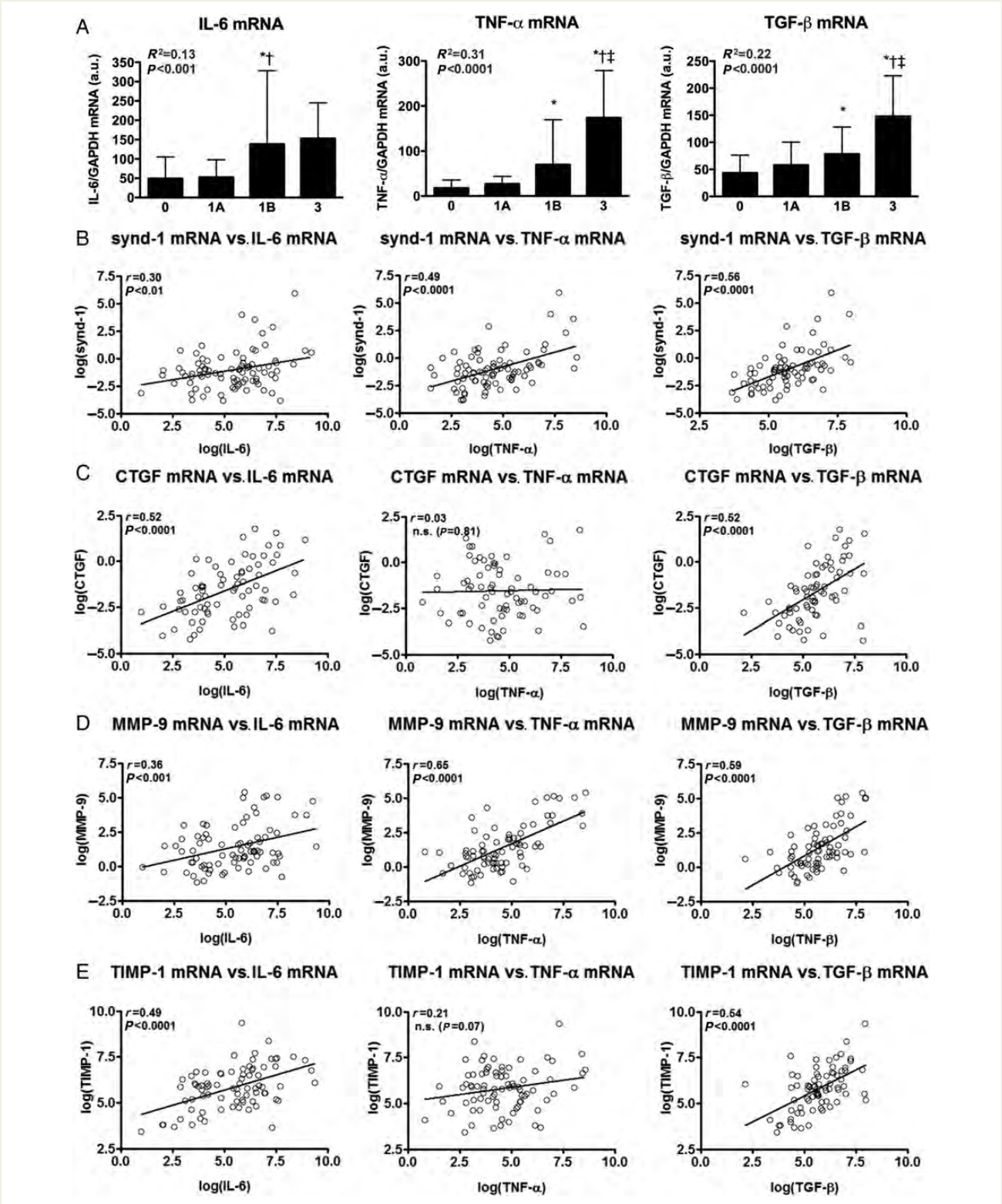

Figure 1 Intragraft transcript levels of in relation to the expression of pro-inflammatory cytokines. (*A*) Intragraft expression levels (mean ± SD) of IL-6, TNF-α, and TGF-β progressively increase with and significantly relate to the International Society of Heart and Lung Transplantation grade of cardiac allograft rejection. (*B*–*E*) Intragraft transcript levels of (*B*) syndecan-1, (*C*) CTGF, (*D*) MMP-9, and (*E*) TIMP-1 in relation to the mRNA expression levels of IL-6, TNF-α, and TGF-β. $^{*}P < 0.05$ vs. Grade 0; $^{\dagger}P < 0.05$ vs. Grade 1A; $^{\ddagger}P < 0.05$ vs. Grade 1B; $n = 102$, including $n = 38$ for Grade 0 collected from 27 patients, $n = 31$ for Grade 1A from 22 patients, $n = 25$ for Grade 1B form 15 patients and $n = 8$ for Grade 3 from 5 patients.

