Original paper

The potential role of ultrasonic strain imaging and immunophenotyping in diagnosing acute rejection after heart transplantation

Tomas Daukšas [a,*], Rita Sudikienė [b,c], Radvilė Malickaitė [b], Jelena Čelutkienė [b]

[a] *Faculty of Medicine, Vilnius University, Vilnius, Lithuania*
[b] *Clinic of Cardiovascular Diseases, Faculty of Medicine, Vilnius University, Vilnius, Lithuania*
[c] *Clinic of Children's Diseases, Faculty of Medicine, Vilnius University, Vilnius, Lithuania*

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Summary

Background: There has been a continued search for an accurate noninvasive technique for detecting sub-clinical acute rejection in heart transplant recipients.

Ultrasonic deformation imaging – strain/strain rate (SR) – is sensitive in detecting sub-clinical abnormalities in regional myocardial function and could potentially be sensitive tool to detect changes in deformation induced by graft rejection. There is an evidence of the importance of immunophenotyping in determining transplant rejection as well.

Aim: to assess the potential role of cardiac ultrasound velocity/strain imaging and immunological testing (alterations in peripheral blood T-cells subsets activation) in the detection of acute allograft rejection proven by endomyocardial biopsy.

Patients and methods: A retrospective observational study was carried out involving 28 patients (22 men and 6 women) who underwent a total of 167 routine follow up endomyocardial biopsies with correlative cardiac ultrasound and immunophenotyping data. Myocardial velocity derived from pulsed wave tissue Doppler imaging (PW-TDI) was calculated in the longitudinal direction in basal lateral segment of left ventricle (LV) in 4-chamber view and in the radial direction in basal posterior LV segment in long parasternal axis view. Global systolic strain by speckle tracking was calculated in the longitudinal, radial and circumferential directions.

Results: According to the International Society of Heart and Lung Transplantation criteria, 90 biopsies (Group 1) had grade 0, 1R or 2R rejection, and 30 biopsies (Group 2) had grade 3R rejection. The results of the forward selection revealed that the best indicator to predict the rejection was the amount of CD4+/HLA-DR+ cells. Univariate logistic regression analysis showed that global radial systolic strain performs better in terms of receiver-operator-characteristic curves (ROC) than the rest of the measurements (area-under-curve 0.83, where a cut-off value of 32.4% had 91.7% sensitivity and 77.8% specificity).

Conclusions: One of the best non-invasive parameters in the detection of acute sub-clinical rejection appears to be the expression of CD4+/HLA-DR+ cells. Among ultrasound markers the best predictor of acute rejection is global radial systolic strain.

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Introduction

Timely detection of acute sub-clinical allograft rejection is a continuous imperative after heart transplantation. Right ventricular endomyocardial biopsy currently remains the gold standard for diagnosing rejection but carries all the potential risks and drawbacks of an invasive procedure. In the search for an alternative sensitive noninvasive method to diagnose sub-clinical rejection, techniques such as cardiac ultrasound, gamma scintigraphy, phosphorus-31 nuclear magnetic resonance spectroscopy, serological and immunological testing have all been tried [1].

Regional strain imaging is a new cardiac ultrasound modality which allows the detection of abnormalities in regional contractile function. It

* Corresponding address: Tomas Daukšas, Faculty of Medicine, Vilnius University, M.K. Čiurlionio str. 21, LT-03101 Vilnius, Lithuania. Tel.: +37061473160

E-mail: tomas.dauksas@yahoo.com (T. Daukšas).

is an accurate method for noninvasive quantification of systolic deformation, and has been shown to detect changes in regional systolic function at an earlier sub-clinical stage than either conventional echocardiography [1–3].

Allograft rejection is mediated by T-cells that recognize allogeneic major histocompatibility complex (MHC) molecules via the direct and indirect pathway. The direct pathway involves T-cells that react against MHC/peptide complexes expressed on the surface of donor antigen-presenting cells (APCs). In contrast, T-cells involved in the indirect pathway recognize peptides derived from processing and presentation of allogeneic MHC molecules by self (recipient) APCs. To explore the relative contribution of these two pathways to rejection, we have evaluated the response of peripheral blood T-cells [4].

Thus, the purpose of our retrospective observational study was to assess the potential role of cardiac ultrasound velocity/strain imaging and immunological testing (alterations in peripheral blood T-cells subsets activation) in the detection of acute allograft rejection proven by endomyocardial biopsy.

