Myocardial Titin and Collagen in Cardiac Diastolic Dysfunction:
Partners in Crime!

Running title: Hamdani et al.; Titin and collagen in diastolic dysfunction

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High myocardial diastolic stiffness has usually been attributed to excessive myocardial collagen deposition. Over the last decennium stiff cardiomyocytes were also identified as important contributors to high myocardial diastolic stiffness especially in heart failure (HF) with preserved ejection fraction (HFPEF)\(^1\)\(^-\)\(^3\). Cardiomyocyte stiffness relates to elasticity of the giant cytoskeletal protein titin, which spans the sarcomere from Z-disc to M-line and functions as a bidirectional spring responsible for early diastolic recoil and late diastolic distensibility of cardiomyocytes\(^4\). In HFPEF patients and in HFPEF animal models\(^5\), the observed rise in cardiomyocyte stiffness was always accompanied by increased deposition of collagen and it therefore remained unclear if impaired elasticity of titin could be solely responsible for high myocardial diastolic stiffness and HFPEF. In this issue of Circulation however, Chung et al. provide compelling evidence for titin being the sole perpetrator in the diastolic left ventricular (LV) dysfunction of a HFPEF mouse model\(^6\). They generated mice with a deletion of nine immunoglobulin (Ig)-like domains from the proximal tandem Ig segment of titin’s spring region (IG KO). This deletion extended the remaining titin spring segments and increased overall titin stiffness. Despite unaltered myocardial collagen content or composition, the IG KO mice developed HFPEF evident from a reduced exercise tolerance, an enlarged left atrium and a steeper LV end-diastolic pressure-volume relationship. The elegant study of Chung et al. therefore clearly establishes myocardial titin to be able to sufficiently compromise diastolic LV function to induce HFPEF.

**The Adjustable Spring Properties of Titin**

Titin, with a molecular mass of up to 3800 kDa, spans half-sarcomeres from the Z-disk to the M-band and contains a molecular spring segment, the I-band region, which supports early diastolic recoil and late diastolic resistance to stretch (**Figure 1A**). The I-band region has a complex...
structure comprising three extensible segments: (1) tandem immunoglobulin (Ig)-like domain regions with a proximal and a distal segment, (2) a PEVK segment, and (3) a unique sequence that is part of the N2B element (N2-Bus) and expressed only in cardiac titin. In the study by Chung et al., nine immunoglobulin (Ig)-like domains from the proximal tandem Ig segment were deleted. This shortened the overall length of titin and increased titin stiffness. Changes in titin stiffness have also been reported following isoform shifts or posttranslational modifications like phosphorylation or oxidation. The two main titin isoforms expressed in human (and other mammalian) hearts are N2B, which is the shorter and stiffer isoform, and N2BA, which is the longer and more compliant isoform\(^4\). Higher expression of the N2BA titin isoform was reported in eccentrically remodeled hearts of ischemic or dilated cardiomyopathy\(^4\). In contrast, only a minor shift towards the N2BA titin isoform was observed in concentrically remodeled hearts of HFpEF patients (Figure 1B)\(^7\). Titin also adjusts cardiomyocyte stiffness through post-translational modifications like phosphorylation or oxidation (Figure 1C-D). Protein kinase A (PKA)\(^8\) and protein kinase G (PKG)\(^9\) phosphorylate titin at its N2B segment. This allows beta-adrenergic stimulation, nitric oxide or natriuretic peptides to acutely lower cardiomyocyte stiffness. In HFpEF, titin is hypophosphorylated\(^7\) probably because of low myocardial PKG activity induced by low NO bioavailability and high oxidative stress\(^3\). Protein kinase C\(\alpha\) (PKC\(\alpha\)) phosphorylates titin at its PEVK region. PKC\(\alpha\) administration raises stiffness of cardiomyocytes isolated from normal myocardium\(^10\) but has no effect on cardiomyocytes retrieved from an animal model\(^11\). Titin can also be phosphorylated by extracellular signal-regulated kinase-2 (ERK2)\(^12\) and at several sites by calcium calmodulin dependent kinase II (CaMKII)\(^13\). Both ERK2 and CaMKII lower cardiomyocyte stiffness. The pathophysiological relevance of phosphorylation by PKC\(\alpha\), ERK2 and CaMKII for diastolic LV dysfunction in
HFPEF remains to be elucidated. Finally, stiffness of cardiomyocytes also rises in the presence of oxidative stress because of formation within the titin molecule of disulfide-bridges, which reduce the contour length of the N2B segment (Figure 1D).

**Interstitial Collagen: A Loose or a Tight Fit?**

In diastolic LV dysfunction, cardiomyocyte stiffness and collagen deposition are often unequally involved. Resting tension of isolated cardiomyocytes stretched to 2.2 μm sarcomere length (F\text{passive}) and myocardial collagen volume fraction (CVF) were simultaneously assessed in several HF animal models\(^6,15,16\) and in patients with HFPEF, heart failure with reduced ejection fraction (HFREF) and aortic stenosis (AS)\(^1,2,17\). Titin IG KO mice\(^6\) and obese ZSF1 rats\(^15\) are at one end of the spectrum with a 2.0 times increase in F\text{passive} and no change in CVF. AS patients are at the other end of the spectrum with no change in F\text{passive} and a 2.4 times increase in CVF\(^17\). HFPEF patients occupy an intermediate position with a 2.0 times increase in F\text{passive} and a 2.3 times increase in CVF whereas HFREF patients come close to AS patients presenting with a 1.5 times increase in F\text{passive} and a 2.6 times increase in CVF.

