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Differential changes in titin domain phosphorylation increase myofilament stiffness in failing human hearts

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Aims Titin-based myofilament stiffness is defined by the expression levels of the cardiac titin-isoforms, N2B and N2BA, and by phosphorylation of the elastic titin domains N2-B unique sequence (N2-Bus) and PEVK. Phosphorylation of the N2-Bus by cGMP-dependent protein kinase (PKG) or cAMP-dependent protein kinase (PKA) decreases titin stiffness, whereas phosphorylation of the PEVK-domain by PKC increases it. We aimed to identify specific sites within the N2-Bus phosphorylated by PKA and PKG and to determine whether differential changes in titin domain phosphorylation could affect passive stiffness in human failing hearts.

Methods and results Using mass spectrometry, we identified seven partly conserved PKA/PKG-targeted phosphorylation motifs in human and rat N2-Bus. Polyclonal antibodies to pSer4185, pSer4010, and pSer4099 in the N2-Bus, and to pSer11878 in the PEVK-region were used to quantify titin-domain phosphorylation by western blot analyses of a set of human donor and failing hearts with similar titin-isoform composition. Passive tension determined in skinned human myocardial fibre preparations was significantly increased in failing compared with donor hearts, notably at shorter sarcomere lengths where titin contributes most to total passive tension. Phosphorylation of Ser4185, Ser4010, and Ser4099 in the N2-Bus was significantly reduced in failing hearts, whereas phosphorylation of Ser11878 in the PEVK-region was increased compared with donor hearts.

Conclusion We conclude that hypo-phosphorylation of the N2-Bus and hyper-phosphorylation of the PEVK domain can act complementary to elevate passive tension in failing human hearts. Differential changes in titin-domain phosphorylation may be important to fine-tune passive myocardial stiffness and diastolic function of the heart.

Keywords Connectin • Diastolic dysfunction • Myocardial stiffness • Passive tension • Phospho-specific antibodies

1. Introduction

The prevalence of chronic heart failure increases continuously in western countries, and although our understanding of the pathophysiology of heart failure continues to grow, the mortality of the disease remains high.[1] Numerous studies have addressed the molecular basis of systolic dysfunction, but the mechanisms that cause diastolic dysfunction are still poorly understood. A major variable of myocardial diastolic function is the left-ventricular (LV) distensibility. Restrictions of the ventricular distensibility, e.g. as a result of myocardial remodelling during heart disease, severely impair the diastolic filling phase and reduce cardiac performance.[2] The underlying passive mechanical properties of the myocardial tissue are determined by two main factors: the collagen-based stiffness of the extracellular matrix, and the titin-based stiffness of the myocytes. Titin is a giant protein with a size of more than 3000 kDa, spanning a half-sarcomere from the Z-disk to the M-line. The mammalian heart expresses two major isoform-types: a rather short and stiff N2B-isoform (3.0 MDa), and longer and more compliant N2BA-isoforms (3.2–3.7 MDa). The isoforms mainly differ in length and composition of the elastic I-band region, which is responsible

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for titin's role as a molecular spring during sarcomere stretch. Since the N2B and N2BA isoforms are co-expressed in a sarcomere, the relative expression ratio of the isoforms partly defines myofilament stiffness.[3,4] During progression to decompensated heart failure, this isoform ratio often shifts towards the longer and more compliant N2BA isoforms and reduces titin-based passive tension, probably as an adaptive response to counterbalance the fibrotic stiffening of the diseased myocardium.[5,6]

A second mechanism to adjust the passive mechanical properties of the sarcomere involves phosphorylation of elastic I-band domains of titin, the so-called N2-B unique sequence (N2-Bus), and the PEVK-region (PEVK, proline, glutamate, valine, and lysine residues). It has previously been demonstrated that titin is phosphorylated by cAMP- and cGMP-dependent protein kinases (PKA, PKG)[7–10] and may cause a drop in titin-based passive tension by up to 20%.[7–9] Results from human tissue further suggested a deficit in PKG-mediated titin phosphorylation in end-stage human failing hearts.[8] Radioactive kinase assays using recombinant titin fragments indicated a site of PKA- and PKG-mediated phosphorylation of the N2-Bus at position Ser4185, which is uniquely expressed in human titin (residue numbers in this study refer to the human titin sequence UniProtKB accession number, Q8WZ42).[8] However, recent evidence suggested PKG-induced titin phosphorylation in dog hearts.[11]

The PEVK-region is phosphorylated by the α-isoform of the Ca^{2+}-dependent protein kinase-C (PKCα) at positions Ser11878 and Ser12022 in a sequence, which is present in cardiac and skeletal muscle titin, and is evolutionary conserved in the titin sequence of many species.[12] Importantly, PKCα-induced phosphorylation of the PEVK region increases titin-based passive tension.[12] In hypertrophic cardiomyopathy (HCM), PKCα expression and activity are often increased,[13] raising the possibility that altered PKCα-signalling affects titin phosphorylation in failing hearts and is responsible for increased myofilament passive tension. Recent evidence indeed suggested that phosphorylation of the PEVK region is altered in heart failure.[14,15]

The fact that PKA- and PKG-induced phosphorylation of the N2-Bus decreases titin stiffness, whereas PKCα-mediated phosphorylation of the PEVK region increases it, implies that phosphorylation of the titin domains may be precisely regulated to modify myofilament passive stiffness in a co-ordinated manner. In our present study, we addressed this question in more detail and extended our characterization of the PKA- and PKG-mediated phosphorylation of titin's N2-Bus region by identifying specific phosphosites using mass spectrometry. We then tested how changes in the PKA- and PKG-dependent phosphorylation status of the N2-Bus compare with changes in the PKCα-dependent phosphorylation of the PEVK region in human hearts with HCM or dilated cardiomyopathy (DCM). In order to specifically study the influence of altered titin phosphorylation on passive fibre stiffness, we selected samples from human donor and end-stage failing hearts with identical cardiac titin-isoform composition. Interestingly, despite the same titin-isoform composition, passive fibre stiffness was significantly increased in failing vs. donor hearts. Our data indicate the presence of a cluster of PKA/PKG-targeted phosphorylation motifs within the N2-Bus, which is hypo-phosphorylated in human DCM and HCM hearts. We further demonstrate that hypo-phosphorylation of the N2-Bus is accompanied by increased phosphorylation of the PEVK-domain, suggesting that differential titin-domain phosphorylation acts complementary and may therefore promote increased passive tension in heart failure.

