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TITIN ISOFORM EXPRESSION IN AORTIC STENOSIS

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ABSTRACT

Background:

Titin is a giant sarcomeric protein that plays a major role in determining passive myocardial stiffness. The shorter N2B isoform results in a higher passive myocardial stiffness than the longer N2BA isoform. We hypothesised that expression of the short N2B isoform would be increased in patients with aortic stenosis compared with healthy controls in response to pressure overload, in order to act as a modulator for the increased demand placed on the left ventricle during the early stages of the hypertrophic response.

Methods:

Myocardial biopsies were obtained from the left ventricle of 19 patients undergoing aortic valve replacement for aortic stenosis who had no significant co-existing coronary artery disease. Left ventricular biopsies were also obtained from thirteen donor hearts for comparison.

Results:

SDS-agarose gels revealed small N2B and large N2BA cardiac titin isoforms with a mean N2BA:N2B ratio that was significantly decreased in the 19 aortic stenotic patients compared with the 13 controls (0.66 ± 0.04 in the normal donor hearts versus 0.48 ± 0.03 in patients with aortic stenosis; $p=0.02$). However, total titin remained unchanged (0.28 ± 0.02 vs. 0.24 ± 0.02 ; $p=0.29$).

Conclusion:

Expression of less N2BA and more N2B titin in response to pressure overload will result in the generation of higher passive tension upon stretch to a given sarcomere length and this might affect cardiac performance.

INTRODUCTION

Aortic stenosis is the commonest valve lesion in industrialised countries and approximately 3% of the population over the age of 75 have severe aortic stenosis. When a pressure gradient develops between the left ventricle and the ascending aorta, LV pressure rises resulting in an increased wall stress. The development of hypertrophy tends to normalise wall stress. Since aortic stenosis develops slowly, hypertrophy develops in proportion to increased intraventricular pressure, and myocardial stress remains normal [1]. The diastolic properties of the left ventricle are affected in aortic stenosis [2-6]. The diastolic abnormality results from a combination of impaired myocardial relaxation and altered chamber compliance because of the effects of increased wall thickness and of structural remodelling of the myocardium [5]. Here we examined whether titin, a major contributor to passive force generated during myocardial stretch, is involved in diastolic abnormalities.

Titin is a giant sarcomeric protein which spans the half-sarcomere from the Z-band to the M-line. When sarcomere length increases during diastole, the I-band region of titin extends and force develops. The shape of the passive force-sarcomere length relation of titin influences ventricular filling during diastole and ventricular emptying during systole. The force generated by titin arises from its extensible I-band region, which consists of three main segment types: (1) the so-called PEVK segment, (2) serially-linked immunoglobulin (Ig)-like domains flanking the PEVK segment [7], and (3) the N2B element [8]. Two major classes of titin isoforms have been found in cardiac muscle and are referred to as N2B and N2BA [8;9]. Because of its shorter extensible I-band region (due to both fewer Ig domains and a shorter PEVK segment), expression of the N2B isoform results in a higher passive myocardial stiffness than that of the N2BA isoform [9].

Recent studies in animal models of dilated cardiomyopathy [10] and pressure overload [11] have revealed adjustments in titin isoform expression that increased passive myocardial stiffness. However, with respect to human studies there is a paucity of data [12;13]. Van Heerebeek et al have demonstrated an increase in the N2B isoform in patients with diastolic heart failure, many of whom were hypertensive with an increase in left ventricular mass index [14], while Makarenko et al demonstrated that there was an upregulation of the compliant N2BA isoform in patients with dilated cardiomyopathy [15].

In this study we assessed titin expression at the protein level utilising gel electrophoresis. We report that left ventricles from patients with aortic stenosis undergoing aortic valve replacement exhibit a shift in titin isoform expression towards the shorter N2B isoform. In order to avoid the confounding effect of ischaemia/infarction on titin isoform expression, we excluded patients with significant coronary disease requiring coronary artery bypass grafting at the time of valve replacement.