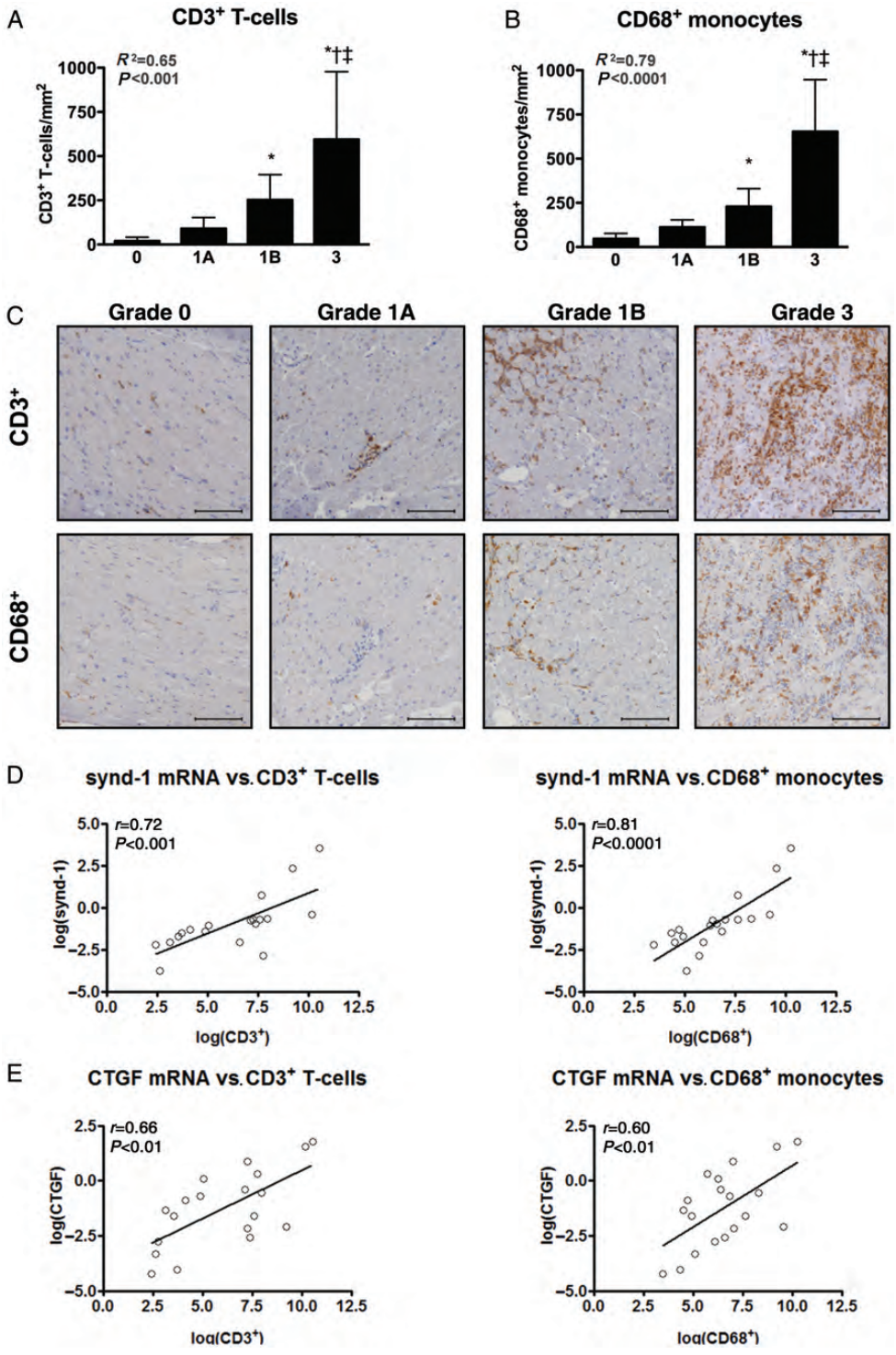

Figure 2 Transcription of synd-1, CTGF, MMP-9, but not TIMP-1 coincide with the influx of T-cells and monocytes in the transplanted heart. Immunohistochemical analysis revealed a significantly increased influx of (*A*) $CD3^+$ T-cells and (*B*) $CD68^+$ monocytes with increasing grade of human cardiac allograft rejection. Data presented as mean ± SD (*C*) Representative microphotographs of CD3 and CD68 immunoreactivity in endomyocardial biopsies displaying cardiac allograft rejection Grade 0, 1A, 1B, and 3. Scale-bar: 100 μm. Transcript levels of (*D*) synd-1, (*E*) CTGF, (*F*) MMP-9, and (*G*) TIMP-1 in relation to the influx of $CD3^+$ T-cells and $CD68^+$ monocytes. $^*P < 0.05$ vs. Grade 0; $^\dagger P < 0.05$ vs. Grade 1A; $^\ddagger P < 0.05$ vs. Grade 1B; $n = 21$ for International Society for Heart and Lung Transplantation Grade 0 collected from 21 patients, $n = 19$ for Grade 1A from 19 patients, $n = 16$ for Grade 1B from 15 patients and $n = 8$ for Grade 3 from 5 patients. For histological analysis, seven Grade 3 endomyocardial biopsies were added from patients that were transplanted prior to the start of our study.