Patients and methods

Patients

The study population consisted of 28 patients (22 men and 6 women, mean age 44 ± 15 years) who underwent heart transplantation and had 167 biopsies carried out in the Cardiology Department of the Vilnius University Hospital Santariškių Klinikos as part of their routine postoperative management. Inclusion criteria: standard echocardiography including tissue Doppler imaging (TDI) (for the calculation of radial and longitudinal velocity) and speckle tracking (for the calculation of radial, longitudinal and circumferential strain) performed within 1 month before the endomyocardial biopsy or at the same day if the rejection is grade 3R; standard echocardiography including TDI and speckle tracking performed within 3 months before or after endomyocardial biopsy if the rejection grade is 0, 1R or 2R (the echo data were retrospectively analyzed for patients who had undergone biopsy and then, depending on the grade of rejection, time-frame for valid echo was set 1 month if the rejection is grade 3R and 3 months if the rejection grade is 0, 1R or 2R); no bacterial and fungal infection found within 3 weeks before and after endomiocardial biopsy, immunophenotyping and CMV/EBV PCR performed within 2 days before or after biopsy. One hundred and twenty observations met the criteria.

Endomyocardial biopsy

All endomyocardial biopsies (EMB) were performed according to the standard procedure used in posttransplant patients in our institution, i.e. a jugular approach, a Scholten bioptome and a minimum of three samples from right ventricle per biopsy session.

As per institution policy, acute rejection is monitored by serial myocardial biopsies performed one week after the transplantation, on biweekly basis till the end of the hospital stay, and approximately bimonthly till the end of the first year after cardiac transplantation. After this, a biopsy is either taken 1 year after heart transplantation or when patients develop abnormal clinical findings. All the biopsies were read by an experienced pathologist who was blinded to the cardiac ultrasound findings.

Cellular rejection was graded using the International Society of Heart and Lung Transplantation 2004 classification of acute cellular rejection in transplant endomyocardial biopsy specimens: grade 0, no evidence of acute cellular rejection; grade IR, interstitial and/or perivascular infiltrate with up to one focus of myocyte damage; grade 2R, two or more foci of infiltrate with associated myocyte damage; grade 3R, diffuse infiltrate with multifocal myocyte damage +/– edema, +/– hemorrhage, +/– vasculitis.

Patients for this study were divided into Group 1 with grade 0, 1R or 2R rejection and Group 2 with grade 3R rejection.

Standard echocardiography

Patients underwent a conventional transthoracic cardiac ultrasound examination together with the acquisition of myocardial velocity and strain imaging data.

The following parameters were measured: left ventricle (LV) ejection fraction (using the Simpson or eye ball method), cardiac output (l/min), stroke volume (ml) (based on Doppler flow measurement), cardiac index (l/min/m^2). PW-TDI velocity was acquired and calculated in the longitudinal direction in basal lateral segment of LV in 4-chamber view and in the radial direction in basal posterior LV segment in long parasternal view. Global systolic strain by speckle tracking was calculated in the longitudinal, radial and circumferential directions.

The echocardiographic studies were performed using a Vivid 7 ultrasound scanner (GE Healthcare, USA). The images were acquired from standard parasternal and apical views. Three heart cycles were stored in a cineloop format and sent to an external workstation for post-processing.

Immunophenotyping

Lymphocyte subpopulation analysis, including T-cell activation markers (CD4+/CD103+, CD8+/CD103+, CD4+/CD134+, CD8+/CD134+, CD4+/CD25+, CD8+/CD57+, CD8+/CD38+, CD8+/HLA-DR+ and CD4+/HLA-DR+) detection, was performed by two-color immunofluorescence technique using mouse anti-human monoclonal antibodies (Becton Dickinson Immunocytometry Systems, BDIS, USA), labeled with fluorescein isothiocyanate and phycoerythrin. Whole blood was incubated with the appropriate antibody combination, then the red blood cells were lysed with a lysing solution (BDIS, USA) and the remaining cells were washed three times using phosphate-buffered saline. At least 10,000 leukocytes were analyzed on a FACSCalibur flow cytometer (BDIS, USA) using SimulSET software.