2. Methods

2.1 Heart muscle tissue

LV tissue from human donor and failing hearts was procured and deep-frozen in liquid nitrogen and stored at −80°C. Tissue samples from failing hearts were obtained from patients with idiopathic DCM ($n = 6$) or HCM ($n = 3$) who underwent heart transplantation due to severe systolic dysfunction (NYHA class III or IV). All available information on the tissue samples, and details on titin-isoform composition are provided in Supplementary material online, *Table S1*. All human tissue collection was in accordance with Australian National Health Medical Research guidelines and approved by the Human Research Ethics Committee of the University of Sydney (reference 7326). The study was also approved by the Ethics Committee at Ruhr University Bochum (registration # 3447-09), and conforms with the principles outlined in the Declaration of Helsinki.

2.2 Expression of recombinant titin fragments

Human cardiac titin fragments (UniProtKB accession number, Q8WZ42), a dog cardiac titin fragment (UniProtKB, F1PV45), and a rat cardiac titin fragment (NCBI accession number AF525412) were expressed in *Escherichia coli* NEB Express and purified by the GST-Fusion System (Amersham Biosciences). The following constructs were generated: human N2-Bus, rat GST-N2-Bus, dog N2-Bus, and human PEVK (constitutively expressed PEVK region encoded by titin exons 219–225). The mouse titin sequence used for the sequence alignment in *Figure 1* refers to NCBI accession number A2ASS6. Details on the recombinant titin fragments are provided in Supplementary material online.

2.3 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography

Recombinant titin fragments were analysed by 10% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) after incubation of [γ^{32}P] ATP and PKA or cGMP-activated PKG as previously described.[8] For titin analyses, cardiac tissue was homogenized in modified Laemmli buffer[16] and proteins were separated by agarose-strengthened 2.0% SDS–PAGE as described.[17,18] For analysis of titin isoform composition, each sample was loaded in a concentration gradient of 5–30 μg protein, and bands were visualized by Coomassie-stain. The gels were scanned using a Fuji-imager LAS 4000 and analysed densitometrically (Multi Gauge V3.2). Average titin-isoform composition was calculated from a minimum of $n = 3$ per experimental condition.

2.4 NanoLC-ESI-MS/MS analysis and identification of phosphopeptides

The PKA- and PKG-phosphorylated titin fragments were excised from the SDS–PAGE and used for digest with trypsin and Glu-C. For mass spectrometric identification, the peptide extracts were separated in a reverse phase nanoHPLC system (U3000, Dionex LC Packings) using a 75 μm I.D. × 2 cm capillary pre-column and an additional 75 μm (I.D.) × 25 cm column (both C18 PepMap, Dionex LC Packings). The nanoHPLC was coupled to the LTQ Velos ETD linear ion-trap mass spectrometer (Thermo Scientific) and the eluted and ionized peptides were analysed by alternating collision-induced dissociation and electron transfer dissociation–tandem mass spectrometry.

The mass spectrometric data were used for searches against the human NCBInr subdatabase (6 340 215sequences in major database) and the in-house triatoma-yeast database of the Medical Proteome-Center (University of Bochum) containing protein sequences from expressed sequence tags of titin and others combined for statistical reasons to the yeast protein sub-database of the NCBI (TaxID 4932, 07/05/2007).[19] The Mascot® V. 2.2 algorithm was used for the database search and the following search parameters were selected: trypsin or V8 (D, E specificity) peptide cleavage, peptide mass accuracy of 0.3 Da (monoisotopic), fragment mass accuracy of

