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Aortic stenosis and aortic regurgitation express different titin isoforms: Differences and relationships with functional and geometric characteristics☆

Vito Mannacio [a,*], Luigi Mannacio [a], Anita Antignano [b], Vincenzo De Amicis [a], Francesco Musumeci [c], Gabriele Iannelli [a]

[a] Department of Cardiac Surgery, University Federico II, School of Medicine, Naples, Italy
[b] Department of Cardiology, Azienda Ospedaliera Santobono-Pausillipon, Naples, Italy
[c] Department of Cardiac Surgery, Azienda Ospedaliera S. Camillo Forlanini, Rome, Italy

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ABSTRACT

Background-Titin represents an important biomechanical sensor which determines compliance and diastolic/systolic function of the left ventricle (LV). To assess the different titin-isoform expression and the relationships with functional and geometric patterns, we analyzed titin-isoform expression and cardiomyocytes contractile function in myocardial biopsy samples of patients undergoing aortic valve replacement (AVR) for aortic stenosis (AS) and for aortic regurgitation (AR).

Method -Specimens, collected from the LV of 35 with AS and 35 with AR undergoing AVR were analyzed for titin-isoform expression and cardiomyocytes force measurement. Ten donor hearts were analyzed as controls for normal values. Results were implemented with preoperative geometry and function assessed by Doppler echocardiography.

Results-Compared to controls, N2BA/N2B titin-isoforms ratio was reduced to 0.24 in AS ($p < 0.001$) but increased to 0.51 in AR ($p < 0.001$). N2BA/N2B titin-isoforms ratio was further reduced in 8 patients with severe (restrictive) diastolic dysfunction (0.17 ± 0.03, $p < 0.001$) but was increased in patients with severe systolic dysfunction (0.58 ± 0.07, $p < 0.001$). As compared to controls, Fpasive was higher in AS (6.7 ± 0.2 vs 4.4 ± 0.4 kN/m^2, $p < 0.001$) but was lower in AR (3.7 ± 0.2 vs 4.4 ± 0.4 kN/m^2, $p < 0.001$). Total force was comparable. Fpassive was significantly higher in AS patients with severe than with moderate LV diastolic dysfunction (7.1 ± 0.5 vs $6.6. \pm 0.6$, $p = 0.004$).

Conclusions-titin-isoform expression differs in AS and AR as adaptive response to different pathophysiologic scenarios. Co-expressing isoforms at varying ratios results in modulation of the passive mechanical behavior of the LV at different degree of dysfunction and allows for compensative adjustment of the diastolic/systolic properties of the myocardium.

1. Introduction

Aortic stenosis (AS) and aortic regurgitation (AR) involve adaptive processes which compensate for pressure or volume overload. These processes are accompanied by structural remodeling substantiated by derangements in the muscular and nonmuscular compartments of the left ventricle (LV). Cardiomyocytes develop distinct structural reshaping of cytoskeletal, modifications in membrane-associated proteins and reassembly of sarcomere components [1–3].

In the last years, several studies emphasized the role of titin within the sarcomeres as the main determinant for the correct alignment of actin and myosin myofilaments and their elastic properties [4,5]. Titin is expressed in two different isoforms, N2B and N2BA, with different molecular and functional characteristics: the N2B isoform is stiffer and less distensible, the longer N2BA isoform is more elastic and compliant [6]. Together with the interstitial elastic and connective components, titin isoform ratio determines not only the rigidity and the diastolic properties of the ventricle, but also the systolic function modulating the Frank-Starling mechanism [7].

☆ All author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

* Corresponding author at: Via S. Domenico 62, 80127 Naples, Italy.
E-mail address: vitoantonio.mannacio@unina.it (V. Mannacio).

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Table 1
Clinical Profile and Echocardiographic Left Ventricular Morphologic and Functional Characteristics of Patients.

	Aortic stenosis ($n = 35$)	Aortic regurgitation (n.35)	Controls ($n = 10$)	*p	**p
Age (y)	61.5 ± 8.5	59.8 ± 9.3	38.7 ± 7.1	0.3	<0.001
Female sex	13(37.1%)	14(40%)	3(30%)	0.9	0.9
BMI (units of kg/m^2)	23.6 ± 4.7	25.4 ± 5.1	25.3 ± 3.2	0.2	0.3
Hypertension	14(40%)	17(48.5%)		0.7	
LV diastolic function					
Mild dysfunction		2(6.6%)			
Moderate dysfunction	25(71.4%)	2(6.6%)			
Severe dysfunction	10(28.6)				
LV systolic function					
Normal	35(100%)	8(22.8%)			
Mild dysfunction		14(40%)			
Moderate Dysfunction		8(22.8%)			
Severe dysfunction		5(14.4%)			
LVEDVI (mL/m^2)	68.3 ± 8.2	118.8 ± 28.5			
LVESVI (mL/m^2)	22.4 ± 3.1	54.2 ± 7.2			
LVEF (%)	66.3 ± 5.4	44.7 ± 12.2			
LVMI (g/m^2)	144.3 ± 12.4	210.8 ± 21.5			
RWT (%)	0.48 ± 0.04	0.35 ± 0.04			
LVPWTd (mm)	11 ± 1.8	10 ± 1.7			
IVSTd (mm)	13 ± 0.5	12 ± 0.3			
LAI diameter (mm/cm^2)	29 ± 2.4	25 ± 3.1			
PASP (mm Hg)	61.5 ± 6.6	29.4 ± 7.6			
AVAI (cm^2/m^2)	0.59 ± 0.05	1.8 ± 0.3			
Peak velocity (m/s)	3.9 ± 0.03	2.1 ± 0.6			
Mean pressure drop (mmHg)	42.8 ± 6.2	14.8 ± 2.2			
Peak gradient (mm Hg)	105.5 ± 18.4	13.3 ± 3.1			
Mean gradient (mm Hg)	55.5 ± 7.2	9.3 ± 2.1			
Velocity ratio (m/s)	0.18 ± 0.03	0.9 ± 0.02			
E/A ratio	1.7 ± 0.6	1.6 ± 0.7			
E/E' ratio	13.3 ± 3.2	16.5 ± 2.1			
Deceleration time (m/s)	167.8 ± 23.6	132 ± 18			
S/D ratio	1.8 ± 0.09	0.8 ± 0.03			
Diastolic flow reversal		Prominent olodiastolic			
Pressure half time (m/s)		182 ± 24			
Regurgitant jet/LVOT (width %$^\zeta$)		0.6 ± 0.05			
Vena contracta (width cm$^\zeta$)		0.6 ± 0.04			
Regurgitant volume (mL/beat)		57.1 ± 4.5			
Regurgitant fraction (%)		55.4 ± 5.1			
Effective regurgitant orifice (cm^2)		27.5 ± 3.2			
EFS%	27.8 ± 2.1	18.8 ± 2.2			
MFS%	21.8 ± 2.1	12.8 ± 3.3			