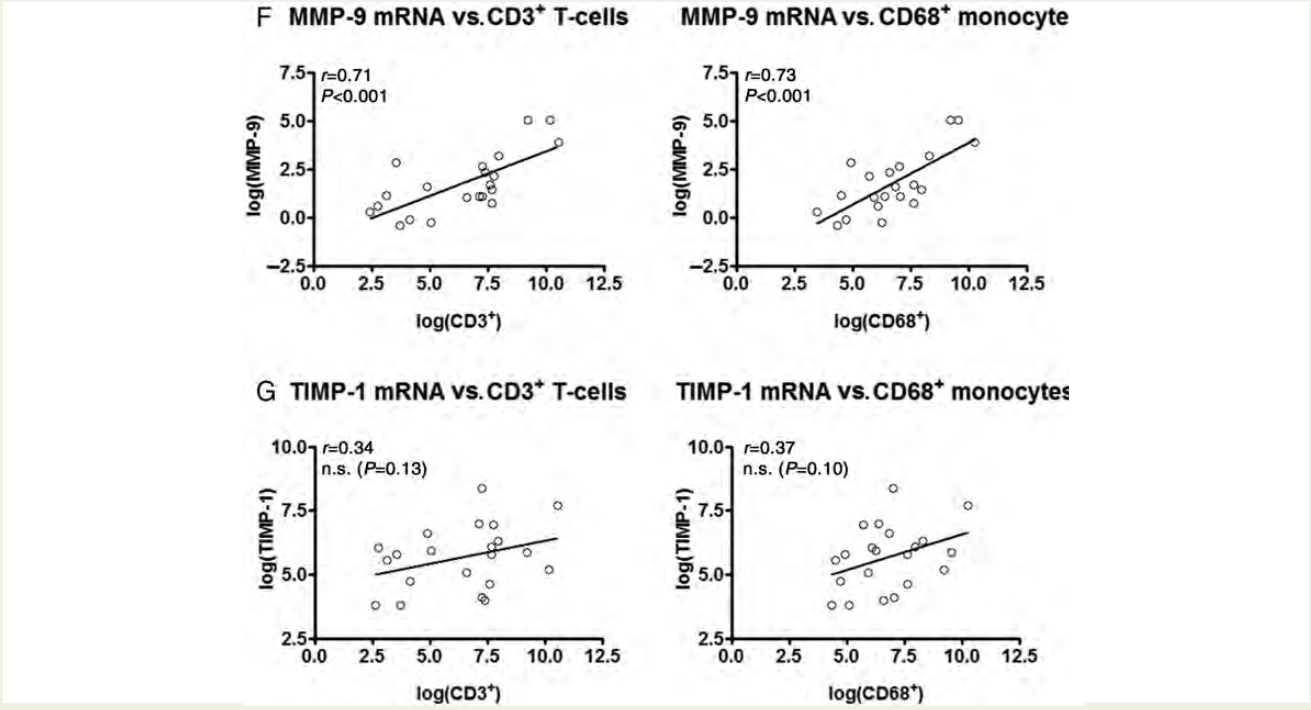

Figure 2 (Continued).

Table 2 Synd-1 and MMP-9 protein levels in relation to rejection grades on endomyocardial biopsy

	Grade 0 ($n = 21$)	Grade 1A ($n = 19$)	Grade 1B ($n = 16$)	Grade 3 ($n = 15$)	ANOVA post-test for linearity	
					R^2-value	*P*-value
Synd-1, %	2.04 ± 0.5	4.07 ± 0.75	5.24 ± 1.34*	12.89 ± 3.36*,**,***	0.692	<0.0001
MMP-9, %	0.12 ± 0.07	0.35 ± 0.57	0.79 ± 0.61*	1.67 ± 2.03*,**,***	0.240	<0.0001

Protein levels are given as percentage of the positive staining area relative to the total tissue area and presented as mean ± SD. $N = 71$, including $n = 21$ for ISHLT Grade 0 collected from 21 patients, $n = 19$ for Grade 1A from 19 patients, $n = 16$ for Grade 1B from 15 patients and $n = 15$ for Grade 3 from 12 patients.
EMB, endomyocardial biopsy; Synd-1, syndecan-1; MMP, matrix metalloproteinase.
*$P < 0.05$ vs. Grade 0.
**$P < 0.05$ vs. Grade 1A.
***$P < 0.05$ vs. Grade 1B.

Grade of CAR (*Table 2*, *Figure 3A*). Strikingly, synd-1 and MMP-9 immunoreactivity were minimally detectable in Grade 0 and 1A, but progressively increased in EMBs displaying Grade 1B or Grade 3 rejection episodes. In Grade 3 EMBs, a 6.3-fold increase for synd-1 and a 13.9-fold increase for MMP-9 protein expression were observed, when compared with ISHLT Grade 0 ($P < 0.0001$ for both; *Table 2*). Synd-1 and MMP-9 highly co-localized with infiltrating mononuclear cells and strongly correlated to the degree of infiltrating $CD3^+$ T-cells and $CD68^+$ monocytes (*Figure 3A–C*). In addition, a diffuse immunostaining for MMP-9 and Synd-1 was found in the interstitial matrix and in areas with extensive fibrosis (*Figure 3A*).

Taken together, our data clearly indicate intragraft Synd-1 and MMP-9 protein expression as potent markers for infiltrating T-cells and monocytes in the transplanted heart.