METHODS

Patients

The patient population included 19 patients with severe aortic stenosis undergoing aortic valve replacement. No patients had significant coronary artery disease requiring

coronary artery bypass grafting at the time of valve replacement, and all had a normal left ventricular ejection fraction. Thirteen samples were obtained from donor hearts who were not used for transplantation but whose valves were used for homografts (control specimens). These controls had normal cardiac dimensions, wall thickness, and left ventricular ejection fraction. Baseline demographics for the patient and control groups are shown in Table 1. We used samples from the oldest subgroup of donors in order to be as close as possible to the age of the aortic stenosis patient group (although perfect age matching was impossible). All patients gave informed written consent and the study protocol was approved by the Local Research Ethics Committee. The investigation conforms with the principles outlined in the Declaration of Helsinki[16].

Echocardiographic Assessment of LV Function

Left ventricular ejection fraction and left ventricular dimensions were determined prior to aortic valve replacement from 2D images using the Biplane Simpson's method and M-mode echocardiography. Baseline echocardiographic data for both the patients and control group are shown in Table 2.

Myocardial biopsies

Full-thickness specimens of the left ventricle were obtained at the time of aortic valve replacement from the apex. Specimens were obtained from the left ventricles of thirteen donor hearts at the time of explantation and were used as control specimens. Samples were frozen in liquid nitrogen and stored at -80°C.

Gel Electrophoresis and Immunoblotting

Myocardial muscle samples were quick-frozen in liquid nitrogen, pulverized, and then rapidly solubilised [17]. The samples were analysed with SDS-agarose gels [18]. Each sample was loaded at 5 different volumes and this was repeated on 2 different gels (10 loading per sample). As protein stain Coomassie Brilliant Blue R250 was used. Wet gels were scanned and analysed with 1D scan software (Scanalytics Inc) [9]. The integrated optical density of N2BA titin, N2B titin, total titin (N2BA + N2B + T2), and myosin heavy chain (MHC) were determined as a function of the volume of the solubilised protein sample that was loaded (a range of volumes was loaded on each gel, typically 5 with 2 gels per sample, resulting in 10 values per heart). The slope of the linear relation between integrated optical density and loaded volume was obtained for each protein. The N2BA:N2B ratio was calculated as the slope of the N2BA titin divided by the slope of the N2B titin. A representative gel sample is shown in Figure 1. Several of the control and aortic stenosis samples were re-run in order to assess variability of the technique when the same tissue is analysed multiple times on different SDS-gels (increasing the number of values to 20 per heart). Essentially identical results were obtained; the coefficient of variance was typically 0.02-0.04.

Simulations of the effect of isoform shift on the titin-based passive force

In order to obtain functional insights through simulations of the effect of the isoform switch on the titin-based passive force, we calculated the force—SL relations of the two titin isoforms using the wormlike chain WLC equation:

$$\frac{Fx(PL)}{k_B T} = \frac{z}{L} + \frac{1}{4(1-z/L)^2} - \frac{1}{4}$$

where F is force (in pN), k_B is Boltzmann's constant, T is absolute temperature, PL and L are the persistence and contour lengths. We serially-linked three WLCs, representing the combined tandem Ig segments, the PEVK, and N2B-U's spring elements. We assumed a PEVK contour length (CL) of 70 nm in N2B titin and 300 nm in N2BA titin; CL of tandem Ig segments were set at 200 nm in N2B titin and 300 nm in N2BA titin; CL of N2B Us was 200 nm for both isoforms (note that these values are based on extensive previous work, both single molecule mechanics and immunoelectron microscopy). The assumed persistence lengths (PL) were 1.3 nm, 12 nm and 0.65 nm, respectively. We then calculated the force-SL relation of a single titin molecule and compared results for N2B titin and N2BA titin. (For additional details on the calculation see Trombitas K *et al.* [19]. We used these relations to determine the average force-SL relation for sarcomeres that coexpress N2B and N2BA titins at levels seen in control and levels seen in AS patients. The results were used to calculate the force change due to the isoform switch.