Values are mean ± SD or numbers (percentage). BMI, Body mass index. LV, Left Ventricle. *p, patients with aortic stenosis vs patients with aortic regurgitation. **p, control patients vs diseased patients. LV systolic and diastolic dysfunction were graded according to the ESC or to the EAE/ASE recommendations respectively [21,23]. Hypertension was blood pressure above 140/90 mmHg.
A: peak velocity during atrial systole; AVAI: aortic valve area index; D: diastolic peak velocity; E: early flow velocity; E′: early diastolic velocity; EFS: endocardial fractional shortening; IVSTd: interventricular-septum thickness in diastole; LAI: Left atrium index; LVEDVI: Left ventricular (LV) end diastolic volume index; LVEF: LV ejection fraction; LVESVI: LV end systolic volume index; LVOT: LV outflow tract; LVMI: LV mass index; LVPWTd: LV posterior wall thickness in diastole; MFS: midwall fractional shortening; PASP: pulmonary artery systolic pressure; RWT: relative wall thickness; S: systolic peak velocity.
ζ at a Nyquist limit of 50–60 cm.

Titin has been the subject of a number of studies in animal models [8,9]. However, paucity of data have been published regarding human myocardium hitherto. Even the normal N2BA/N2B isoform ratio in normal human LV has not been definitively assessed [10–12].

Some recent studies described alterations in titin isoform expression in the LV of patient with congestive heart failure secondary to ischemic disease, dilated cardiomyopathy or pressure overload secondary to AS [10,13–15]. Actually, to date, no data are available regarding the titin isoform behavior in patients with AR. Because of insufficient knowledge, the present study compared titin isoform expression and contractile function in the biopsy samples procured from the LV of patients undergoing aortic valve replacement (AVR) for pure AS or pure AR. Specific morphofunctional echocardiographic characteristics were also analyzed for their possible relationship with titin isoforms. A group of normal donor hearts were also analyzed as controls.

2. Materials and method

2.1. Patients

Population consisted of 70 patients equally distributed between AS ($n = 35$) and AR (n = 35) referred for primary isolated surgical AVR and operated on between January 2011 and July 2016. According to ACC/AHA 2006 Guidelines, indications for AVR in patients with AS were: area <1.0 cm2, mean gradient >40 mm Hg, jet velocity >4.0 m/s in symptomatic patients or area <0.6 cm2, mean gradient >60 mm Hg, jet velocity >5.0 m/s in asymptomatic patients. Indications for AVR in patients with AR were: LV dysfunction (ejection fraction ≤0.50) in symptomatic or asymptomatic patient and normal LV systolic function (ejection fraction >0.50) but severe LV dilatation (end-diastolic dimension >75 mm or end-systolic dimension >55 mm) in asymptomatic patients [16].

General exclusion criteria were: severe comorbidities (dialysis, hepatic failure), autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis, scleroderma, Sjögren's syndrome or psoriatic arthritis) or connective tissue disorders (Marfan syndrome, Ehlers-Danlos syndrome, Loeys-Dietz syndrome), acute aortic dissection, congenital defects of the aortic valve (bicuspid valve or sub-valvular stenosis induced by fixed or dynamic components), atrial fibrillation, contemporary mitral and/or tricuspid

valve stenosis or regurgitation, evidence of coronary stenosis and active endocarditis. We considered as tolerable only a minimal and negligible presence of regurgitation in AS patients or stenosis in AR patients. To avoid significant bias due to age-related titin expression, we included only patients no younger than 55 and not older that 65 years. All AS patients had echocardiography indicative of pressure overload, all AR patients had of volume overload. Further exclusion criterion in patients with AS was reduced LV systolic function (ejection fraction <0.50). All patients underwent preoperative conventional coronary angiography or 64-Slice multidetector computed tomography [17].