Matricellular proteins, proteoglycans, MMPs, TIMPs, and cardiac allograft fibrosis

Intragraft transcript levels of collagen type 1α1 (COLIa1) and type IIIα1 (COLIIIa1) significantly increased with the production of pro-inflammatory/pro-fibrotic cytokines TGF-β and IL-6, the influx of $CD3^+$ T-cells and $CD68^+$ monocytes and increasing grade of rejection (*Figure 4A* and *B*, *Table 3*). Together, indicating a close association between inflammation and collagen production during CAR. A closer look at the components of the TGF-β pathway revealed that TGF-β-receptor-1 and -2 expressions are significantly elevated in Grade 3 rejecting EMBs, whereas those of latent TGF-β-binding proteins (LTBP)-1 to -4 did not show any significant changes (*Figure 4C–E* and Supplementary material

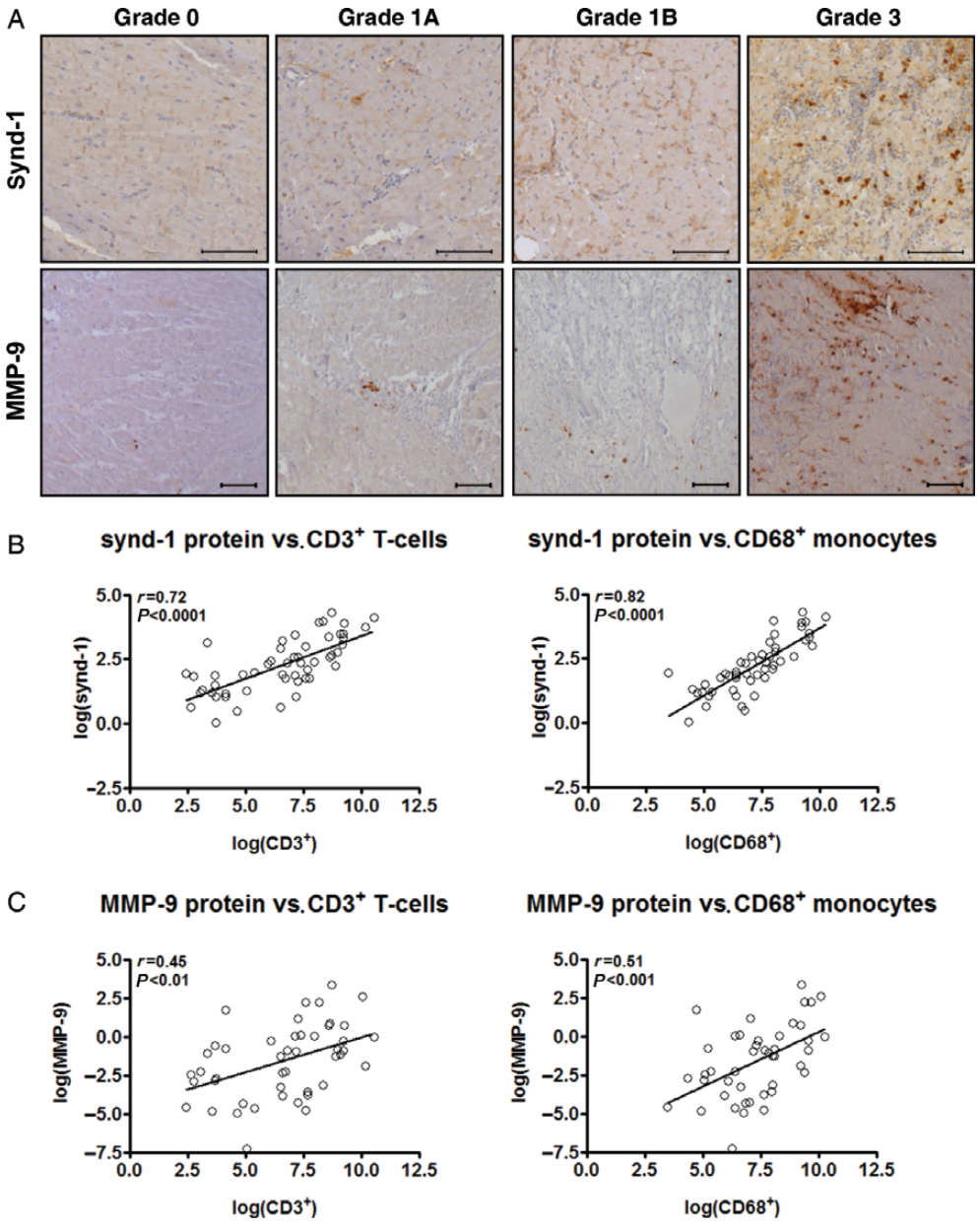

Figure 3 Intragraft synd-1 and MMP-9 protein expression correlates with the influx of T-cells and monocytes in human cardiac allograft rejection. (*A*) Representative microphotographs synd-1 and MMP-9 immunoreactivity in endomyocardial biopsies displaying rejection Grade 0, 1A, 1B, and 3. Scale-bar: 100 μm (*B* and *C*). Immunohistochemical analysis revealed a strong correlation between (*B*) synd-1 and (*C*) MMP-9 and the number of infiltrating $CD3^+$ T-cells and $CD68^+$ monocytes. $N = 71$, $n = 21$ for International Society for Heart and Lung Transplantation Grade 0 collected from 21 patients, $n = 19$ for Grade 1A from 19 patients, $n = 16$ for Grade 1B from 15 patients and $n = 15$ for Grade 3 from 12 patients.

online, *Table S4*). More interesting, however, further analyses revealed that not only the intragraft transcript levels of both TGF-β-receptors, LTBP-1, -2, and -3, but also SPARC, CTGF, TSP-2, MMP-14, TIMP-1, Testican-1, TSP-1, Synd-1, MMP-9, and MMP-2 (from highest to lowest) all positively correlated with the expression levels of COLIa1 and COLIIIa1 (*Table 3*).