Statistical Analysis

All data are expressed as mean \pm SEM. Statistical significance was evaluated by means of a two-tailed Mann-Whitney test.

RESULTS

Patients

The age of the patients with aortic stenosis (19 patients; 7 female, 12 male) was 68 ± 2.2 years, compared to 59 ± 1.6 years for the controls (13 patients; 9 female, 4 male). All patients with aortic stenosis had a left ventricular ejection fraction $> 50\%$. No patients with aortic stenosis had significant coronary artery disease requiring coronary artery bypass grafting at the time of valve replacement. All patients had evidence of left ventricular hypertrophy, as evidenced by an increase in both the interventricular septum and posterior wall dimensions in diastole (1.65 ± 0.03 cm and 1.42 ± 0.12 cm respectively). The left ventricular mass index (LVMI) was also increased in patients with aortic stenosis (164.38 ± 15.22 g/m²) compared with reported normal values of 76 ± 13 g/m² for males and 66 ± 11 g/m² for females[20]. Echocardiographic findings are summarised in Table 2.

Human Cardiac Titin Expression

High-resolution SDS-agarose gels revealed in both control and disease samples prominent N2B and N2BA bands and only limited levels of titin degradation (reflected by the minor T2 bands): see Figure 1. Densitometric gel analysis revealed that the total titin to MHC ratio in patients with aortic stenosis and healthy controls were similar (0.28 ± 0.02 vs. 0.24 ± 0.02 ; $p=0.29$) (Figure 2A).

Titin Isoform Expression

The ratio of N2BA to MHC was similar between the two groups (0.09 ± 0.01 vs. 0.09 ± 0.01 ; $p=0.87$) (Figure 2B), but the ratio of N2B to MHC was significantly higher in the group with aortic stenosis compared with healthy donor hearts (0.17 ± 0.01 vs. 0.13 ± 0.01 ; $p = 0.02$) (Figure 2C).

N2B-to-N2BA Titin Isoform Ratio

The mean ratio of N2BA to N2B titin was 0.66 ± 0.04 in the normal donor hearts versus 0.48 ± 0.03 in patients with aortic stenosis ($p=0.003$) (Figure 2D). Thus, total titin was not different between the two groups, but the mean expression level of the

stiffer N2B isoform was increased at the expense of the more compliant N2BA isoform.

Gender-related Differences in Titin Expression

For all of the parameters above there were no statistically significant differences between male and females in either the control or aortic stenosis group.

The effect of isoform shift on the titin-based passive force

Figure 3A shows the results of the force-SL relation of a single titin molecule and compared results for N2B titin and N2BA titin. These relations were then used to determine the average force-SL relation for sarcomeres that coexpress N2B and N2BA titins at levels seen in control and levels seen in AS patients. Results are shown in Fig. 3B. The results were used to calculate the force change due to the isoform switch, revealing a force increase that ranged from ~6 to ~12% (Fig. 3B).

DISCUSSION

In left ventricular hypertrophy complex adaptive changes take place that affect a wide range of proteins. Morphological changes accompanying left ventricular hypertrophy may also involve the giant sarcomeric protein titin. Studies in animal models have reported an alteration in titin isoform expression in response to pressure-overload [11]. The importance of titin for passive-tension development implies that alteration in isoform expression should have profound effects on mechanical cardiac function. A characteristic of human myocardium is the co-expression of two principal titin isoforms, N2B and N2BA. A shift toward higher N2B titin levels increases cellular passive stiffness, as shown in a canine-model of pacing-induced heart failure [21]. Furthermore, in a study of spontaneously hypertensive rats, the presence of left ventricular hypertrophy correlated with a higher N2B titin isoform expression compared with the N2BA isoform [11].

In the present study, we aimed to investigate whether the titin isoform expression pattern was altered in patients with aortic stenosis and left ventricular hypertrophy as a response to pressure overload of the left ventricle.

