ISSN 0006-2979, Biochemistry (Moscow), 2024, Vol. 89, Nos. 12-13, pp. 2083-2106 The Author(s) 2024. This article is an open access publication.

REVIEW

Troponins and Skeletal Muscle Pathologies

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Received May 2, 2024
Revised November 19, 2024
Accepted December 1, 2024

Abstract—Skeletal muscles account for ~30-40% of the total weight of human body and are responsible for its most important functions, including movement, respiration, thermogenesis, and glucose and protein metabolism. Skeletal muscle damage negatively impacts the whole-body functioning, leading to deterioration of the quality of life and, in severe cases, death. Therefore, timely diagnosis and therapy for skeletal muscle dysfunction are important goals of modern medicine. In this review, we focused on the skeletal troponins that are proteins in the thin filaments of muscle fibers. Skeletal troponins play a key role in regulation of muscle contraction. Biochemical properties of these proteins and their use as biomarkers of skeletal muscle damage are described in this review. One of the most convenient and sensitive methods of protein biomarker measurement in biological liquids is immunochemical analysis; hence, we examined the factors that influence immunochemical detection of skeletal troponins and should be taken into account when developing diagnostic test systems. Also, we reviewed the available data on the skeletal troponin mutations that are considered to be associated with pathologies leading to the development of diseases and discussed utilization of troponins as drug targets for treatment of the skeletal muscle disorders.

DOI: 10.1134/S0006297924120010

Keywords: skeletal muscle, troponin, ageing, myopathy, biomarker, monoclonal antibodies

INTRODUCTION

In mammals, striated muscles are represented by two tissue types: skeletal and cardiac. Skeletal muscles accounts for ~30-40% of the total body weight and perform important functions such as movement, respiration, and thermogenesis [1, 2]. They are involved in the major share of glucose, lipid, and protein metabolism. Thus, skeletal muscle damage or pathology could have a significant impact on various aspects of whole-body functioning, leading to impaired quality of life and, in severe cases, disability or death.

Skeletal muscle dysfunction can be caused by mechanical injuries, myopathies, and various diseases that are accompanied by muscle atrophy and rhabdomyolysis. The most common causes of mechanical damage to skeletal muscles include vigorous physical activity, surgery, trauma, and prolonged compression syndrome [3]. The term “myopathy” describes any skeletal muscle disease of various etiologies. These pathologies are frequently accompanied by abnormalities of the skeletal muscle structure and development of metabolic disorders in the tissue [4]. Myopathies are classified as either congenital (inherited) or acquired (secondary). Inherited myopathies include: muscular dystrophies (associated with mutations in the genes of certain cytoskeletal proteins of skeletal muscle, e.g., Duchenne muscular dystrophy or Becker muscular dystrophy); congenital myopathies (e.g., nemaline myopathy, core myopathy); metabolic myopathies (associated with mutations in the genes of various enzymes involved in carbohydrate and lipid metabolism);

Abbreviations: ALS, amyotrophic lateral sclerosis; cTnI, cardiac troponin I; cTnT, cardiac troponin T; fsTnC, fast skeletal troponin C; fsTnI, fast skeletal troponin I; fsTnT, fast skeletal troponin T; ss/cTnC, slow skeletal/cardiac troponin C; ssTnI, slow skeletal troponin I; ssTnT, slow skeletal troponin T.

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mitochondrial myopathies (associated with mutations in mitochondrial oxidative phosphorylation proteins); and channelopathies [5-8]. Muscular dystrophies are considered as one of the most common and severe inherited myopathies, some of which, such as Duchenne muscular dystrophy, are characterized by progressive muscle weakness and atrophy, eventually leading to death from cardiopulmonary arrest [9]. Acquired myopathies include: inflammatory myopathies (most commonly of autoimmune nature, such as dermatomyositis and polymyositis), myopathies associated with various infections (caused by bacteria, fungi, or viruses), toxic myopathies (e.g., caused by drug or alcohol poisoning or myotoxic drugs such as statins and fibrates), myopathies associated with various diseases (rheumatic diseases, malignant tumors, and endocrine diseases), idiopathic myopathies, and endocrine myopathies [10-13].

Another serious pathology of skeletal muscle is rhabdomyolysis. It is the process of skeletal muscle breakdown accompanied by the release of intracellular components into the bloodstream, which can be life-threatening in severe cases. When more than 100 g of muscle tissue is simultaneously damaged, concentration of myoglobin in the blood reaches a critical value, and a significant amount of protein is deposited in renal tubules. This process could provoke acute renal failure, which is the main cause of fatal outcomes in rhabdomyolysis [12, 14]. In children, the major factors leading to rhabdomyolysis are viral myositis, trauma, rheumatic diseases, high-intensity exercise, and medications [12]. In adults, rhabdomyolysis most often occurs due to alcohol or drug abuse, medications, trauma, malignant neuroleptic syndrome, or immobility [5, 12].