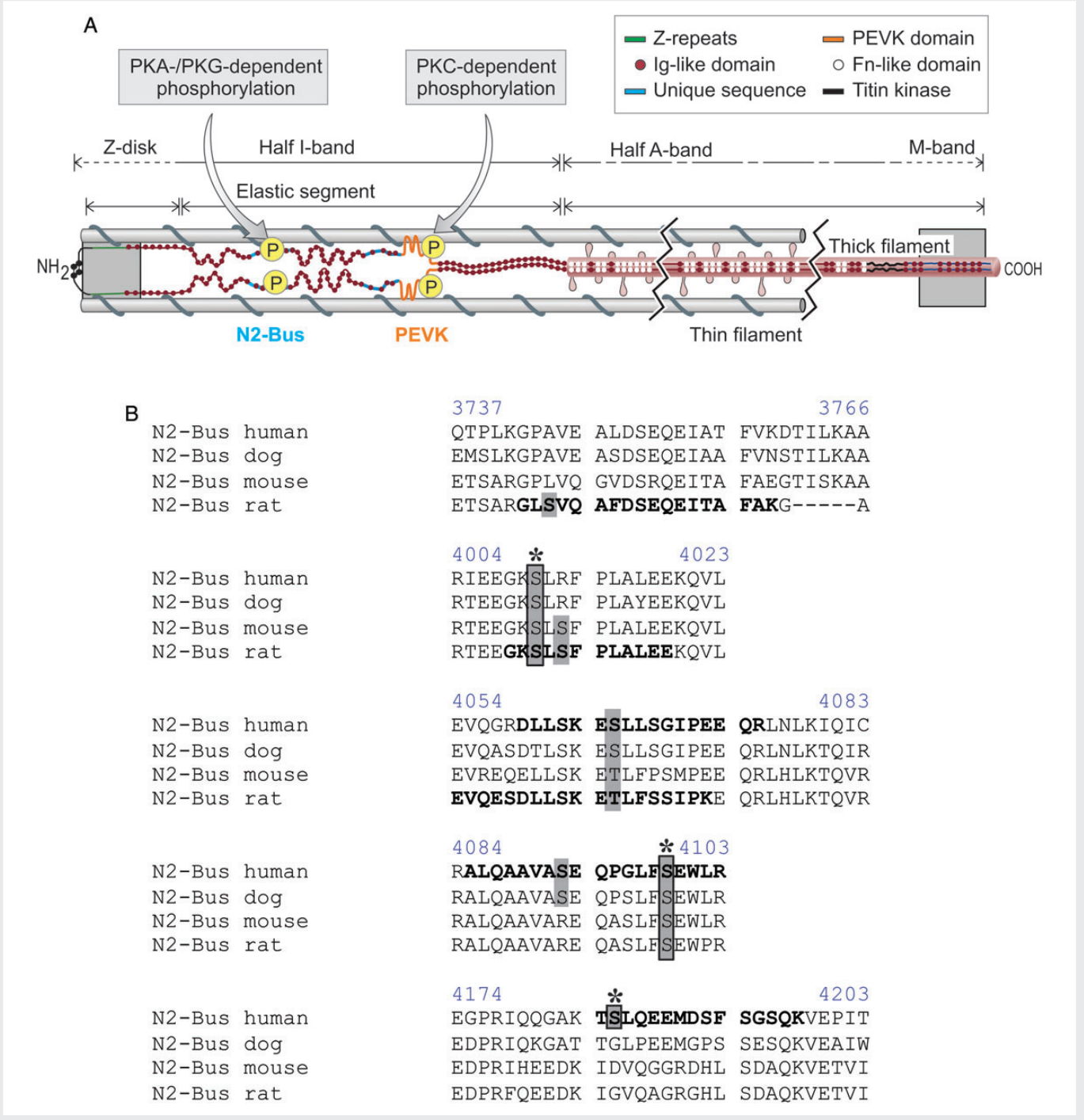

Figure 1 Identification of phosphosites in two spring elements of human and rat titin. (*A*) Domain architecture of N2BA-titin isoform in a cardiac half-sarcomere. Arrows point to phosphorylation by PKA and PKG in the N2-Bus, and by PKCα in the PEVK-region. (*B*) Sequence alignment of human, dog, mouse, and rat titin N2-Bus fragments. Numbers indicate the amino acid position in full-length human titin according to UniProtKB accession number, Q8WZ42. Bold sequences represent the phosphopeptides identified by NanoLC-ESI-MS/MS analysis; phosphosites are highlighted in grey. Asterisks mark the positions detected by the phosphospecific antibodies.

0.4 Da (monoisotopic), variable modification due to oxidation of methionine and phosphorylation of serine, threonine, and tyrosine and maximally three missed cleavage sites. The charge states of all abundant fragment ions were checked according to their isotopic pattern and compared with theoretical ions. A detailed description of the method is provided as Supplementary material online. The amino acid sequence of the rat titin N2-Bus was aligned to that of the human N2-Bus and the identified phosphorylation sites were numbered according to the nomenclature of the full-length human titin sequence (UniProtKB accession number Q8WZ42).

2.5 Phosphosite-directed antibodies

Polyclonal antibodies were generated in rabbits immunized with peptides containing phospho-Ser4185 (EGPRIQQGAKT**S**LQEEMDS), -Ser4010 (VRIEEGK**S**LRFPC), or -Ser4099 (QANLF**S**EWLRNID), all from the

N2-Bus, or phospho-Ser11878 (CEVVLK**S**VLRKR) from the PEVK-region. All antibodies but anti-pSer4185, which was made by Immunoglobe, Germany, were made by Eurogentech, Belgium. Specificity of the affinity-purified antibodies was tested by western blot using recombinant fragments of the human N2-Bus and PEVK-region. The N2-Bus fragment was phosphorylated by cGMP-activated PKGα (Biaffin), the PEVK fragment by activated PKCα (Promega), as previously described.[8,12]

2.6 Analysis of titin phosphorylation levels

Titin isoforms were separated by agarose-strengthened 1.8% SDS–PAGE. Total titin phosphorylation was analysed using the fluorescence-based phosphoprotein stain ProQ diamond (Invitrogen) in comparison to the total protein stain Sypro Ruby (Invitrogen). Staining and imaging were performed as previously described.[18] To study titin domain phosphorylation, proteins were transferred onto a PVDF-membrane first probed with the phosphospecific antibodies (α-pPEVK/ α-pN2B) and then with antibodies recognizing total N2-Bus or PEVK (α-PEVK/ α-N2B). See Supplementary material online for details of the membrane stripping method. Bands were visualized using a Fuji-LAS 4000 imaging system and signal intensity was analysed densitometrically (Multi Gauge V3.2). To detect differences in titin domain phosphorylation levels, we determined, for each sample and with each set of phosphosite-directed antibodies, the signal intensity of phospho-titin and total-titin. The ratio of *phospho:total* titin was then used to normalize the phosphorylation status of failing heart samples to that of donor heart samples. For details see Supplementary material online and Supplementary material online, *Figure S3*.