We have demonstrated that total titin expression (the sum of both isoforms and titin degradation products) relative to myosin heavy chain was similar in the donor hearts (0.24 ± 0.02) to previously reported values for human control myocardium (0.23 ± 0.03) [12] and for various animal models (0.2 to 0.25) [9]. We also found that total titin is not significantly different in patients with aortic stenosis and left ventricular hypertrophy (0.28 ± 0.02) compared with control subjects.

In large mammals, total titin consists of the relatively large N2BA and smaller N2B isoforms. We found that N2BA:N2B expression ratio of human control myocardium from donor hearts is 0.66 ± 0.04 , a value slightly higher than that previously reported by Neagoe et al [13] and Nagueh et al [22]. However, the number of samples obtained from donor hearts was higher in our study than the aforementioned studies (13 donor hearts compared with 6 and 12 respectively), the average age of the patients from whom tissue was obtained was higher (mean age 59 ± 1.6 years vs. 46 ± 2.4 years and 46.8 ± 5.2 years) and differences in sample location (apex in our study compared with mid-wall regions in the aforementioned study), possibly explaining the present result. In the patients with aortic stenosis, there was significantly increased N2B titin expression, resulting in a N2BA to N2B ratio of 0.48 ± 0.03 . These findings are in keeping with those from a study of titin isoform expression in the spontaneously hypertensive rat [11].

We obtained functional insights into the effect of the isoform switch through single molecule simulations (Figure 3). This revealed a force increase in patients with aortic stenosis that ranged from ~6 to ~12%. These relative force changes are of a similar magnitude to those found by others who have measured passive mechanics in myocardial tissue of control and cardiomyopathic hearts[13;15] and they are likely to be sufficiently large to have functional consequences.

The interaction of the heart with the systemic vasculature, termed ventricular-arterial (V-A) coupling, is a key determinant of cardiovascular performance. The capacity of the body to augment cardiac output, regulate systemic blood pressure, and respond appropriately to elevations in heart rate and preload depends on both the properties of the heart and the properties of the vasculature into which the heart ejects blood. Normal physiological V-A coupling matches these properties so that maximal (or near maximal) cardiac work, power, and chamber efficiency are all achieved, while maintaining blood pressures and cardiac outputs within a physiological range [23;24]. End-systolic elastance (Ees) is a measure of end-systolic left ventricular stiffness, whereas arterial elastance (Ea) is a measure of impedance (being influenced by static and pulsatile afterload and by heart rate), and is calculated as the ratio of systolic pressure/stroke volume). Over an Ea/Ees ratio spanning 0.3 to 1.3, left ventricular stroke work and cardiac metabolic efficiency is near optimal, whereas both decline at much higher or lower ratios. Ea/Ees ratios have been reported in normal humans, and are generally between 0.7-1.0. This shift in titin isoform expression in patients with aortic stenosis may be expected to increase left ventricular end systolic elastance (Ees), and therefore maintain the coupling ratio (Ees/Arterial elastance) within the normal range in the face of an increased left ventricular afterload. Shapiro et al have studied this concept in an elderly canine model of hypertension[25]. In keeping with our current data, the authors demonstrated that chronic increases in Ea were coupled to chronic increases in LV systolic stiffness, coupled with a reduction in expression of the more compliant N2BA isoform.

Study limitations

Chronically diseased hearts show a greatly altered transmural gradients of titin expression ratio [10;21], unlike human donor hearts[15], enforcing the fact that matching the sample location is critical. It is known that the apex expresses relatively less N2BA than the free LV wall. Transmural gradients have been described in canine, but not in human models. Thus considering that we work with human samples this issue is of less concern. Furthermore, all samples were derived from the apex and were full thickness samples. It is unlikely therefore that differences in sample location or transmural gradient are a major factor in our work. Our work only pertains to the intracellularly-based titin forces, but in light of the contribution of both titin and the extracellular matrix to myocardial wall stiffness important work that focuses on collagen as well as several other factors remains to be done in future follow-up studies.