For both CMV and EBV analysis DNA has been extracted from whole blood using QIAamp® DNA Blood Mini Kit on fully automated QIAcube instrument (Qiagen, Germany). Detection and quantitation of viral DNAs was performed by real-time PCR using *artus* CMV RG PCR Kit and artus EBV RG PCR Kit (Qiagen, Germany) on Rotor-Gene® 3000 instrument.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (version for Windows). Continuous values are reported as mean ± SD (standard deviation). In order to find out the best predictor for rejection we have applied two methods: forward logistic regression and receiver operating curves.

Plots of ROC curves were also used to determine sensitivity and specificity. Statistical significance was inferred for $p < 0.05$.

Results

Comparing ultrasound markers between the two groups, all of them, except global circumferential systolic strain, were lower in Group 2. However, only LV ejection fraction, radial myocardial velocity and global radial systolic strain were significantly different ($p < 0.05$) (Table 1). Except the amount of CD4+/CD103+ and CD4+/CD25+ cells, majority of the immunophenotypic measurements were increased in the group with a rejection. Significant differences were observed in the amount of CD8+/HLA-DR+ and CD4+/HLA-DR+ cells ($p < 0.05$) (Table 1).

Predictive ability for detecting rejection was determined by univariate logistic regression analysis, where each variable was considered as independent. The results of univariate analysis are summarized in Table 2. Thus, according to univariate analysis, LV EF ejection fraction, radial myocardial velocity, global radial systolic strain, amount of CD8+/CD57+, CD8+/HLA-DR+, CD4+/HLA-DR+ were independently associated with rejection. Secondly, multivariate analysis was used. Because the number of variables com-

Table 1.
Differences of study parameters between the two groups

Characteristic	Group 1	Group 2
No. of EMB	90	30
Stroke volume (ml)	63.71 ± 19.28	56.45 ± 13.76
Ejection fraction (%)	54.35 ± 8.41	50.08 ± 10.93*
Cardiac output (l/min)	5.06 ± 1.45	4.46 ± 0.93
Cardiac index (l/min/m^2)	2.65 ± 0.69	2.39 ± 0.52
Radial myocardial velocity (cm/s)	9.97 ± 1.56	8.55 ± 1.16*
Longitudinal myocardial velocity (cm/s)	10.28 ± 2.54	9.50 ± 2.23
Global radial systolic strain (%)	37.40 ± 13.85	25.80 ± 7.87*
Global longitudinal systolic strain (%)	17.05 ± 5.05	15.78 ± 4.49
Global circumferential systolic strain (%)	14.85 ± 5.71	15.72 ± 6.42
Amount of CD4+CD103+ (%)	1.85 ± 4.52	1.4 ± 1.42
Amount of CD8+CD103+ (%)	2.76 ± 3.33	4.45 ± 3.9
Amount of CD4+CD134+ (%)	5.85 ± 4.24	7.1 ± 4.5
Amount of CD8+CD134+ (%)	5.17 ± 5.50	6.1 ± 4.5
Amount of CD4+CD25+ (%)	29.62 ± 10.14	26.65 ± 12.74
Amount of CD8+CD57+ (%)	14.53 ± 9.26	20.6 ± 10.41
Amount of CD8+CD38+ (%)	26.38 ± 9.76	31.55 ± 12.54
Amount of CD8+/HLA-DR+ (%)	12.26 ± 7.8	20.26 ± 11.68*
Amount of CD4+/HLA-DR+ (%)	4.94 ± 1.90	6.65 ± 2.87*

Values are *n* or mean ± SD; *$p < 0.05$; EMB – endomyocardial biopsy.