2.7 Mechanical measurements

For passive tension measurements, small muscle strips (length, 3–4 mm; diameter, 400–500 μm) were prepared from LV tissue of de-frozen human hearts and skinned overnight in relaxing solution supplemented with 40 μg/mL leupeptin, 30 mM 2,3-butanedione monoxime, 1 mM dithiothreitol, and 0.5% w/v triton-X-100, as previously described.[6] The skinned tissue was extensively washed in relaxing solution without triton-X-100 and small fibre bundles with a diameter of 200–300 μm and a length of 1.0–2.5 mm were dissected. Force measurements on skinned human fibre bundles were performed with a muscle mechanics workstation (Scientific Instruments, Heidelberg, Germany) as previously described.[6] Briefly, samples were bathed in relaxing solution at room temperature and mounted between stainless steel clips attached to a motor arm and a force transducer. Fibres were stretched from slack SL (average, 1.9 μm) in six steps to ∼2.4 μm. SL was measured by laser diffraction. Passive force was indexed to cross-sectional area ('passive tension') by measuring the fibre diameter and assuming a circular cross-section.

2.8 Statistics

Statistical significance of passive tension–sarcomere length relation was tested using two-way ANOVA. Normalized data were tested using Student's *t*-test with Bonferroni correction. To test for statistically significant differences in phosphorylation levels, we used the unpaired Student's *t*-test. *P*-values <0.05 were considered as significantly different and are represented in figures by asterisks.

3. Results

3.1 Identification of PKG- and PKA-targeted phosphorylation motifs in titin N2-Bus

In order to identify phosphorylation motifs, we generated recombinant constructs of human, rat, and dog N2-Bus and analysed their phosphorylation by protein kinases PKA and PKG (*Figure 1A*). Autoradiographic assays confirmed PKA- and PKG-mediated phosphorylation of the N2-Bus from all three species (Supplementary material online, *Figure S1*). The phosphorylated human and rat N2-Bus constructs were further used to identify PKG- and PKA-targeted phosphorylation motifs by tandem mass spectrometry. The amino acid sequence of the rat, mouse, and dog titin N2-Bus was aligned to the respective human N2Bus sequence (*Figure 1B*). Identified phosphorylation sites are numbered according to the full-length human titin sequence (UniProtKB accession number, Q8WZ42).

In the human N2-Bus, we identified phosphorylation sites at positions Ser4065 (PKA-induced), Ser4092 (PKG-induced), Ser4099 (PKG-induced), and Ser4185 (PKA- and PKG-induced) (*Figure 1B*). This result confirms the PKA- and PKG-sensitive phosphorylation site Ser4185, which was previously found by us using autoradiography.[8] The newly identified PKG-dependent phosphorylation site at Ser4092 is conserved in human and dog, but not in rat and mouse. PKG-phosphorylated Ser4099 is located in a region with a high sequence homology conserved in dog, mouse, rat, and human. Ser4065 is conserved in human and dog titin, but is replaced by a threonine in rat and mouse titin. Mass spectrometry also identified this site as a target for PKA-dependent phosphorylation in rat titin. In the rat N2-Bus, we further detected phosphosites at position Ser3744 (PKA- and PKG-induced), Ser4010 (PKA-induced), and Ser4012 (PKA-induced) (*Figure 1B*). Ser3744 is only in the rat sequence, whereas Ser4010 is conserved in all four species and Ser4012 is expressed in mouse and rat, but not dog and human titin. A summary of N2-Bus phosphopeptides identified by mass spectrometry is provided in *Table 1*; a representative spectrum is shown in Supplementary material online, *Figure S2*.

Table 1 Phosphorylated motifs in titin's N2-Bus region

Sample	Kinase	Phosphopeptide	Position
N2-Bus rat	PKA/PKG	GL**pS**VQAFDSEQEITAFAK	Ser 3744
N2-Bus rat	PKA	GK**pS**L**pS**FPLALEE	Ser 4010/4012
N2-Bus rat	PKA	EVQESDLLSKE**pT**LFSSIPK	Thr 4065
N2-Bus hum	PKA	DLLSKE**pS**LLSGIPEEQR	Ser 4065
N2-Bus hum	PKG	ALQAAVA**pS**EQPGLF**pS**EWLR	Ser 4092/Ser 4099
N2-Bus hum	PKA/PKG	T**pS**LQEEMDSFSGSQK	Ser 4185

Phosphopeptides identified by tandem mass spectrometry of recombinant N2-Bus construct incubated with PKA or PKG. PKA/PKG indicates that both kinases phosphorylated the fragment. For the peptides containing two putative phosphorylation sites, the fragmentation spectra indicated the presence of mono- as well as di-phosphorylated peptides.

To characterize titin domain phosphorylation in tissue samples, we used phosphospecific antibodies to Ser4010, Ser4099, and Ser4185 in the human N2-Bus, as well as Ser11878, a known PKCα-dependent phosphosite in the PEVK-region. Specificity of the phosphospecific antibodies was tested on recombinant constructs of the N2-Bus phosphorylated with PKG or PKA, recombinant PEVK-construct phosphorylated with PKCα, and the respective non-phosphorylated controls (representative examples in *Figure 2*). Whereas the α-pN2Bus and α-pPEVK antibodies selectively detected the phosphorylated but not the non-phosphorylated constructs, the respective sequence-specific antibodies detected both the phosphorylated and the non-phosphorylated constructs; they thus served as a loading control.