Conclusion

In patients with aortic stenosis and left ventricular hypertrophy, N2B titin is upregulated, at the expense of the more compliant N2BA isoform. This shift in isoform expression may act as a modulator for the increased demand placed on the left ventricle during early stages of the hypertrophic response to pressure overload.

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Conflicts of interest

None.

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TABLE 1. Baseline Characteristics

	Donor Hearts	Aortic Stenosis
Sample size	n=13	n=19
Age	59 ± 1.6	67 ± 2.1
Gender	4 Male / 9 Female	12 Male / 7 Female
LVEF	> 50%	> 50%

TABLE 2. Echocardiographic data in patients with Aortic Stenosis

	Aortic Stenosis	Controls
BSA (m²)	1.96 ± 0.05	NA
IVSd (cm)	1.65 ± 0.04	0.85 ± 0.5
LVEDd (cm)	4.7 ± 0.1	5.7 ± 1.7
PWd (cm)	1.4 ± 0.04	0.88 ± 0.4
LVMI	163.2 ± 17.4	NA
Peak AV Grad (mmHg)	81.9 ± 6.3	4.9 ± 0.3

BSA: Body surface area; IVSd: Intraventricular septal dimension in diastole; LVEDd: Left ventricular dimension in end-diastole; PWd: Posterior wall thickness in diastole; LVMI: Left ventricular mass index; Peak AV Grad: Peak aortic valve gradient on echocardiography

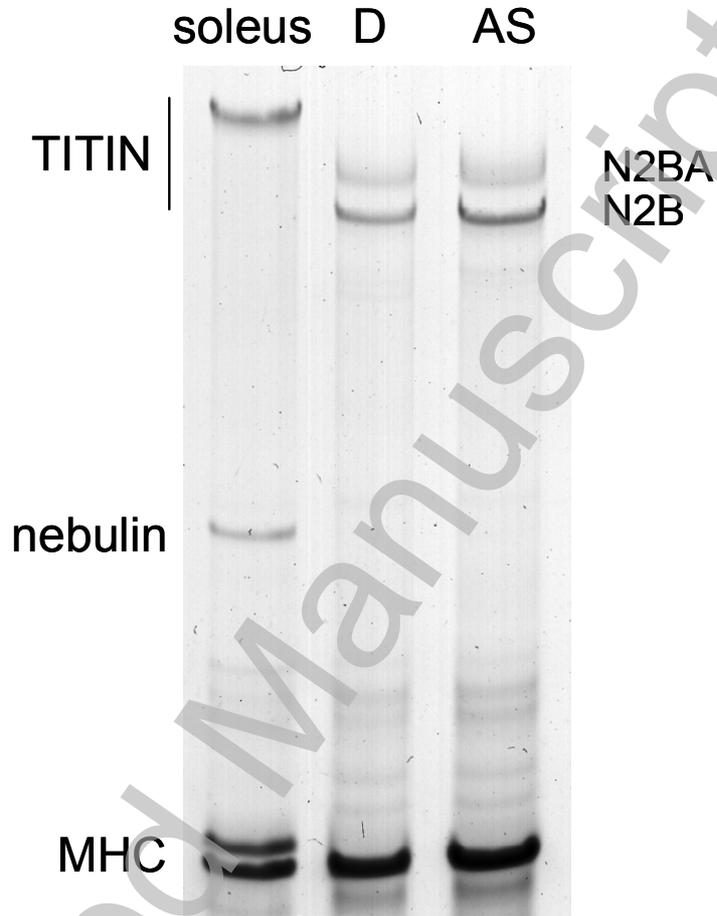
FIGURE LEGENDS

Figure 1: A representative gel sample from a donor heart (D) that functions as a control and a patient with aortic stenosis (AS). The left band represents a gel sample from soleus muscle. Soleus titin and nebulin function as molecular weight (MW) size markers (3.7 MDa and 0.8 MDa, respectively).