Ten normal hearts obtained from donors for transplantation were analyzed as reference values for normal titin distribution and force measurements. Body mass index of controls patients was similar to that of diseased patients but age was significantly low (range 17 to 52 years, mean 38.7 ± 7.1). The cause of death was as follow: head trauma ($n = 5$), gunshot wound ($n = 2$), intracranial hemorrhage ($n = 3$). Controls had normal cardiac dimensions, wall thickness, and ejection fraction. All donor hearts were implanted. Main clinical and demographic characteristics of patients and controls are reported in Table 1.

The study complies the declaration of Helsinki. The Institutional Research Ethics Committee approved this study and all patients provided written informed consent.

2.2. Echocardiographic measurements and calculations

All patients had comprehensive transthoracic echocardiography. When it was assessed as poor or inadequate, the transoesophageal approach was employed. AS was graded according to EAE/ASE recommendations [18]. AR was graded according to ASE [19]. Reference limits of LV size, geometry and function were assessed according to ESC recommendations [20]. Peak and mean aortic gradients were calculated from continuous wave Doppler measurements using the modified Bernoulli equation and the aortic valve area with the standard continuity equation. The LV function was evaluated by the ejection fraction (LVEF) calculated by the Simpson rule. The LV mass was calculated from LV linear dimensions by means of the ASE-recommended formula (LVmass = 0.8×(1.04 [(LVIDd+PWTd+SWTd)^3-(LVIDd)^3]) + 0.6 g) and normalized to body surface area (LVMI). Calculation of relative wall thickness [RWT = 2xPWTd (Posterior Wall Thickness in diastole)/LVIDd (LV internal diameter in Diastole)] permitted categorization of an increase in LV mass as either concentric (≥0.42) or eccentric (≤0.42) hypertrophy. Patterns of LV remodeling based on end diastolic volume, wall mass, and RWT were classified following the scheme proposed by Gaasch.[21] Pulse wave tissue Doppler imaging was performed in the apical views to acquire mitral annular velocities and support 2D images for LV diastolic process.

Systolic and diastolic dysfunction of the LV were graded as mild, moderate or severe [20,22].

2.3. Myocardial biopsies

All myocardial biopsies were taken as full-thickness specimens from the apex of the left ventricle by means of an our patented device. Diseased hearts were sampled during AVR before cannulation for cardiopulmonary bypass, donor hearts just before to be explanted. In addition to the apex, the first 3 donor hearts were also sampled from the mid lateral wall to evaluate whether possible changes in the N2BA/N2B ratio from apex to lateral wall might be a potential source of variability in results.

Biopsy products from patients and controls were handled carefully and divided into samples of 5–8 mg each. Three to 4 were analyzed within 1 to 3 h for cardiomyocytes force measurements, other were snap-frozen in liquid nitrogen. All samples were evaluated blindly to patient identity or condition.

2.4. Titin isoform separation

For titin isoform measurement, tissue samples were homogenized in 100 to 200 μL of Tris sodium dodecyl sulfate (SDS) buffer (pH 6.8) containing 8 g/mL leupeptin (Roche Diagnostics, Monza, Italy) and 10 mL phosphatase inhibitor cocktails DMSO solution, 10 μL/mL (Sigma-Aldrich, St Luis, MO, USA) and were heated (3 min at 95–99 °C) and centrifuged (10 min, 13.000 g at 0 °C). Each sample was applied on agarose-strengthened 2% SDS-polyacrylamide gels and stained with Coomassie brilliant blue R250. The optical volume of protein bands was determined with UN-SCAN-IT 6.1 densitometric software (Silk Scientific, Orem, UT, USA). Titin isoform expression was determined as the proportion of N2BA and N2B isoforms (N2BA + N2B = 100%). Titin degradation products were identified as T2 band. Because multiple tissue samples were analyzed per heart, we first evaluated the individual mean isoform expression in each patient and then the "mean of means" for group of patients.

Several randomly selected samples from the normal donor and from AS and AR patients were rerun in order to assess the variability of the technique. Essentially the coefficient of variance was low (0.02–0.04).

2.5. Force measurements in isolated cardiomyocytes

Force measurements were performed in single, mechanically isolated cardiomyocytes following the protocol described by Falcão- Pires et al. and according to our previous study [13,14,23]. Biopsy samples were dissected into small strips in relaxing solution (in mmol/L: free Mg 1, KCl 100, ethylene glycol tetra-acetic acid 2, Mg-ATP 4, and imidazole 10; pH 7.0) and minced. To avoid titin degradation, solutions were supplemented with 40 μg/mL protease inhibitor leupeptin. To remove all membrane residuals, samples were incubated for 5 min in relaxing solution supplemented with 0.2% Triton X-100. Under an optical microscope, a single myocyte was suspended between a force transducer and a piezoelectric actuator. Single myocyte-sized preparations were carefully selected on the basis of uniformity of the striation pattern. Force measurements were performed at a sarcomere length of 2.2 μm and at 15 °C. Myocytes were subjected to relaxing and activating solutions at various $Ca2^+$ concentrations (pCa = −10log[$Ca2^+$]). Starting from pCa 9.0 m (maximal relaxation), exposed to a series of solutions with intermediate pCa, maximal activation was obtained at pCa 4.5. The maximal activation was used to calculate maximal calcium-activated isometric force (Fmax). From the resulting force-pCa curve, the Ca2+ concentration generating 50% of maximal force (pCa50) was determined using sigmoid regression analysis (SigmaPlot). Fig. 2-a. Once a steady state force level was reached, the length of the myocyte was reduced by 20% within 2 ms using the piezoelectric motor (slack test). The distance between the baseline and the steady force level was the total force (Ftotal). The cell was than restretched and returned to the relaxing solution, in which a second slack test of 10-s duration was performed to determine passive force (Fpassive). Maximal calcium activated tension (Factive) was calculated by subtracting Fpassive from the Ftotal at saturating [Ca2+] (pCa 4.5). To assess the behavior of Fpassive, following the baseline force-pCa measurements, cardiomyocytes were incubated for 40 min at 22° in relaxing solution supplemented with the catalytic subunit from bovine heart of protein kinase A (PKA, 100 U/mL; Merck S.p.a., Milan, Italy) and 6 mmol/L dithiothreitol (Merck S.p.a., Milan, Italy).