Next, transmural interstitial fibrosis was visualized and analysed by Sirius red staining (*Figure 4F–H*). Surprisingly, total collagen deposition did not reflect the mRNA expression profiles of COLIa1 and COLIIIa1 (*Figure 4F*). Whereas the total amount of deposited collagen showed a trend towards increase in Grade 1A–B compared with Grade 0, no differences were seen in Grade 3 compared with Grade 0 EMBs, suggesting that the increased collagen expression might be counterbalanced by extensive proteolytic degradation in Grade 3 rejecting EMBs (Supplementary material online, *Figure S5*). In addition, a significant

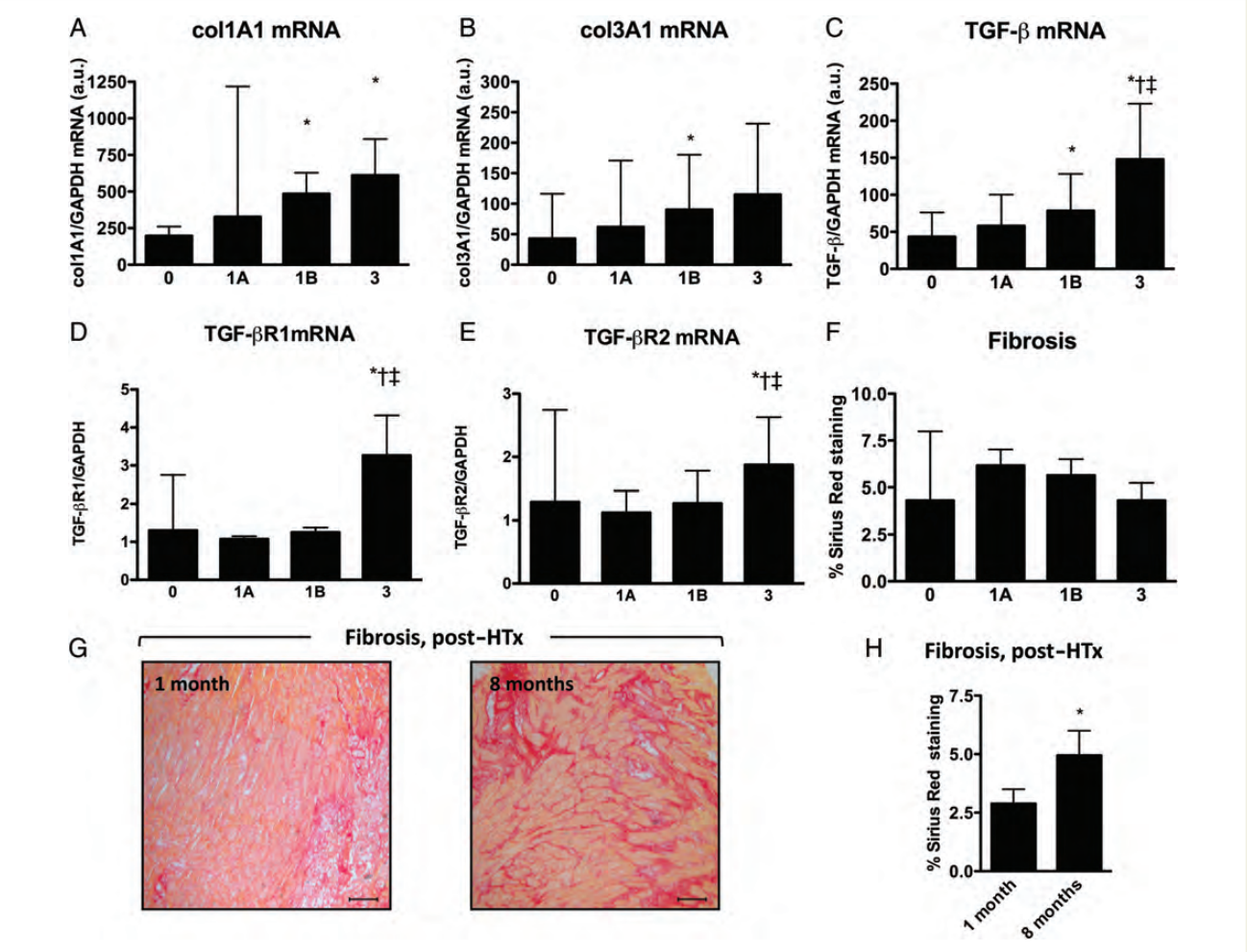

Figure 4 Cardiac allograft fibrosis. Intragraft transcript levels of (*A*) collagen type Iα1 (Col1a1) and (*B*) type IIIα1 (Col3a1) progressively increased with increasing grade of cardiac allograft rejection. (*C*–*E*) Intragraft transcript levels of (*C*) TGF-β, (*D*) TGF-β receptor-1 (TGF-βR1), and (*E*) TGF-β receptor-2 in respect to the grade of cardiac allograft rejection. (*F*) Sirius red stained collagen deposition with respect to the different grades of cardiac allograft rejection. (*G*) Representative Sirius red stained Grade 3 rejecting endomyocardial biopsies at 1 and 8 months after HTx. Scale-bar: 100 μm. (*H*) Cardiac allograft fibrosis is significantly increased at 8 months, when compared with 1 month after heart transplantation (*$P < 0.01$). *$P < 0.05$ vs. Grade 0 or vs. 1 month post-heart transplantation; †$P < 0.05$ vs. Grade 1A; ‡$P < 0.05$ vs. Grade 1B.

increase in collagen deposition was noted 8 months after transplantation, when compared with 1 month (*Figure 4G* and *H*).