Table 2.
The results of univariate analysis for prediction of acute rejection

Variable	Coefficient (Standard error)	Odds ratio (95% CI)	*p* value
Stroke volume (ml)	–0.027 (0.021)	0.974 (0.934; 1.014)	0.201
Ejection fraction (%)	–0.059 (0.027)	0.942 (0.894; 0.993)	0.026
Cardiac output (l/min)	–0.305 (0.257)	0.737 (0.445; 1.221)	0.236
Cardiac index (l/min/m^2)	–0.400 (0.465)	0.670 (0.270; 1.666)	0.389
Radial myocardial velocity (cm/s)	–0.936 (0.400)	0.392 (0.179; 0.858)	0.019
Longitudinal myocardial velocity (cm/s)	–0.164 (0.150)	0.849 (0.633; 1.138)	0.273
Global radial systolic strain (%)	–0.082 (0.036)	0.921 (0.858; 0.989)	0.024
Global longitudinal systolic strain (%)	–0.068 (0.073)	0.934 (0.809; 1.078)	0.352
Global circumferential systolic strain (%)	0.026 (0.060)	1.026 (0.913; 1.154)	0.662
Amount of CD4+CD103+ (%)	–0.041 (0.096)	0.960 (0.795; 1.159)	0.671
Amount of CD8+CD103+ (%)	0.135 (0.089)	1.145 (0.963; 1.362)	0.126
Amount of CD4+CD134+ (%)	0.067 (0.066)	1.069 (0.939; 1.218)	0.311
Amount of CD8+CD134+ (%)	0.035 (0.055)	1.035 (0.930; 1.153)	0.525
Amount of CD4+CD25+ (%)	–0.025 (0.026)	0.976 (0.927; 1.027)	0.343
Amount of CD8+CD57+ (%)	0.064 (0.032)	1.067 (1.002; 1.136)	0.044
Amount of CD8+CD38+ (%)	0.044 (0.027)	1.045 (0.991; 1.102)	0.103
Amount of CD8+/HLA-DR+ (%)	0.086 (0.034)	1.090 (1.020; 1.165)	0.011
Amount of CD4+/HLA-DR+ (%)	0.314 (0.133)	1.368 (1.054; 1,776)	0.018

The values: standard error; odds ratio (lower limit of 95% CI; upper limit of 95% CI); *p* value for checking the hypothesis that the regression coefficient statistically significantly differed from 0 (i.e., variable allows to predict biopsy results).

Table 3.
The results of forward selection model for prediction of acute rejection

Variable	Coefficient (Standard error)	Odds ratio (95% CI)	*p* value
Amount of CD4+/HLA-DR+ (%)	0.557 (0.253)	1.745 (1.062; 2.868)	0.028

The values: regression coefficient (standard error); odds ratio (lower limit of 95% CI; upper limit of 95% CI); *p* value for checking the hypothesis that the regression coefficient statistically significantly differed from 0 (i.e., variable allows to predict biopsy results). Model obtained using 20 observations.

Table 4.
ROC analysis of independent predictors of rejection

Variable	AUC	Standard deviation	*p* value	Number of observations
Ejection fraction (%)	0.636	0.068	0.048	101
Amount of CD4+/HLA-DR+ (%)	0.674	0.078	0.035	54
Amount of CD8+/CD57+ (%)	0.696	0.078	0.017	54
Amount of CD8+/HLA-DR+ (%)	0.708	0.079	0.013	53
Radial myocardial velocity (cm/s)	0.755	0.093	0.023	32
Global radial systolic strain (%)	0.833	0.068	0.001	39

The values: AUC – area under ROC curve; SD – standard deviation; *p* value for checking the hypothesis that the regression coefficient statistically significantly difference from 0 (i.e., variable allows to predict biopsy results).

pared with the number of observations is large, a forward selection model was applied.

According to the results of the forward selection, the best indicator to predict the rejection is the amount of CD4+/HLA-DR+ (Table 3).

Finally we estimated areas under receiver operating curves (AUCs) for each of the variables obtained by previous analysis. The AUC of global radial systolic strain was the largest (0.83, see Table 4), where a cut-off value of 32.4% had 91.7% sensitivity and 77.8% specificity.

We have also built logistic regression model which employs both CD4+/HLA-DR+ and radial systolic strain. The AUC of this model was 0.933. The best possible sensitivity and specificity were 90.0% and 92.3%, respectively. Model was built using 23 observations. Model summary is presented in Table 5.

Discussion

To our best knowledge, no study was carried out addressing the question of the potential role of combined ultrasonic strain imaging and immunophenotyping techniques in diagnosis of

Table 5.
Logistic regression model which uses two best independent predictors

Variable	Coefficient (Standard error)	Odds ratio (95% CI)	*p* value
Amount of CD4+/HLA-DR+ (%)	0.436 (0.275)	1.546 (0.902; 2.650)	0.134
Radial strain	0.234 (0,111)	1.264 (1.017; 1.571)	0.034

The values: regression coefficient (standard error); odds ratio (lower limit of 95% CI; upper limit of 95% CI); *p* value for checking the hypothesis that the regression coefficient statistically significantly differed from 0 (i.e., variable allows to predict biopsy results). Model obtained using 23 observations.

acute rejection after heart transplantation. Therefore, we can refer to the results of limited number of studies, which investigate the use of tissue Doppler imaging and speckle tracking techniques in diagnosing allograft rejection.