2.6. Statistics

Values are given as percentages or mean ± SD. The normal distribution of the continuous values was tested by means of the Anderson-Darling test. A p value >0.05 was considered indicative of normal distribution. One-way Analysis of Variance (ANOVA) was performed among AS, AR and control groups to compare continuous variables normally distributed (age, N2BA/N2B isoform ratio, Ftotal and Fpassive). Significant differences revealed by ANOVA were analyzed by Newman-Keuls method. Patients with AS and patients with AR, subgrouped as having moderate or severe diastolic dysfunction or normal/mild or moderate/severe systolic dysfunction respectively, were compared for N2BA/N2B isoform expression between subgroups and with controls by means of unpaired t-test for normally distributed values or Wilcoxon rank-sum test for not normally distributed values (N2BA/N2B isoform ratio in AS patients with severe diastolic dysfunction). Nominal variables were processed by means of the χ^2 test or the Kruskal-Wallis one-way test of variance when appropriate.

Force measurements were averaged from 3 to 4 samples from each heart and this mean value was taken as a single datum point for the calculation of the overall means. Pearson's correlation coefficient was used to assess the association between titin isoform expression and echocardiographic data and titin isoform expression and force measurement data. Simple linear regression analysis was used for modeling the relationship titin isoform ratio (explanatory variable) and RWT or LVEF (scalar dependent variable). A p value <0.05 was considered significant for all measurements. Data were analyzed by SPSS version 15 for Windows (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Echocardiography

Table 1 summarizes preoperative echocardiographic characteristics. Quantitative parameters regarding measurements in AR patients should be viewed with caution and only in the context of the other signs of severity because of the intrinsic limitations of quantitative measurement. All patients with AS had normal or reduced LV dimensions and increased LVMI. Moderate LV diastolic dysfunction occurred in 25 patients (71.4%), severe in 10 (28.6%). All AR patients had LV dilatation. Eight patients (22.8%) showed preserved LV function, 14 patients (40%) had mild LV systolic dysfunction, moderate or severe LV systolic dysfunction occurred respectively in 8 (22.8%) and 5 patients (14.4%). Increased LVMI was associated to reduced RWT (eccentric LV hypertrophy) in 11 patients (31.4%). According to the scheme of LV remodeling based on volume, mass, and geometry, all AS patients were classified as having concentric hypertrophy. Among AR patients, 9 (25.7%) were classified as having physiologic hypertrophy, 26 (74.3%) eccentric hypertrophy.

3.2. Titin isoforms expression and ratio

Densitometric analysis of gels revealed that N2B and N2BA bands were prominent in all samples and only limited levels of titin degradation

(reflected by the minor T2 bands) were present regardless they were taken from controls, AS or AR patients. Fig. 1-a-b. Total titin content (N2BA + N2B + T2) per unit of tissue was higher by ≈7% in AS patients and lower by ≈5% in AR patients compared to controls. However, the changes did not reached statistical significance ($p = 0.3$ and $p = 0.4$ respectively).

Mean N2BA/N2B isoform expression were 30 ± 5/70 ± 5 (ratio 0.43 ± 0.02) in controls, 18 ± 5/82 ± 5 (ratio 0.24 ± 0.02) in AS and 35 ± 5/65 ± 5 (ratio 0.51 ± 0.04) in AR. There was statistically significant difference among groups as determined by one-way ANOVA, ($p < 0.001$). The Newman-Keuls post-hoc test confirmed that differences in N2BA/N2B isoform ratio among each study group were all statistically significant.

In AS samples the mean expression of the stiffer N2B isoform was increased at the expense of the more compliant N2BA isoform. In contrast, in AR samples the N2BA titin isoform was more dominant, accompanied by a down-regulation of N2B titin isoform. Of note, we did not find significant differences in titin isoform expression between the apex and mid lateral wall of the LV in the 3 normal donor hearts tested for the possible regional gradient. The N2BA/N2B distribution in the apex was 30.2/69.8% (ratio 0.43) and in the mid lateral wall was 32.8/67.2 (ratio 0.48) ($p = 0.9$). The distribution of N2BA/N2B titin isoform ratios obtained from the apex of LV in controls and diseased hearts and specific relationship with the degree of diastolic or systolic dysfunction are depicted in Table 2.