Synd-1 and MMP-9 intragraft transcript levels discriminate rejecting from non-rejecting endomyocardial biopsies

Finally, we set out to determine whether measuring intragraft transcript levels of one or more ECM-related macromolecules could potentially discriminate rejecting from non-rejecting EMBs. For this purpose, EMBs displaying ISHLT Grade 3 were considered as 'rejecting', whereas EMBs that exhibit ISHLT Grade 0, 1A, and 1B as 'non-rejecting'. Next, receiver operating characteristic (ROC) curve analysis was performed for the four parameters that on univariate analysis had a *P*-value <0.01 (*Figure 5*). These included the 2log transformed intragraft transcript levels of synd-1 ($P < 0 < 0001$), CTGF ($P < 0.01$), MMP-9 ($P < 0.0001$), and TIMP-1 ($P < 0.01$). Strikingly, ROC curve analyses showed the most significant discrimination with an area under the curve of 0.969 for 2log(synd-1) [95% confidence interval (CI), 0.915–1.000; $P < 0.0001$] and 0.981 for 2log(MMP-9) (95% CI: 0.954–1.000; $P < 0.001$; *Figure 5*). An optimal cut-off was calculated at −0.44 for 2log(synd-1), representing a specificity of 80.0% and sensitivity of 100% and at 3.81 for 2log(MMP-9), representing a specificity of 95.4% and sensitivity of 100%.

Taken together, these analyses indicate that the intragraft transcript levels of synd-1 and MMP-9 show potential as a decision-making tool in clinical practice to diagnose human CAR based on EMBs. Nevertheless, future analysis using larger cohorts of EMBs should be performed to confirm our results.

Table 3 Intragraft transcript levels in relation to collagen synthesis

	Col Ia1 mRNA		Col IIIa1 mRNA	
	r-value	*P*-value	*r*-value	*P*-value
Proteoglycans and matricellular protein transcript levels				
Synd-1	0.45	<0.0001	0.43	<0.0001
Synd-4	0.15	0.298	0.16	0.188
TSP-1	0.57	<0.0001	0.54	<0.0001
TSP-2	0.76	<0.0001	0.72	<0.0001
CTGF	0.80	<0.0001	0.77	<0.0001
SPARC	0.91	<0.0001	0.84	<0.0001
Testican-1	0.58	<0.0001	0.66	<0.0001
MMP and TIMP transcript levels				
MMP-1	0.09	0.649	0.07	0.566
MMP-2	0.40	<0.001	0.40	<0.001
MMP-9	0.42	<0.0001	0.40	<0.0001
TIMP-1	0.63	<0.0001	0.61	<0.0001
MMP-14	0.64	<0.0001	0.65	<0.0001
TIMP-2	0.27	<0.05	0.26	<0.05
TIMP-3	0.08	0.934	0.16	0.206
TIMP-4	−0.10	0.506	−0.04	0.678
Pro-inflammatory cytokine and TGF-β-related transcript levels				
IL-6	0.41	<0.0001	0.38	<0.0001
TNF-α	0.12	0.452	0.20	<0.05
TGF-β	0.67	<0.0001	0.72	<0.0001
TGF-β-R1	0.41	<0.0001	0.40	<0.0001
TGF-β-R2	0.21	<0.05	0.22	<0.05
LTBP-1	0.33	<0.01	0.30	<0.01
LTBP-2	0.38	<0.0001	0.38	<0.0001
LTBP-3	0.42	<0.0001	0.40	<0.0001
LTBP-4	−0.06	0.532	0.07	0.949
$CD3^+$ leucocytes and $CD68^+$ monocytes				
$CD3^+$ leucocytes	0.65	<0.001	0.59	<0.01
$CD68^+$ monocytes	0.59	<0.005	0.52	<0.01

Synd-1, syndecan-1; TSP, thrombospondin; CTGF, connective tissue growth factor; SPARC, secreted protein acidic and rich in cysteine; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinases; IL, interleukin; TNF, tumour necrosis factor; TGF-β, transforming growth factor-β; TGF-β-R, TGF-β-receptor; LTBP, latent TGF-β-binding protein.

Discussion

Although discoveries of the last decade have made it clear that changes in the cardiac ECM modulate key inflammatory and reparative pathways and thereby co-determine the structural and functional integrity of the heart,[3,4] to date, no attempts have been made to investigate the intragraft expression of matricellular proteins, proteoglycans, MMPs and TIMPs in relation to inflammation, fibrosis, and the degree of CAR in the human transplanted heart. Our study demonstrates that—out of 15 ECM-related macromolecules—synd-1 and MMP-9 are the only two that consistently correlate with the degree of human CAR, the expression of pro-inflammatory cytokines TNF-α, IL-6, and TGF-β, infiltrating $CD3^+$ T-cells and $CD68^+$ monocytes, and collagen transcription in the transplanted heart. Furthermore, their protein expression robustly co-localized with areas of inflammation and sites of active remodelling, indicating synd-1 and MMP-9 as potent intragraft markers for the inflammatory response in human cardiac allografts. On the other hand, we also reveal that a multitude of additional non-structural ECM proteins, including SPARC, CTGF, TSP-1 and -2, Testican-1, MMP-2, -14 and, TIMP-1 closely associate with intragraft collagen transcription. More importantly, ROC curve analysis demonstrated that intragraft synd-1 and MMP-9 transcript levels might have potential as a decision-making tool in clinical practice to diagnose Grade 3 rejecting EMBs.