Tissue Doppler-derived strain and SR imaging

TDI, also known as tissue velocity imaging, is currently accepted as a sensitive and sufficiently accurate echocardiographic tool for quantitative assessment of cardiac function [5,6]. Several tissue Doppler velocity parameters appeared to be useful for the diagnosis and prediction of long-term prognosis in major cardiac diseases [7–9]. Myocardial time-velocity curves can be obtained either online as spectral pulsed TDI, known as PW-TDI, or reconstructed offline from two-dimensional (2D) color coded TDI images, known as color TDI loops. In addition to velocity and displacement (tissue tracking) measurements, due to the relationship between velocity and SR, TDI also allows the reconstruction of strain (and SR) curves and color coded images.

Non-Doppler speckle-tracking derived 2D strain imaging

Non-Doppler 2D-strain imaging derived from speckle tracking is a newer echocardiographic technique for obtaining strain and SR measurements [2,4,10–13]. It analyzes motion by tracking speckles (natural acoustic markers) in the 2D ultrasonic image. These acoustic markers are statistically equally distributed throughout the myocardium and their size is about 20–40 pixels. These markers ("stable" speckles) within the ultrasonic image are tracked from frame to frame.

By tracking these speckles, strain and SR can be calculated. The advantage of this method is that it tracks in two dimensions, along the direction of the wall, not along the ultrasound beam, and thus is angle independent [2]. The necessity of high image quality is a major limitation for routine clinical applicability in all patients. At present, the optimal frame rate for speckle tracking seems to be at least 50–70 frames per second, which is lower compared to TDI (>180 frames per second).

Although speckle-tracking derived 2D-strain and TDI derived strain calculations do not give the same values (2D strain imaging gives lower SR values), strain and SR measurements obtained by these two different imaging techniques correlate well [14].

A series of studies have shown standard gray scale echocardiography (either 2D or M-mode) to have a low sensitivity in detecting changes in either systolic or diastolic function due to acute rejection. Our case-control study showed that LV ejection fraction was the only standard echocardiography parameter, which was independently associated with rejection. The changes in transmitral Doppler flow indices are rather non-specific in detecting rejection as they are markedly influenced by other variables such as heart rate, age and loading conditions [15]. Furthermore, the heart of a transplanted patients is denervated. This usually results in a sinus tachycardia which can give rise to a pseudorestrictive ventricular filling pattern similar to that which occurs during acute rejection [16].

Myocardial velocity imaging has recently been shown to be an adjunct to assess the function of the transplanted heart [17]. Nevertheless, there are no already known markers which are being used to confirm the rejection of transplant.

The velocity values obtained after transplantation are markedly influenced by the exaggerated overall motion of the transplanted heart. This is a major limitation when using velocities to describe regional function in such hearts. Nevertheless, the results of our study proved that radial myocardial velocity is one of the best predictor of acute rejection (AUC = 0.755; *p* value = 0.023). Myocardial oedema which appears during acute rejection would result in decreased systolic deformation as such interstitial fluid would be incompressible. Thus strain imaging might be sufficiently sensitive to detect sub-clinical changes in regional systolic function. Our study showed that in a transplanted heart, a significant reduction in global radial systolic strain could be reliable early sign of rejection.

Bader et al. found that in patients with acute antibody-mediated rejection (AMR), LV fractional shortening was significantly reduced compared

with those with no AMR (mean ± SD, 31.8 ± 8.9% vs. 36.0 ± 7.1%; $p = 0.02$). This study concluded, that although one echocardiographic parameter was statistically different in the setting of rejection, lack of consistency and overlap between nonrejection and rejection groups does not permit definitive noninvasive diagnosis of cardiac allograft rejection using this imaging modality [18].

In a study by Mankad et al., a tissue Doppler peak to peak mitral annular velocity > 135 mm/s was found to be valuable in detecting early rejection. High TDI velocities had a good ability to exclude rejection. That study, however, included a limited number of TDI studies ($n = 89$), with only 14 episodes of rejection, mostly in the mild range [19].

A study by Vivikananthan et al. has shown promising results with myocardial performance index as an indicator of rejection. However, this study also is limited by its retrospective nature and small sample size [20].