Analyzed by linear regression, N2BA/N2B ratio and RWT were closely related only in patients with AS (R2 = 0.71, $p < 0.001$) compared to the modest correlation revealed in that with AR (R2 = 0.43, $p = 0,05$). Hence, the higher LV concentric hypertrophy is accompanied by a switching toward the higher expression of the stiffer N2B isoform whereas the LV eccentric hypertrophy imply increased level of the more compliant N2BA isoform no proportionally related. Finally, the comparative analysis of N2BA/N2B ratio in controls versus patients with normal/mild LV systolic dysfunction and in controls versus those with moderate/severe systolic dysfunction revealed that titin isoform expression was significantly deranged irrespectively from LV systolic function. Table 2. However, at linear regression analysis there was a close relationship between N2BA/N2B ratio and LVEF ($R^2 = 0.86$, $p = 0.004$) as the change in titin isoform ratio in AR group was mainly driven by the severity of the LV systolic function. Fig. 1-c-d.

3.3. Mechanical properties measurements

The average force-pCa relations obtained for controls, AS and AR patients are shown in Fig. 2-a. At pCa 4.5, force reached a clear plateau. Therefore, in subsequent experiments, pCa 4.5 was assessed as the maximal force. Furthermore, force-pCa2+ characteristics of isolated cardiomyocytes were measured to determinate the pCa50 in all groups. pCa50, corresponded to the [Ca2+] at which Ftotal-Fpassive reached 50% of the value observed at maximal activation. Accordingly, in subsequent experiments, pCa50 was used for measuring submaximal force. Analyzed by one-way ANOVA, Ftotal at pCa 4.5 was not statistically different among AS (19.8 ± 3.3 kN/m^2) and AR (18.8 ± 3.1 kN/m^2) and controls (21.2 ± 2.8 kN/m2) cardiomyocytes ($p = 0.37$). Fig. 2-b. Calculated pCa50 were similar in controls, AS patients AR patients (5.5 ± 0.4, 5.4 ± 0.3 and 5.3 ± 0.3 kN/m2 respectively, $p = 0.09$).Fig. 2-c. By contrast, As compared to controls Fpasive was higher in AS specimens (6.7 ± 0.2 vs 4.4 ± 0.4 kN/m^2, $p < 0.001$) whereas it was lower in AR specimens (3.7 ± 0.2 vs 4.4 ± 0.4 kN/m^2, $p < 0.001$). Fig. 2-d. The Newman-Keuls post-hoc test confirmed that differences in Fpassive

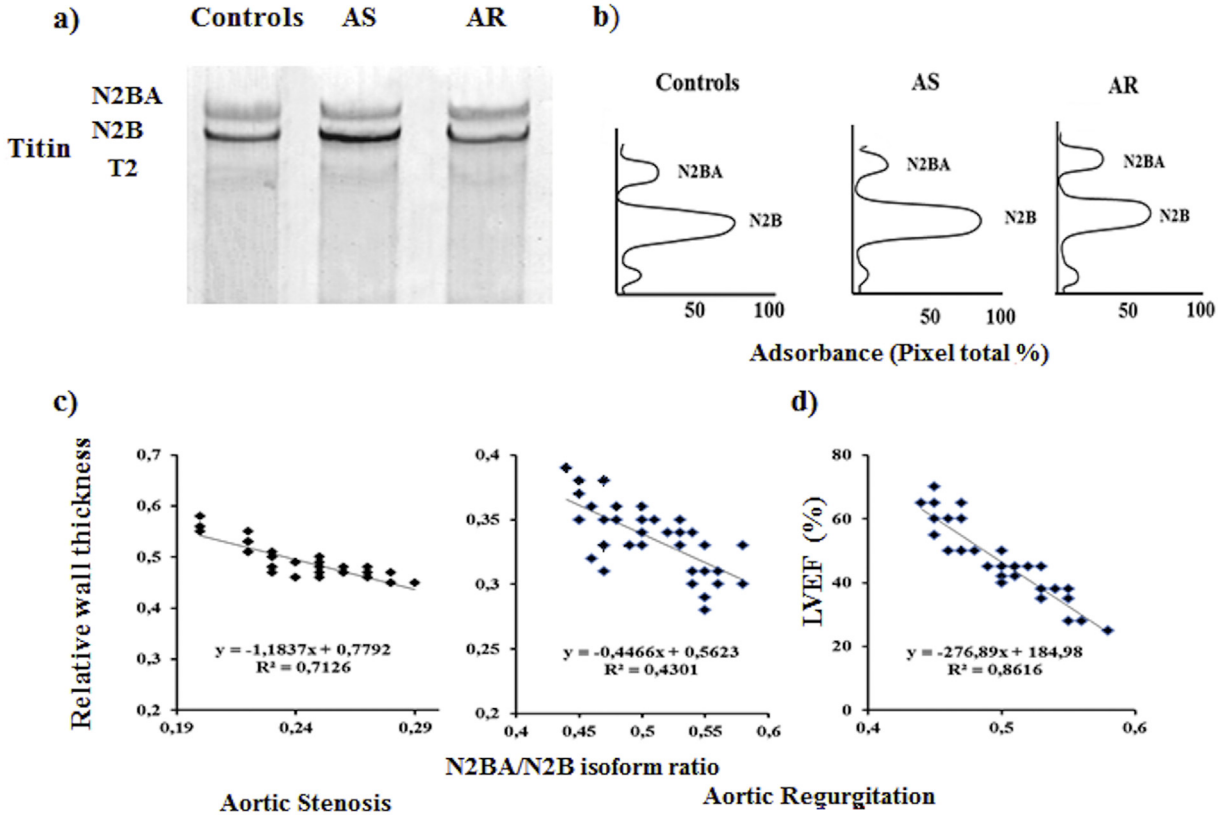

Fig. 1. a) Representative gels of samples from the apex of the left ventricle of patients with aortic stenosis (AS), aortic regurgitation (AR) and normal donor heart. T2, titin degradation compound. Note, the titin T2 degradation products are resolved into one band. The amount of the T2 bands appeared not to vary in controls, AS and AR samples. c) Linear regression analysis of N2BA/N2B isoform ratio for correlation to relative wall thickness of the left ventricles of patients with aortic stenosis and aortic regurgitation. d) Linear regression analysis of N2BA/N2B isoform ratio for correlation to Left ventricular ejection fraction (LVEF) of the left ventricle of patients with aortic regurgitation.