Figure 2. A-D: Titin expression in donor and aortic stenosis hearts based on analysis of SDS-agarose gels. Total titin and N2BA titin (relative to myosin heavy chain/MHC) expression are indistinguishable between donor and aortic stenosis hearts (2A and B), whereas N2B expression (relative to MHC) is significantly increased in the aortic stenosis group (2C). N2BA:N2B expression ratio is significantly lower in aortic stenosis patients (2D).

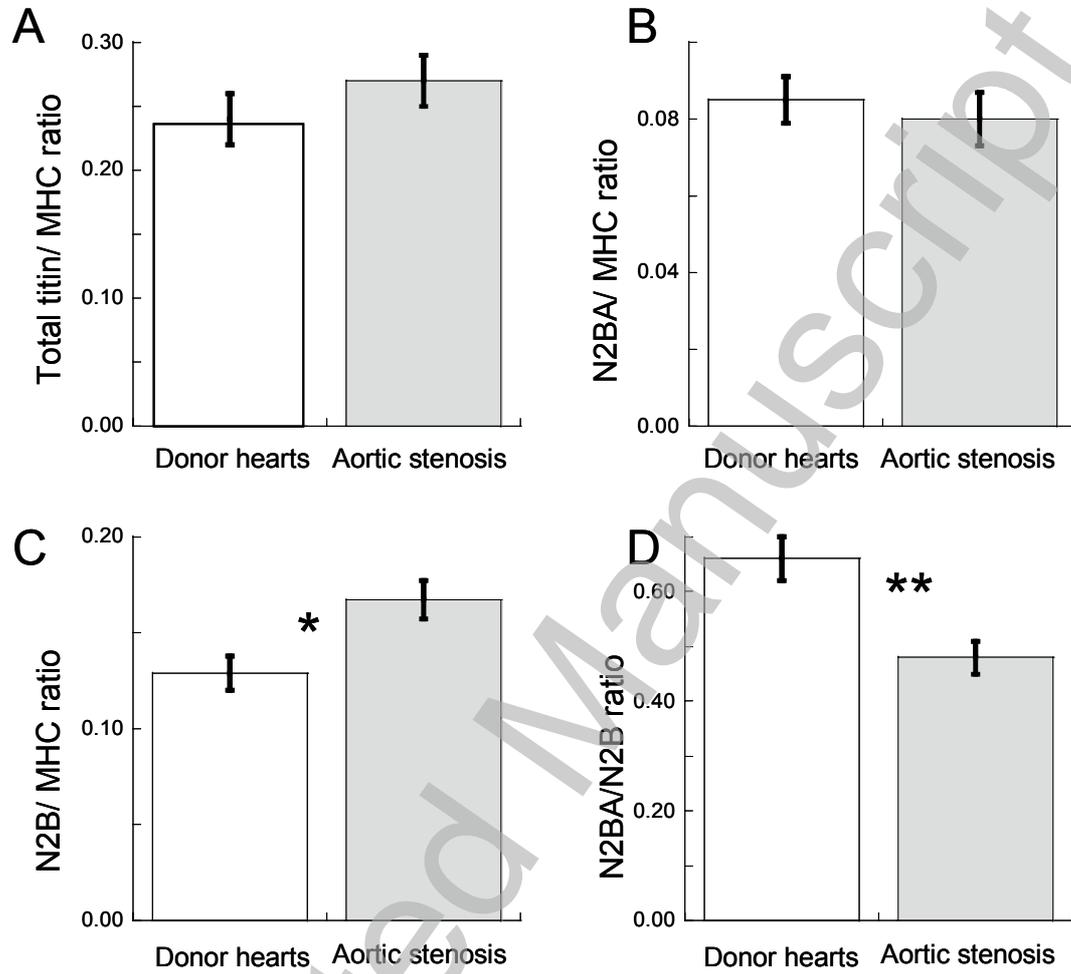
Figure 3. A: Predicted force-SL relations of single N2B (red) and N2BA molecule (black). **B:** Average force per titin molecule in control (black) and aortic stenosis sarcomere (blue) and increase in titin force in aortic stenosis over control (red).