Comparing the deformation parameters obtained from patients who underwent routine endomyocardial biopsies, Marciniak et al. found significantly lower LV longitudinal and radial peak systolic strain and SR values in patients with acute rejection ≥grade 1B in comparison to those with biopsies graded between 0 and 1A [1]. In patients with biopsy-proven acute rejection episodes ≥grade 3, Dandel et al. found a significant ($p < 0.05$) reduction of LV systolic and diastolic radial, circumferential and longitudinal global peak strain and SR values in comparison to the values measured before rejection. In our study radial myocardial velocity and global radial systolic strain were also significantly lower in the group with 3R grade rejection ($p < 0.05$). Dandel et al. claim that systolic and diastolic global strain rate reduction appeared to be more sensitive for the early detection of acute rejection than the reduction of systolic and early diastolic global strain values. A sudden drop of ≥15% of the radial global strain rate in heart transplanted patients appeared highly predictive for acute biopsy-proven rejection [21]. Our study results show that global radial systolic strain had the largest AUC – 0.83, where a cut-off value of 32.4% had 91.7% sensitivity and 77.8% specificity.

Immunophenotyping

In recent years the application of flow cytometry has progressed rapidly, nevertheless, there have been only few attempts to correlate post-transplant immunophenotypic monitoring of peripheral blood mononuclear leukocytes with rejection episodes in heart transplant recipients. While investigating innate immunity it was shown, that pre-operative neutrophil surface adhesion molecule CD11b expression after *in vitro* lipopolysaccharide stimulation correlated with rejection grade detected in the first endomyocardial biopsy sample [22]. Some data suggests that cytotoxic lymphocyte markers, such as CD57 and CD56, were higher expressed on lymphocytes in transplanted patients compared with controls [23]. Post-transplant immunophenotype is shown to be dependent on immunosuppression used, for example, in patients treated with mycophenolate mofetyl a significant reduction of the B-cell count was observed in comparison to a healthy control group and patients under therapy with the other purine synthesis inhibitor azathioprine [24]. Besides, the percentages of CD38-positive B-cells, activated T-cells (CD4/CD25, CD8/CD38) and HLA-DR-expressing NK-cells were reduced during therapy with mycophenolate mofetil [24].

Recently great emphasis is given to the control mechanisms of alloimmune response, including impact on the regulatory cells, adhesion molecules and cell-cell interaction co-receptors. For example, greater amount of regulatory subpopulation of T-cells (Tregs) expressing CD4 and CD25 were shown in cardiac allograft vasculopathy (CAV) patients versus no CAV patients [25]. Besides, changes of regulatory cell number has been seen during extracorporeal photopheresis (ECP): in experimental setting it increases the frequency of FoxP3/CD4/CD25 splenic T-cells, and its effects can be transferred to untreated recipients using minimal numbers of CD4/CD25 T-cells, indicating that CD4/CD25 T-cells may play a key role in the immunomodulatory effects of ECP.

It was shown that adhesion molecule CD103 is expressed not only on T-cells in lung and gut, but also on allograft-infiltrating T-cells, besides, human CD103/CD8 T-cells can be induced by stimulation with alloantigens *in vitro* [26]. In experimental models it was shown that T-cell costimulatory molecule CD134 (OX40) can be expressed by functionally different T-cell subsets, e.g., activated T-effector-cells and both natural and induced Foxp3+ Tregs [27]. Rat cardiac transplantation model confirmed that CD134-CD134L blockade on allograft rejection in fully MHC-mismatched setting alone did not prolong graft survival compared with that of untreated recipients. However, in combination with B7 blockade, long-term allograft survival was achieved in all recipients, suggesting that CD134–CD134L is a critical pathway in alloimmune responses [28].

Nevertheless, little information exists regarding the relation between the expression of regula-

tory and co-stimulatory molecules on the surface of peripheral blood leukocytes and echocardiographic data in humans.

This methodology described in the article can be easily repeated, is non-invasive and less costly compared with biopsy; furthermore, we could use immunophenotyping when strain imaging brings a suspicion of acute rejection.

Conclusion

One of the best non-invasive parameters in the detection of acute sub-clinical rejection appears to be the expression of CD4+/HLA-DR+. Among ultrasound markers the best predictor of acute rejection is global radial systolic strain.

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