An accumulating body of evidence suggests that CAR surveillance based on intragraft gene-expression profiling my help to improve clinical management of transplant patients.[12–14] More specifically, these studies advocate that the use of gene-expression profiling might require fewer EMBs and could potentially provide a more uniform readout with greater sensitivity and limited sampling error when compared with the current histolopathological standard to identify and classify CAR.[13] Strikingly, our results indicate that synd-1 and MMP-9 intragraft transcriptional profiling may represent a novel quantitative measure to discriminate rejecting (ISHLT Grade 3) from non-rejecting hearts using a single EMB. Hence, if confirmed in a larger cohort of patients this approach could reduce the inter-reader, subjective variability, as well as grading-inconsistencies among the various transplant centers.[15] Furthermore, future development an intragraft transcriptional RT–PCR 'array', that not only includes synd-1 and MMP-9, but also other markers of acute CAR (such as FOXP3, Granzyme B, Perforin, and Fas-L),[16,17] as well as markers of cardiac allograft vasculopathy (i.e. TSP-1, IFN-γ-inducible CXCR3 chemokines IP-10, and I-TAC, etc.)[14,18]; markers of reduced long-term graft survival (i.e. VEGF, IL-6, TNF-α, and TGF-β)[5,19,20]; and others may allow us to detect certain molecular qualities of the transplanted heart that do not meet the eye under the microscope. Taken together, this approach could potentially provide an extended readout that not only determines the degree of CAR, but also reflects the efficacy of the immunosuppression, that identify patients at risk for the development of CAR and vasculopathy, and thereby co-determine the long-term clinical management of HTx patients.

Interestingly, our study is—to our knowledge—the first to support the concept that pro-inflammatory cytokines, T-cells and monocytes are closely associated with or even in part responsible for the induction of several ECM-related macromolecules in the human transplanted heart. It is well established that CAR is the result of a highly complex, multiplayer immunologic reaction in which pro-inflammatory cytokines, T-cells, and monocytes play a prominent role.[1,5,14] Our results extend these findings with the observation that the expression of TNF-α, IL-6, and TGF-β, but also the influx of $CD3^+$ T-cells and $CD68^+$ monocytes strongly relate to each other and to the grade of human CAR. Moreover, the presence of these pro-inflammatory cytokines T-cells and/or monocytes is strongly paralleled by an increased expression of primarily synd-1, MMP-9, CTGF, and TIMP-1. These observations are in agreement with previous reports, ranging from the expression and functional implications of synd-1 and MMP-9 in a wide

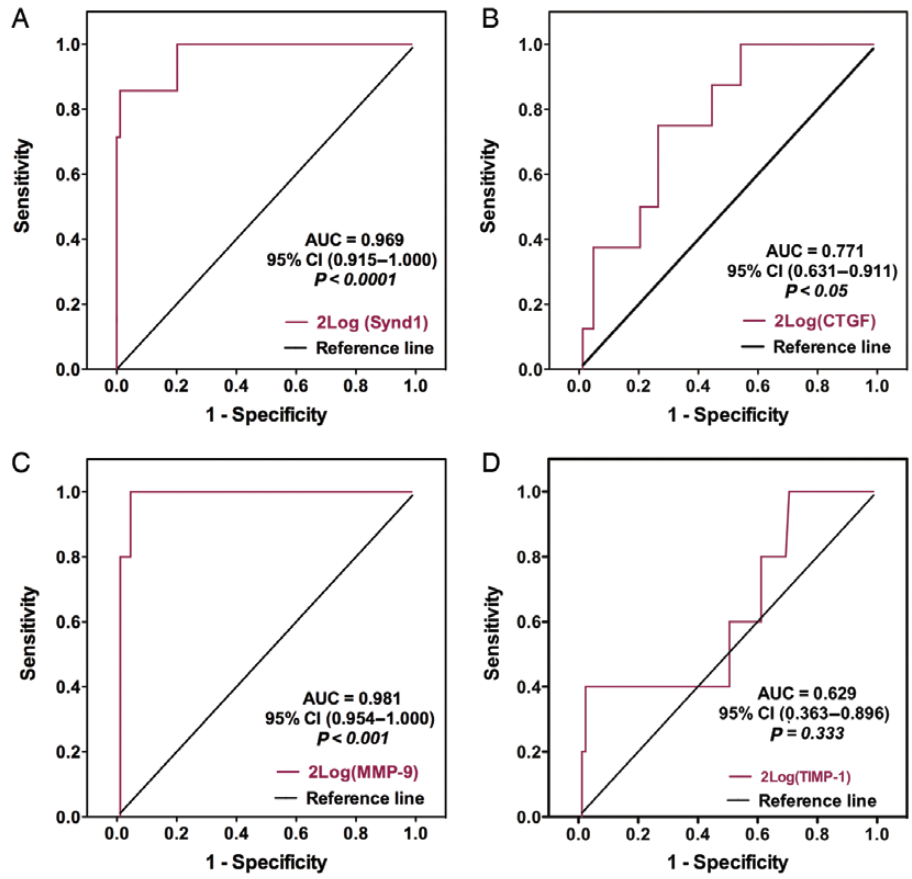

Figure 5 Intragraft synd-1 and MMP-9 mRNA profiling as potential biomarkers for the grade of cardiac allograft rejection. Receiver operating characteristic curves for (*A*) 2log(synd-1), (*B*) 2log(MMP-9), (*C*) 2log(TIMP-1) and (*D*) 2log(CTGF). For this model, Grade 3 was considered as 'rejection', whereas Grade 0, 1A, and 1B as 'non-rejection' and base-2 logarithmic (2log) transformed intragraft transcript levels were used. AUC, area under the curve; 95% CI, 95% confidence intervals.