FIGURES
Figure 1

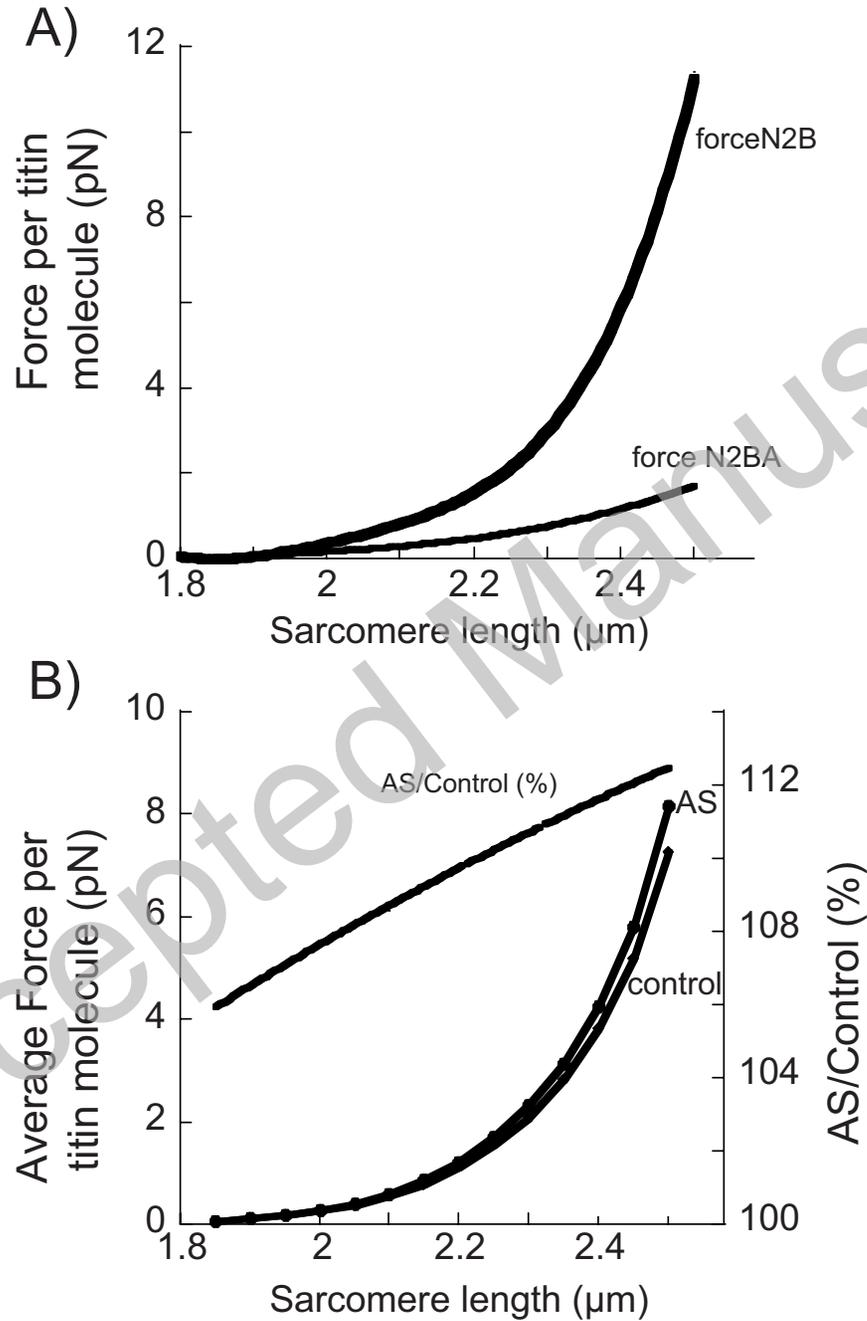


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Figure 2



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Figure 3