3.2 Titin isoform composition and total titin phosphorylation in human donor and failing hearts

To selectively analyse the influence of titin domain phosphorylation on myocardial stiffness, we chose a set of human donor (donor, $n = 8$) and heart failure samples (DCM, $n = 6$; HCM, $n = 3$) with essentially identical titin-isoform composition. The titin isoform-expression pattern was determined on Coomassie-stained 2% SDS–PAGE gels. In human donor hearts, the mean percentages were 60.5 ± 0.5% N2B and 39.5 ± 0.5% N2BA (*Figure 3A*). In tissue samples from HCM hearts, titin was expressed as 63 ± 3.5% N2B, and 37 ± 3.5% N2BA, and in DCM hearts as 60 ± 2.1% N2B and 40 ± 2.1% N2BA (*Figure 3A*). Detailed information on titin-isoform expression in the individual samples is provided in Supplementary material online, *Table S1*.

Analysis of the overall phosphorylation status of titin using the phosphoprotein stain ProQ diamond showed a reduction in the overall phosphorylation of N2B-titin in the HCM, but not the DCM samples, compared with controls (*Figure 3B*). Phosphorylation of N2BA-titin was unchanged in HCM and DCM samples compared with controls.

3.3 Passive tension in skinned fibres from human donor and failing hearts

In order to determine passive tension of the different groups of heart samples, we analysed skinned fibre preparations from a subset of the tissue samples [$n = 2$ DCM, $n = 2$ HCM (summarized as failing hearts), and $n = 4$ donor hearts]. Relaxed skinned fibres were subjected to a step-wise stretch-protocol stretching the fibre to a maximum of 130% slack length. Fibres from DCM and HCM hearts (*Figure 3C*) showed very similar passive tension values, and the data are therefore summarized as failing hearts in the presented graph. The passive length-tension–sarcomere length relationship of fibres from failing hearts was significantly higher compared with donor hearts. Titin has been shown to dominate total myofibre passive stiffness at low to intermediate SLs, up to ∼2.2 μm.[20] Within this SL range, passive tension was increased over two-fold in fibres from failing compared with donor hearts (*Figure 3D*).

3.4 Site-specific titin phosphorylation changes in human failing hearts

We next determined site-specific phosphorylation within the titin spring elements of human heart samples using phosphospecific antibodies to Ser4010, Ser4099, and Ser4185 in the N2-Bus, and Ser11878 in the PEVK region (*Figure 4*). Signal intensities from western blots were analysed densitometrically, and the ratio of pN2Bus/N2Bus and pPEVK/PEVK was used to compare the relative site-specific phosphorylation of titin in HF vs. donor hearts.

In tissue from heart failure patients, the relative phosphorylation of N2B-titin was significantly reduced by 35 ± 5% (DCM) and 38 ± 6% (HCM) at Ser4010, by 27 ± 3% (DCM) and 27 ± 5% (HCM) at Ser4099, and 23 ± 7% (DCM) and 26 ± 8% (HCM) at Ser4185, compared with donor hearts (*Figure 4A–C*). The differences were less pronounced for N2BA-titin, which showed a reduction in the relative phosphorylation by 33 ± 19% (DCM) and 33 ± 11% (HCM) at Ser4010, and by 8 ± 7% (DCM) and 15 ± 12% (HCM) at Ser4185. At Ser4099, the relative phosphorylation of N2BA-titin was not significantly changed in failing DCM or HCM samples, compared with donor hearts (*Figure 4B*). A selection of further representative western blots is shown in Supplementary material online, *Figure S3*.

In contrast, relative phosphorylation of the PEVK region at Ser11878 was significantly increased by 23 ± 7% in the N2B-titin isoform from DCM samples, and by 26 ± 8% in HCM samples (*Figure 4D*). Again, the observed changes in titin PEVK phosphorylation were less pronounced in the N2BA-isoform, with increased relative phosphorylation of Ser11878 by 12 ± 7% in DCM and 11 ± 8% in HCM samples.

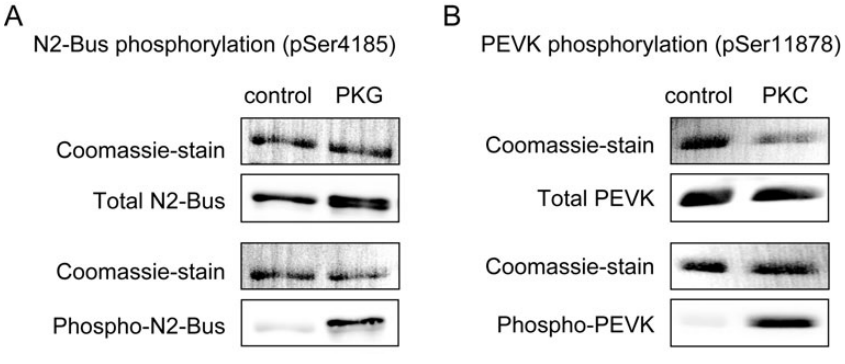

Figure 2 Representative Coomassie-stained gels and western blot images of control and kinase-treated recombinant fragments of human titin N2-Bus (left panels) and PEVK-region (right panels). Membranes were probed with the phosphospecific antibodies (α-pPEVK/ α-pN2B) to Ser11878 and Ser4185 and with antibodies recognizing total N2-Bus or PEVK (α-PEVK/α-N2B).