Apart from unequal involvement in different HF phenotypes, cardiomyocyte stiffness and collagen deposition can also vary widely within a single HF phenotype. When HFPEF patients were classified in accordance to myocardial CVF (low: <5%; intermediate: 5 to 10% and high: >10%), they were equally distributed over the 3 classes of CVF\(^1\). Furthermore, LV end-diastolic pressure (LVEDP), circumferential LV end-diastolic wall stress and radial myocardial stiffness modulus were similarly elevated in the 3 classes of CVF. This finding obviously implied CVF not to be the sole contributor to diastolic LV dysfunction in HFPEF and suggested high cardiomyocyte stiffness to be equally harmful.

The relative importance of cardiomyocyte stiffness and collagen deposition cannot be
inferred from simple measurements of CVF and cardiomyocyte $F_{\text{passive}}$ at 2.2 $\mu$m sarcomere length but requires an integrated assessment of elasticity of cardiomyocytes and of extracellular matrix over the entire physiological distension range of cardiac muscle. The study by Chung et al. in this issue of Circulation$^6$ and two other recent studies$^{15,16}$ nicely illustrate how this can be achieved. In these studies, passive length-tension relations of skinned cardiac muscle strips were obtained before and after KI/KCl administration, which extracts thick and thin filaments and leaves titin unanchored. The passive length-tension relation after KI/KCl therefore corresponds to elasticity of the extracellular matrix. By subtracting this relation from the relation before KI/KCl administration, the elasticity attributable to titin can be calculated. In normal myocardium (Figure 2A), titin accounts for cardiac muscle elasticity at low and intermediate sarcomere lengths. Collagen starts to contribute at high sarcomere lengths and at the outer limit of the physiological range of sarcomere lengths (2.3 $\mu$m) the contributions of collagen and titin equalize. When titin is stiffer than normal (Figure 2B), equalization occurs beyond the outer limit and the fit of interstitial collagen around cardiomyocytes becomes “loose” over the physiological range of sarcomere lengths. When there is excessive collagen deposition (Figure 2C), equalization occurs at intermediate sarcomere length and the fit of interstitial collagen becomes “tight”. When both titin and extracellular matrix are stiffer, equalization again occurs at the outer limit of physiological sarcomere lengths and the relative importance of titin and collagen resembles the normal situation. This last situation was nicely illustrated in mice which developed HF after transverse aortic constriction$^{16}$. These mice had a twofold rise in cardiomyocyte-based stiffness, a threefold rise in extracellular matrix-based stiffness and equalization of the contributions of titin and collagen at the outer limit of the physiological range of sarcomere lengths. The foregoing shows interstitial collagen deposition, which varies widely
between and within HF phenotypes, to exert its restraining effect on LV filling in a titin-dependent fashion.

**Therapeutic Implications**

A stiffer titin has previously been observed to increase cardiomyocyte stiffness in HFPEF\(^1,^7\). The study of Chung et al.\(^6\) in this issue of Circulation provides proof of principle that stiffer titin suffices to induce HFPEF without any involvement of the extracellular matrix. This observation turns titin into a prime therapeutic target in HFPEF. Phosphorylation by PKA or PKG effectively lowers stiffness of titin\(^1,^3,^7\). Increasing myocardial PKA activity by beta-adrenergic stimulation is however obsolete because of risk of arrhythmic death. Raising myocardial PKG activity is a valid alternative because myocardial PKG activity and cGMP content are respectively two and eight times lower in HFPEF myocardium\(^3\). The very low cGMP content does not seem to be substantially improved by blocking cGMP breakdown as evident from the RELAX trial\(^18\), in which sildenafil failed to significantly raise plasma cGMP levels or to ameliorate diastolic LV dysfunction. Restoring myocardial cGMP content probably requires supply of cGMP to be boosted either by increasing myocardial NO bioavailability or by raising natriuretic peptide signalling. The former can be achieved by stringent control of metabolic comorbidities\(^19\) and the latter by the novel angiotensin receptor neprilysin inhibitor LCZ696, which inhibits the breakdown of natriuretic peptides and was recently shown in a phase II study to improve diastolic LV dysfunction of HFPEF patients\(^20\). Finally, as insight in posttranslational modifications of titin progresses, other new therapeutic inroads will appear, which reduce stiffness of titin and improve diastolic LV dysfunction in HFPEF.

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Figure Legends:

Figure 1. Adjustable spring properties of titin. A. Structural components and phosphorylation sites of N2BA Titin. The orange box shows the Ig domain deleted in the study of Chung et al.⁶; B-D. Modulation of cardiomyocyte stiffness (passive-length tension relation) by titin isoform shift (B), by titin phosphorylation (C) and by oxidation (D). N2-Bus, unique sequence; S, sulfide; PKA, protein kinase A; PKG, protein kinase G; PKCα, protein kinase Cα; ERK2, extracellular signal-regulated kinase-2; CaMKII, calcium calmodulin dependent kinase II; P, phosphorylation site.

Figure 2. Titin- and matrix-based components of the myocardial passive length tension relation. Myocardial passive length tension relation and its components in normal myocardium (A), in the presence of a stiffer titin (B) and in the presence of a stiffer matrix (C).
Figure 1
Figure 2
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