variety of inflammatory disorders (reviewed in by Teng *et al.*[7] and Ram *et al.*[21]); the association of synd-1-positive monocytes and MMP-9 expression with rejection of liver, paediatric kidney, and/or pulmonary allografts[22–26]; increased CTGF and MMP-9 transcript levels in a mouse model of CAR[5,10]; to the immunomodulatory properties of synd-1,[7,23] MMP-9,[10] CTGF,[5] and TIMP-1[9] towards T-cells and macrophages. In addition, two-independent studies revealed that in a mouse model of acute CAR, MMP-9 mediates the influx of mononuclear cells into the allografts and regulates the activation and expansion of alloreactive T-cells, whereas the systemic use of a broad-spectrum MMP-inhibitor (GM6001) enhanced allograft survival by directly suppressing the infiltration of inflammatory cells in a mouse model of CAR.[10,11] Similarly, the use of CTGF neutralizing antibodies limited graft infiltration by T-cells in a mouse model of chronic CAR.[27] On the other hand, our group recently revealed that gene-overexpression of synd-1 or TIMP-1 protects the heart against exaggerated inflammation and adverse remodelling in a mouse model of myocardial infarction.[6,28] However, whether up-regulation of synd-1 and TIMP-1 aims to limit the inflammatory influx, and thereby protects the transplanted heart should be the subject of further animal studies. Taken together, our findings are likely to be indicative of a functional role for synd-1, MMP-9, CTGF, and TIMP-1 in the complex inflammatory pathways underlying human CAR and might, therefore, represent potential therapeutic targets to prevent human CAR.

Besides inflammation, cardiac allograft fibrosis has recently been recognized as an important limiting factor towards graft function and long-term survival.[5] We reveal that the production of pro-inflammatory cytokines IL-6 and TGF-β; components of the TGF-β pathway TGF-β-R1 and 2, LTBP-1, -2, and -3; the influx of T-cells and monocytes; as well as the transcription of synd-1, TSP-1 and -2, CTGF, SPARC, Testican-1, MMP-2, -9, -14, TIMP-1, and -2 all strongly relate to the expression of Collagen Ia1 and IIIa1 in the transplanted hearts. Moreover, collagen transcript levels progressively increased with the degree of CAR. Interestingly, IL-6 and TGF-β were previously reported to have a negative impact on graft survival through their chemotactic and pro-fibrotic effects.[5] Interestingly, not only IL-6 and TGF-β, but also synd-1, TSP-1 and -2 CTGF, TIMP-1 and -2 either promote fibroblast proliferation or regulate ECM production by regulating collagen synthesis, assembly and/or maturation in the diseased

heart.[2,4–6,9] In addition, both animal and clinical studies indicated that TSP-1, CTGF, MMP-2, -14, TIMP-2, and TGF-β might directly affect or are associated with the development of cardiac allograft vasculopathy.[5,18,29,30] The present study translates these findings to the human clinical situation and indicates that the TGF-β pathway, synd-1, TSP-1 and -2, CTGF, SPARC, testican-1, MMP-2, -9, -14, and TIMP-1 and -2 might withhold important targets and mechanistic insights to limit the deleterious fibrotic response, cardiac allograft vasculopathy, and progressive loss of function of the human transplanted heart.

An intriguing observation is that total collagen deposition within our cohort of EMBs did not reflect the increasing transcript levels of COLIa1 and COLIIIa1 with increasing grade of CAR. This discrepancy between mRNA and protein could be the result of increased MMP-9-mediated collagen degradation as suggested by the strongly increased MMP-9 transcript, protein, and MMP2/9 *in situ* proteolytic activity levels observed in Grade 3 rejecting EMBs (Supplementary material online, *Figure S5*).[31] In this regard, it might be of future interest to evaluate intragraft as well as circulating markers of collagen metabolism, including N-terminal propeptides of pro-collagen I and III (PINP and PIIINP) and carboxy-terminal telopeptides, in relation to the degree of human CAR.[31–33] Nevertheless, more in-depth investigations will be required to unveil the significance of this phenomenon.

Several limitations should be noted when interpreting our results. As mentioned before, it is important to note that due to our limited number of ISHLT Grade 3 rejecting EMBs, further studies are be mandatory to confirm our results. Furthermore, the amount of endomyocardial tissue obtained did not permit protein analysis by multiple techniques. For this reason, we chose to perform semi-quantitative analysis based on immunohistochemistry, because this permitted the analysis of several protein expressions as well as their localization in the same tissue. The immunohistochemical techniques used in this study have been validated by previous studies from our laboratory.[31]

Despite the limitations of our study, we identified synd-1 and MMP-9 as novel surrogate markers for the degree of inflammation in the transplanted heart. More importantly, we reveal that synd-1 and MMP-9 intragraft transcriptional profiling represents as a novel quantitative tool to discriminate rejecting (ISHLT Grade 3) from non-rejecting hearts. Furthermore, our data point towards important molecular interactions between the immunologic response and the induction of non-structural matrix proteins and collagen expression in the human transplanted heart. Therefore, future efforts to elucidate the interplay between these factors may direct the development of improved diagnostic tools and targeted therapies that limit CAR, vasculopathy, and progressive loss of function of the transplanted heart.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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