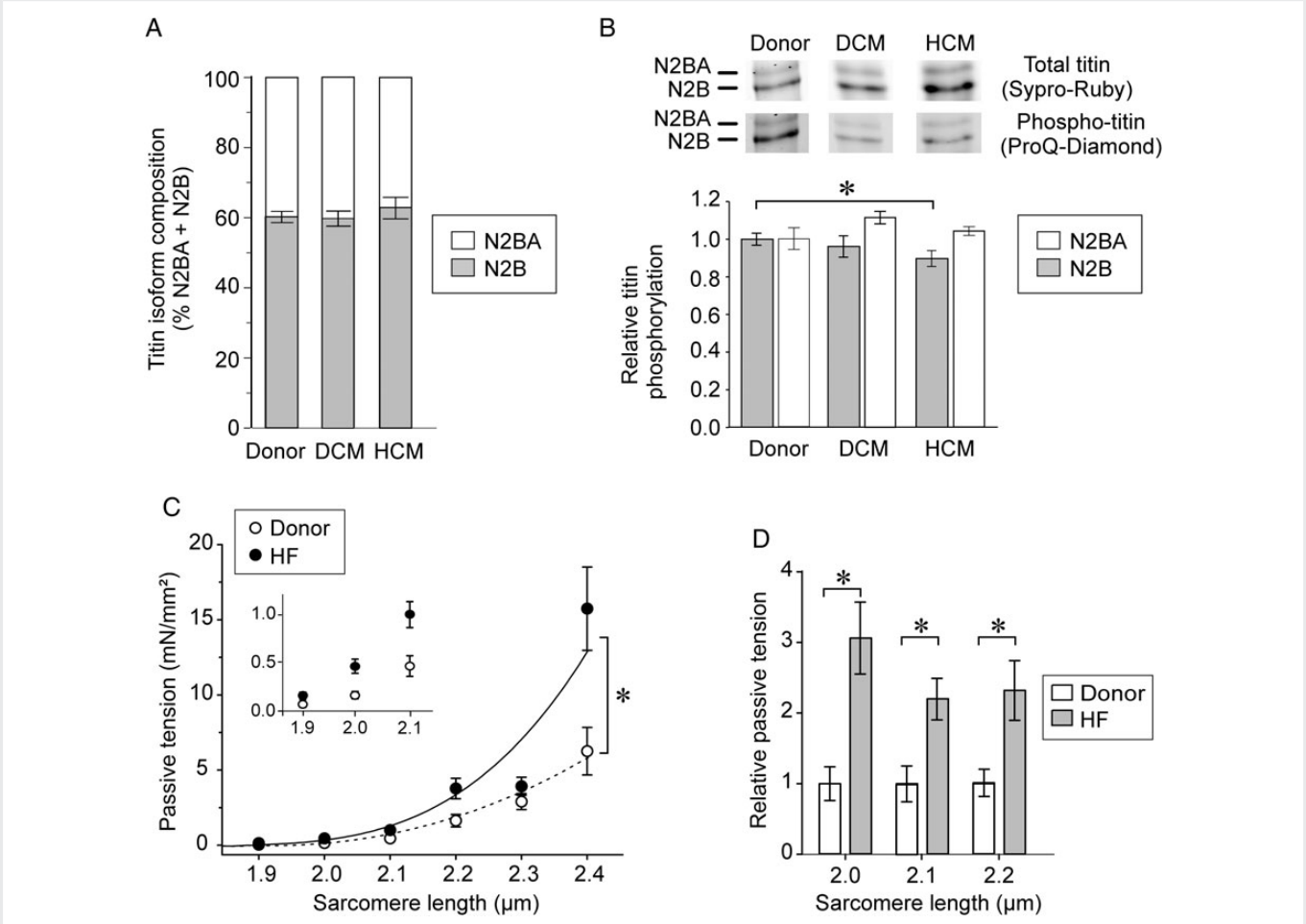

Figure 3 Average titin-isoform composition (N2BA + N2B = 100%) in LV samples from human donor, and end-stage failing DCM and HCM hearts (*A*). (*B*) Relative phosphorylation status of titin isoforms N2BA and N2B in human donor and failing hearts determined on 2% SDS–PAGE gels stained with Sypro Ruby (Total titin) or ProQ diamond (Phospho-titin). Representative gel lanes (top panels) and means ± SEM (*n*, 3–8 individual experiments, each averaging 5–10 gel-lanes per sample). Passive tension-sarcomere length relationship of skinned LV fibres from donor (open symbols) and failing hearts (HF; filled symbols) (*C*). Inset: Magnified section showing differences in passive tension values at 1.9, 2.0, and 2.1 μm sarcomere length. Lines represent polynomial fits to the data (means ± SEM; asterisk indicates a statistically significant difference in the overall passive tension-sarcomere length relationship from failing compared with donor hearts, $P < 0.05$ in two-way ANOVA). Normalized passive tension (indexed to donor) at low sarcomere lengths (2.0–2.2 μm) of failing (grey) and donor heart samples (white) (*D*). Data are means ± SEM [*n*, 3–6 different fibre bundles per sample from $n = 2$ DCM, $n = 2$ HCM (shown as failing hearts), and $n = 4$ donor hearts]. Asterisks indicate statistically significant differences ($P < 0.05$ in Student's *t*-test with Bonferroni correction).

4. Discussion

Titin phosphorylation has been observed at different domains and by various kinases, including ERK1/2, cdc2, CaMKIIδ, PKA, PKG, and PKCα (for review see Krüger and Linke[21] and Hidalgo and Granzier[22]). Interestingly, phosphorylation of the N2-Bus domain by PKA and PKG reduces titin stiffness, whereas phosphorylation of the PEVK-region by PKCα increases it. In our present work, we identified seven mostly novel PKA or PKG-dependent phosphorylation motifs within the N2-Bus using tandem mass spectrometry.

Titin domain-phosphorylation status and passive fibre stiffness were determined in a selected set of human donor and failing heart samples with very similar titin-isoform composition. Despite the identical titin-expression pattern, passive tension was substantially increased in fibres from failing compared with those from donor hearts, especially in the SL range up to ~2.2 μm, at which titin stiffness dominates the passive mechanical properties of myocardium.[20] We provided evidence that the observed increase in passive tension of the failing human heart samples can arise from altered titin phosphorylation. Results from western blot analyses revealed a significantly lower phosphorylation level of Ser4010, Ser4099, and Ser4185 within the N2-Bus domain in failing compared with control hearts, suggesting a hypo-phosphorylation of the N2-Bus in HF. In addition to PKA and PKG, the hypo-phosphorylation of the N2-Bus may partly be mediated by extracellular signal-regulated kinase-2 (ERK2), which has recently been shown to phosphorylate Ser4010 *in vitro*, or by reduced phosphorylation by CaMKII.[23,15] Considering that phosphorylation of the N2-Bus by PKA or PKG lowers myofilament passive tension by up to 20%,[9,8] the reduced phosphorylation of this region in human HF likely contributes to the observed increase in passive fibre tension.