Table 2
Densitometric Analysis of Gels: Titin-isoform Expression.

	N2BA/N2B isoform	N2BA/ N2B ratio
Controls	30 ± 5/70 ± 5	0.43 ± 0.02
Aortic Stenosis	18 ± 5/82 ± 5	0.24 ± 0.02
Moderate LV diastolic dysfunction	19 ± 4/81 ± 5	0.23 ± 0.02
Severe LV diastolic Dysfunction	15 ± 6/85 ± 5	0.17 ± 0.03
Aortic Regurgitation	35 ± 5/65 ± 5	0.51 ± 0.04
Normal LV systolic function	32 ± 4/68 ± 4	0.47 ± 0.03
Mild LV systolic dysfunction	33 ± 3/67 ± 5	0.49 ± 0.03
Moderate LV systolic dysfunction	34 ± 5/66 ± 5	0.51 ± 0.04
Severe LV systolic dysfunction	37 ± 5/63 ± 5	0.58 ± 0.07
Aortic stenosis vs aortic regurgitations vs controls		$p < 0.001$
Aortic stenosis		
Moderate vs severe LV diastolic dysfunction		$p < 0.001$
Moderate LV diastolic dysfunction vs controls		$p < 0.001$
Severe LV diastolic dysfunction vs controls		$p < 0.001$
Aortic regurgitation		
Normal/mild vs moderate/severe LV systolic dysfunction		$p < 0.001$
Normal/mild LV systolic dysfunction vs controls		$p < 0.001$
Moderate/severe LV systolic dysfunction vs controls		$p < 0.001$

Comparative evaluation of N2BA/ N2B isoform ratios among groups and within groups according to LV function.

among each study group were all statistically significant. By Pearson's correlation, AS patients showed a significant relationship between the higher Fpassive and the lower N2BA/N2B titin isoforms ratio ($r = -0.77$, $p < 0.001$). Interestingly, Fpassive was significantly higher in AS patients with severe rather than with moderate LV diastolic dysfunction (7.1 ± 0.5 vs 6.6. ± 0.6, $p = 0.004$). By contrast, in AR patients, the lower Fpassive showed only a weak correlation with higher N2BA/N2B titin isoforms ratio ($r = 0.2$, $p = 0.04$). Furthermore, in AR patients Fpassive was independent from either the degree of LV systolic dysfunction ($p = 0.7$) or the presence of severe LV dilatation ($p = 0.6$). Fig. 2-d shows individual Fpassive in each patients and controls. After PKA, in controls Fpassive dropped from 4.4 ± 0.4 to 3.4 ± 0.3 kN/m2kN/m2. The drop in Fpassive was larger in AS patients than in AR patients ($p < 0.001$) because Fpassive fell to a similarly lower level in AS patients (3.5 ± 0.3 kN/m2) and in AR patients (3.3 ± 0.2 kN/m2). Fig. 2-d. Interestingly, Ftotal at maximal activation did not change after PKA treatment in all groups whereas pCa50 fell in all groups but the differences reached statistical significance only AS patients. Fig. 2-b-c. By Pearson's correlation, the higher Fpassive drop was strictly related with the higher titin N2B isoform content ($r = -0.88$, $p < 0.001$).

4. Discussion

The present study yielded the following information: compared to controls (a) the total titin content was tendentially increased in AS cardiomyocytes and tendentially reduced in AR cardiomyocites although the relative differenced did not reached statistical significance; (b) the N2BA/N2B isoforms ratio was lower in AS patients but was higher in AR patients; (c) compared with controls, at a sarcomere length of 2.2 μm, Ftotal at maximal [$Ca2^+$] in both AS and AR cardiomyocytes was comparable as well as submaximal force (pCa50), but the Fpassive was higher in AS patients and lower in AR patients; (d) after