In cardiac myocytes, cGMP-PKG signalling is a well-established counterpart of the hypertrophic program, and abnormal PKG-signalling has

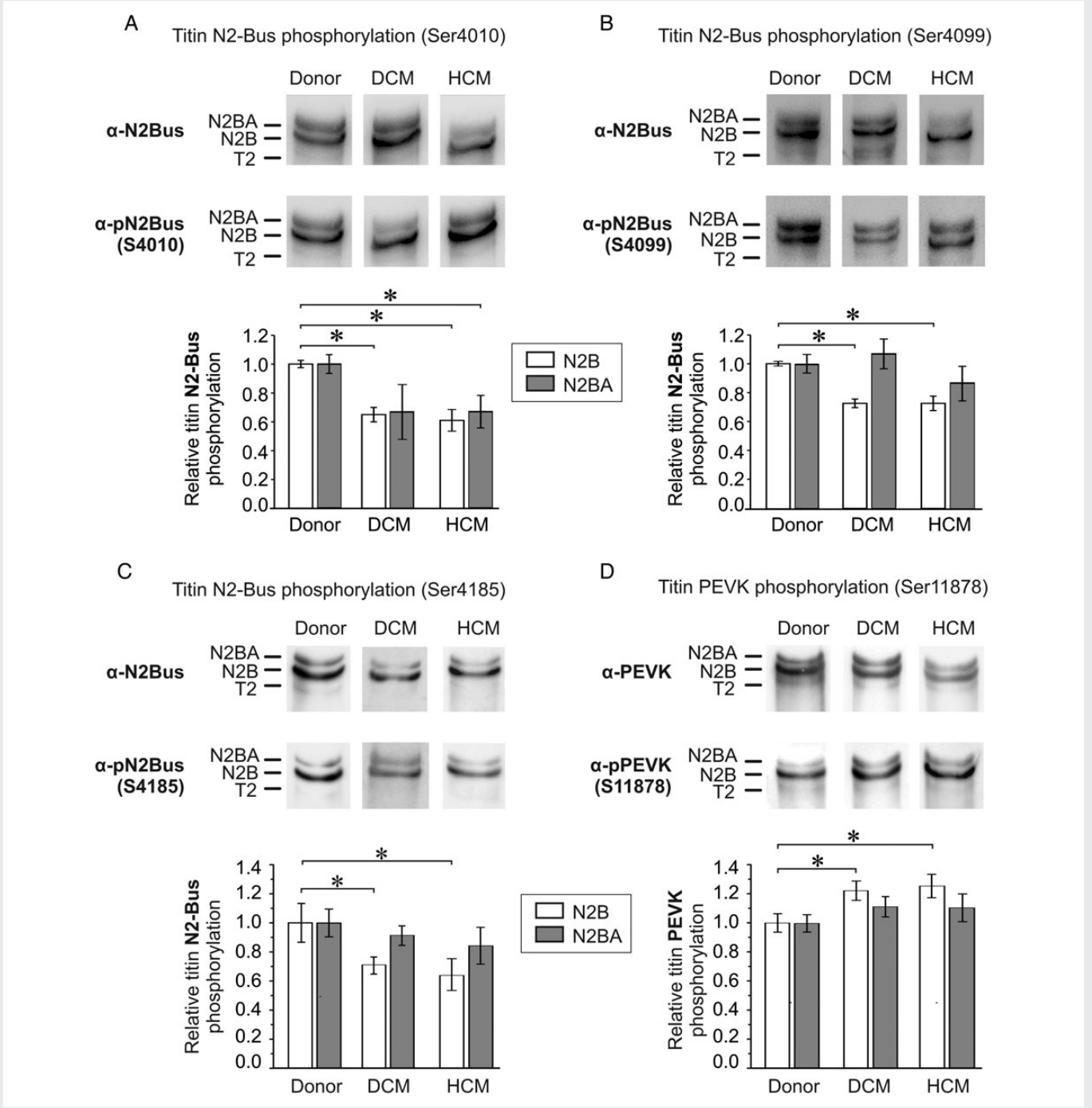

Figure 4 Titin phosphorylation analysed by western blot at Ser4010 (*A*), Ser4099 (*B*), and Ser4185 (*C*) in the N2-Bus, and at Ser11878 in the PEVK-region (*D*). Representative western blots (top of each panel) show titin bands visualized by antibodies to total PEVK/N2-Bus (α-PEVK/α-N2Bus) and the respective phosphosite-directed antibodies (α-pPEVK/α-pN2Bus). Bar graphs (bottom of each panel) show the results of densitometric analyses of titin-isoform bands (N2B, N2BA) for each phosphospecific antibody in human donor ($n = 8$), DCM ($n = 6$), and HCM ($n = 3$) hearts. Data were normalized to donor hearts; means ± SEM, of a minimum of three titin lanes per heart sample. Asterisks indicate statistically significant differences compared with donor tissue ($P < 0.05$ in Student's *t*-test).

been associated with ventricular hypertrophy and heart failure in animal models as well as in human patients (reviewed in Tsai and Kass[24]). In cardiac myocytes from heart failure patients with preserved ejection fraction, increased passive myocyte stiffness was recently associated with low myocardial PKG activity.[25] Assuming that impaired PKG-signalling accounts for part of the differences in titin N2-Bus phosphorylation, increasing the availability of cGMP by inhibiting phosphodiesterase-5 (e.g. by sildenafil) may be a useful strategy to recover PKG-dependent titin phosphorylation, reduce passive tension, and thereby restore cardiac dysfunction. The feasibility of such an approach has recently been shown in an experimental dog model with diastolic dysfunction, where acute cGMP enhancing treatment with sildenafil and BNP increased

titin phosphorylation, lowered titin-based cardiomyocyte stiffness, and thus improved LV diastolic distensibility.[11]

The high abundance of PKA phosphorylation motifs in the N2-Bus suggests that PKA-signalling may play an important role in the healthy human heart to dynamically adjust cardiomyocyte passive tension on a beat-to-beat basis, e.g. in response to β-adrenergic signalling. In turn, chronically impaired β-adrenergic signalling during heart failure could reduce titin N2-Bus phosphorylation levels, as observed in our present study, and increase cardiomyocyte passive tension. This idea is in line with a previous study showing that PKA-treatment of skinned cardiac myocytes from patients with heart failure and a preserved ejection fraction effectively lowers passive myocyte stiffness to that of control cells.[26] In early stages of cardiac insufficiency, over-activation of the β-adrenergic system is a common compensatory mechanism of the heart. In the long run, this compensatory response may cause a neurohormonal overstimulation and lead to down-regulation of the β-receptor density,[27,28] and a subsequently disturbed PKA-signalling pathway.[29] Such impaired PKA-activation could result in chronic hypophosphorylation of titin N2-Bus and thereby contribute to increased passive myocardial stiffness during progression of HF.

In addition to the reduced N2-Bus phosphorylation, we detected a substantial increase in the relative phosphorylation of Ser11878 in the PEVK-region of titin from failing human hearts. Phosphorylation of this serine by PKCα has previously been reported to elevate titin stiffness.[12] Hence, the hyperphosphorylation of the PEVK-domain observed in human HF should add further to increased fibre stiffness. Our findings are in accordance with previous studies suggesting enhanced phosphorylation of Ser11878 in end-stage failing hearts,[15] and as a result of pressure overload-induced heart failure in a mouse model with transverse aortic constriction.[30] In contrast to the β-receptor system, the abundance of α-1 receptors is usually unaltered during heart failure.[31] Consequently, a disease-induced excess of catecholamines should result in elevated basal, α-1 receptor-mediated activation of PKCα by G_q proteins and phospholipase C. Over-activation of PKCα could also be induced by hypertrophic signalling cascades initiated during heart failure.[32,33] We therefore conclude that modification of PKCα-signalling likely modulates titin PEVK phosphorylation during progression of heart failure and may therefore affect myofilament stiffness.

Unfortunately, we do not have full information on the medication of the patients prior to transplantation. We thus cannot exclude the possibility that the observed changes in the titin phosphorylation status are partly due to pharmacological treatment, e.g. with beta-blockers or ACE-inhibitors.

We found significant changes in the phosphorylation status of the N2-Bus and PEVK-region only in the stiff N2B-, but not in the more compliant N2BA-titin isoform. A similar observation has previously been reported by Borbely *et al.*,[26] showing that in HF patients, titin phosphorylation, determined by ProQ-diamond staining, was reduced mainly in the N2B-titin isoform. A possible explanation is that promoting the stiffness of the shorter and stiffer N2B-titin molecule might be more effective than modulation of the more compliant N2BA, especially as it has higher relative expression levels in the human heart. A further explanation is the fact that unlike N2B-titin, the N2BA-isoform is barely stretched in the physiological sarcomere length range, thus limiting the accessibility of the phosphorylation motifs.

The observation that phosphorylation of the N2-Bus by PKA or PKG leads to an increase of the persistence length of this titin region and decreases myofilament passive tension,[8] whereas phosphorylation of the PEVK domain by PKCα reduces the persistence length and increases passive tension[12] may seem rather contradictory at first sight. A possible explanation is based on the amino acid sequences of the respective titin segments: the N2-Bus contains a high amount of acidic (negatively charged) amino acids, resulting in a low isoelectric point of this region. In contrast, the PKC-targeted region of the PEVK domain is composed of a high amount of basic residues and has a much higher isoelectric point. Introduction of an additional negative charge, such as a phosphate group, into an already negatively charged environment could lead to intramolecular electrostatic repulsion. This, in turn, could influence the intrinsically disordered structure of the N2-Bus and thereby increase the distensibility of the N2-Bus domain, reflected by an increased persistence length. In contrast, introduction of a negatively charged phosphate residue into the basic environment of the PEVK-region would prevent electrostatic repulsion and potentially generate additional intramolecular ionic interactions. Such intramolecular interactions likely reduce the distensibility of the PEVK domain, reflected by a reduced persistence length. According to this view, the presence of multiple phosphorylation sites within each region should increase the phosphorylation-induced effect on the molecular elasticity of titin, and may thus allow a dynamic and potentially non-linear modulation of titin stiffness.

In summary, our study indicates the presence of multiple PKA- and PKG-dependent phosphorylation motifs in the N2-Bus which may modulate titin stiffness upon co-ordinated hypo- or hyperphosphorylation. In contrast to previously published patient cohorts, the samples selected for our present study did not show a titin-isoform shift, but differential changes in the phosphorylation of the N2-Bus and PEVK spring elements that elevate passive tension. Further studies are needed to address the question how titin phosphorylation is controlled during progression of systolic and diastolic cardiac dysfunction, and to elucidate the role of titin phosphorylation in modulating myocardial stiffness in failing hearts.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared

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