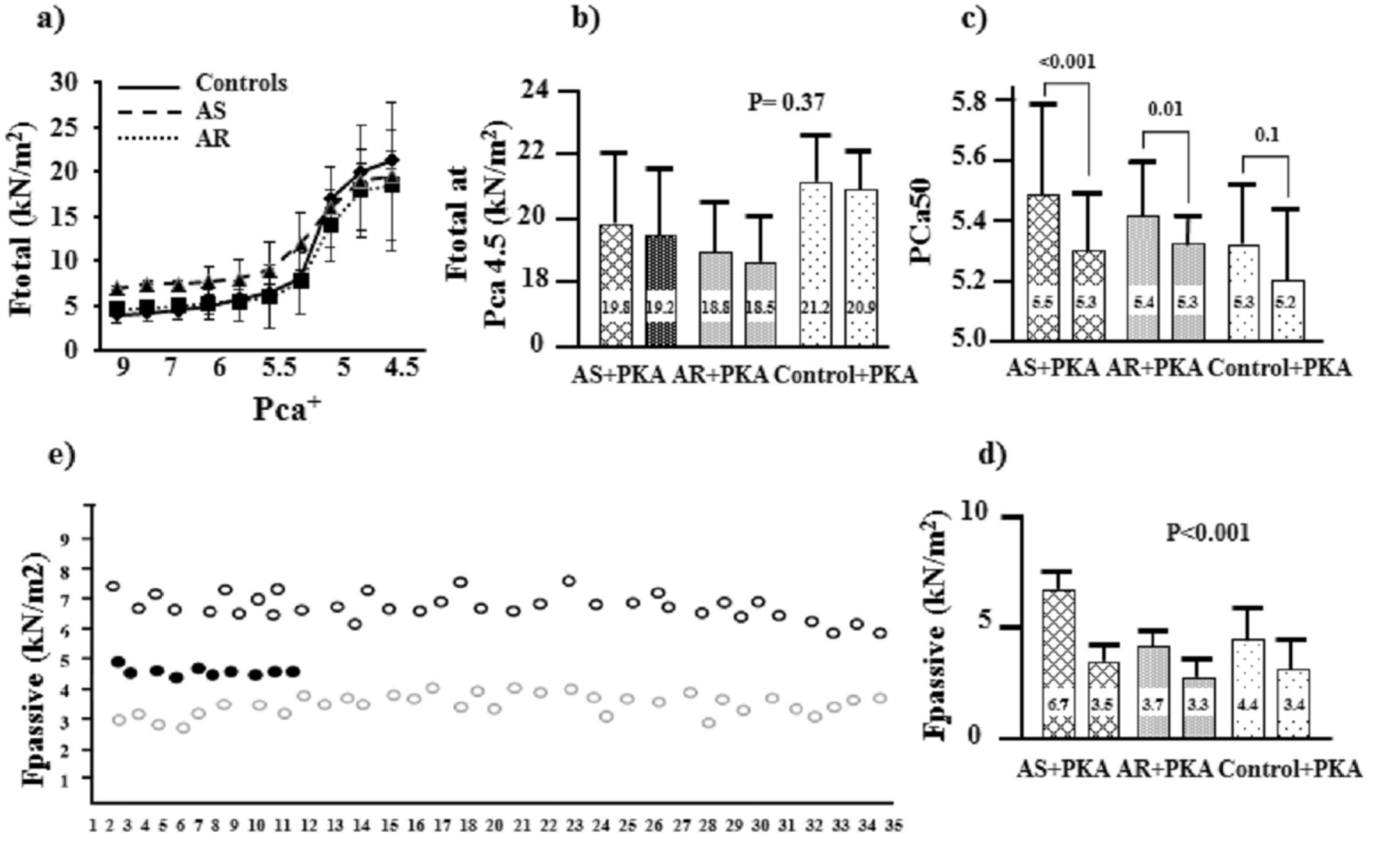

Fig. 2. (a) Mean Ftotal-pCa relation for cardiomyocytes of control, aortic stenosis (AS) and aortic regurgitation (AR) groups. pCa ($_{10}$log[Ca^{2+}]) ranged from 9.0 (relaxing) to 4.5 (maximal activation). (b) Bare graph display of Ftotal at maximal activation in cardiomyocytes from controls, AS and AR hearts. Bare graph compares also Ftotal after in vitro administration of PKA. (c) Bare graph display of PCa50 in cardiomyocytes from controls, AS and AR hearts. Bare graph compares also PCa50 after in vitro administration of PKA. d) Bare graph display of Fpassive in cardiomyocytes from controls, AS and AR hearts. Bare graph compares also Fpassive after in vitro administration of PKA. (e) Fpassive in individual cardiomyocytes from 10 normal donor hearts (open circles), 30 hearts with AS (black filled circles) and 30 hearts with AR (grey open circles). Each individual value is the mean value obtained from 3 to 4 samples analyzed. Note, the highest Fpassive was registered in patients 1,6,10,14,18, 22 all whom had severe diastolic dysfunction.

administration of PKA to the cardiomyocytes, the drop in Fpassive was larger in AS; (e) different titin isoform expressions are consistent with the systolic/diastolic properties of the LV; (f) results obtained from normal donor hearts, contributes to a clearer definition of normal titin isoform expression and behavior in human.

The role of titin in the stiffening of cardiac wall has been evaluated by several authors in mammalian models. Earlier studies established that titin isoform shift is usually associated to pathological situations cardiac overload. In a canine model of pacing-induced dilated cardiomyopathy, Wu reported a titin-isoform shift after four weeks of stimulation [8]. More recently, Hamdani showed abnormal calcium sensitivity of cardiomyocytes and the shift toward the stiffer and less distensible N2B isoform in dogs with hypertension-induced LV hypertrophy [24]. Consistent with these results, Warren described less expression of N2BA and greater of N2B titin isoform in hypertensive rat ventricles [9]. Disease-induced changes in the titin expression have been described also in humans with chronic heart diseases. The possible mechanisms involved in the disease-induced titin isoform expression is still unclear, however it has been suggested that systolic and diastolic heart failure express distinct post-translational changes in the sarcomeric proteins resulting in altered titin isoform expression and phosphorylation. The titin behavior in patients with chronically diseased aortic valve has been questioned only by a few studies hitherto. As very little is known about patients with AR, titin composition and its relationships with cardiomyocytes contractile function in patients with AS has been analyzed only by Falcao-Pires and Williams [14,15]. However, they reported contradictory results not only in their patients with AS (titin isoform ratio increased to 0.61 ± 0.07 and reduced to 0.48 ± 0.03 respectively) but also in their normal controls (0.39 ± 0.05 and 0.66 ± 0.04 respectively). These remarkable discrepancies probably rely on the different experimental/clinical contexts where titin isoforms expression was studied: Falcao-Pires analyzed perioperative LV endomyocardial biopsies resected during cardioplegic arrest whereas in the Williams's study it is unclear whether patients with coexisting AR were also included and the time point for biopsy during the AVR procedures.

As rule, in adults with AS the LV adapts to systolic pressure overload through a hypertrophic process resulting in increased LV wall thickness [13,25]. Thus, the inverse relation between systolic wall stress and ejection fraction is maintained. Until wall stress will be normal, the ejection fraction will be preserved. Titin, as the main determinant of passive tension of the cardiomyocytes, has been reported to be one of the main determinant of the ventricular compliance. Our results support the hypothesis that the abundance of the stiffer N2B and the reduced ratio between the N2BA and N2B isoforms in AS are probably the main, although not the only, contributors to the high diastolic stiffness which influences the heart filling. When wall stress normalizes because of hypertrophy, systolic performance also normalizes. Thus, LV ejection performance could remain in the normal range for a long time.

By contrast, in chronic AR, the LV responds to the volume load with different compensatory mechanisms including an increased end-diastolic volume and chamber compliance as well as a combination of eccentric and concentric hypertrophy all of which accommodate the higher volume without significant changes in filling pressures. The lower myocardial stiffness and the increased compliance of the LV, likely due to the prevalence of the more distensible N2BA isoform, could allow for faster filling and larger end-diastolic volumes for a given filling pressure. This can function as an adaptation mechanism, as the increase in the LV compliance allows an increase in the telediastolic volume and, consequently, an increase in cardiac output, according to the Frank-Starling mechanism. However, this balance cannot be maintained indefinitely and the transition toward LV deterioration is progressive. This evolution, due to impairment of Frank-Starling mechanism, should be probably ascribed to the decreased sensitivity of myofilaments for $Ca2^{+}$ due to the lower development of passive tension in the presence of increased N2BA isoform [11].

In this study, Fpassive in control subjects was 35% lower than in AS patients whereas it was 16% higher in AR patients. Different Fpassive of cardiomyocytes could be ascribed to impaired calcium metabolism or to modified myofilament or cytoskeletal proteins. Taking into account that altered calcium metabolism must be excluded in our cardiomyocytes due to disrupted integrity of sarcolemmal and sarcoplasmic membranes, the differences in Fpassive could be attributed to altered expression or phosphorylation of cytoskeletal proteins. Assumed that titin has phosphorylation sites, in agreement with previous studies, we found that its phosphorylation by PKA lowered Fpassive in isolated cardiomyocytes, especially in case of overexpression of the stiff isoform of titin (N2B).[14,23,26] Furthermore, the fall in Fpassive after PKA was larger in AS patients than in AR patients. Fig. 2-c. This larger fall after PKA was consistent with the larger N2B isoform expression in cardiomyocytes of AS patients. Given that the increased expression of the N2B isoform is associated with increased titin rigidity, this rigidity can be reduced by the phosphorylation of titin by PKA to levels very similar to those found in controls. The decrease in Fpassive after PKA treatment was directly proportional to the initial rigidity of the cardiomyocytes, which is in agreement with the idea that the increase in cardiac rigidity is directly related not only to alterations in the relative expression of the isoforms, but also to the state of hypophosphorylation of the rigid isoform of titin. Accordingly, due to the lower rigidity of the cardiomyocytes of patients with AR, the same treatment with PKA did not result in significant Fpassive. These findings are particularly relevant, as they can also be the target of therapeutic intervention in patients with AS or AR to improve the diastolic filling or the systolic function of the ventricles.

5. Limitations

The statistical power of the study may be hampered by the small size of the AS and AR populations. All biopsy samples were derived from the apex which could have overlooked the possible myocardial tissue heterogeneity. However, consistent with previous studies, we failed to detect a significant heterogeneity in our samples obtained from the apex or the mid-lateral wall (only a negligible difference of about 5–7% has been found). The main strength of this study consists in the high homogeneity of each group carefully selected and the abundance of each specimen. Furthermore, contrarily to previous study which evaluated biopsy samples obtained from transplant recipients or from the right ventricle of patients with clinical suspicion of cardiomyopathy, all our biopsy consisted of full thickness specimens taken from the apex of the left ventricle. Finally, the mean age of the donor hearts was significantly lower compared to AS or AR patients, which could be a not negligible limitation assuming that titin based stiffness might also change with the age. Nonetheless, we hypothesized that our control samples were quite indicative as it may be almost impossible to collect these biopsy samples from donors of similar age than diseased patients.

6. Conclusions

Titin isoform expression differs in AS and AR as adaptive response to different pathophysiologic scenarios. LV myocardium in AS and AR differs in both titin isoform expression and function.

suggesting AS and AR to be associated with distinct cardiomyocyte abnormalities. Co-expressing isoforms at varying ratios results in modulation of the passive mechanical behavior of the LV at different degree of dysfunction and allows for compensative adjustment of the diastolic/systolic properties of the myocardium.

Conflict of interest

None.

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