Myopathies are accompanied by muscle atrophy, a process of loss of muscle mass and strength when catabolism of muscle proteins predominates over anabolism. In addition to myopathies, other diseases and pathologies such as chronic heart failure, chronic obstructive pulmonary disease, cancer, chronic kidney disease, Alzheimer's disease, and infectious diseases could also lead to the development of muscle atrophy [15, 16]. Muscle atrophy could also be caused by immobility and muscle disuse due to paralysis or prolonged bed rest [3].

A loss of muscle mass also occurs during the ageing process – sarcopenia [from Greek words *sarx* (flesh) and *penia* (deficiency)]. This condition is manifested by the decrease in the cross-sectional area and number of muscle fibers, accumulation of adipose or connective tissue in the skeletal muscle, and progressive loss of the muscle mass and strength. All of this entails poor physical performance and, if prolonged, geriatric frailty [17]. Sarcopenia is characterized by the increased risk of soreness, falls, limitations in daily activities, poor prognosis after surgery and, ultimately, high mortality [16]. Prevalence of sarcopenia reaches a level up to 13% in the individuals aged 60-70 years and up to 50% in the individuals aged over 80 [17]. Presence of diabetes increases the risk of sarcopenia by 2-fold, and of geriatric frailty – by 1.5-4-fold [18]. Thus, importance of these problems grows with the increase in life expectancy. To date, nutritional adjustment and exercise have been used to stop progression of sarcopenia, but also therapeutic strategies are being developed to prevent muscle damage [16-18].

Skeletal muscle is the primary tissue responsible for insulin-dependent glucose uptake; hence, loss of muscle mass in sarcopenia could lead to the development of insulin resistance, and ultimately type 2 diabetes. In addition, the skeletal muscle adiposis, which accompanies sarcopenia, could also contribute to dysfunction [17, 19]. In sarcopenia and type 2 diabetes, functional impairments could develop necessitating diagnosis and timely treatment. So, monitoring the decrease in skeletal muscle mass is of high importance, especially in the patients with diabetes.

Three types of skeletal muscle fibers have been identified in humans: slow-twitch (type 1) and fast-twitch (types 2A and 2X; other mammals also have type 2B fast-twitch fibers) [20, 21]. The type 2A fast-twitch muscle fibers are more resistant to fatigue than the type 2X and contain more enzymes of oxidative metabolism [22]. Fiber composition of muscles depends on the function they perform: muscles responsible for maintaining body posture are mainly composed of slow-twitch fibers, while muscles required for movement are predominantly composed of fast-twitch fibers [21].

Development of some pathologies could result in the damage to muscle fibers of only certain type. For example, predominantly fast-twitch muscle fibers are affected, especially type 2X fibers, in the Duchenne muscular dystrophy [23]. Muscle damage induced by administration of statins primarily influences fast-twitch fibers, while fibrates intake impacts slow-twitch fibers [24, 25]. Intense eccentric muscle contractions also primarily damage fast-twitch fibers [26]. Many pathological conditions entailing muscle atrophy are characterized not only by deterioration of certain muscle fiber types, but also by a fiber-shift from one type to another. For instance, denervation or immobility of a limb, spinal cord injury, or prolonged bed rest may lead to slow-to-fast fiber type shift [27]. The reverse process, fast-to-slow fiber type shift, occurs as a result of starvation, glucocorticoid administration, cachexia, and sarcopenia [27]. In addition, fast-twitch and slow-twitch muscle fibers have been shown to have different abilities to regenerate after injury. Studies in rats have shown that while muscles consisting mainly of fast-twitch fibers (e.g., *extensor digitorum*) regenerate

efficiently, muscles composed predominantly of slow-twitch fibers (e.g., *soleus*) do not regenerate completely, developing fibrosis [28].

Various instrumental methods have been used to diagnose skeletal muscle injuries. These include magnetic resonance imaging, computed tomography, and dual-energy X-ray absorptiometry [29]. These approaches allow non-invasive detection of changes in the skeletal muscles (edema, replacement with adipose and connective tissue, muscle atrophy), identification of specific areas of damage, and even quantification of changes in the structure of skeletal muscles [30-34]. However, disadvantage of using instrumental methods is the need for expensive equipment and specialized locations for manipulation. An alternative is determination of blood concentrations of biomarkers – most commonly skeletal muscle proteins – that are released into the bloodstream when muscle fibers are damaged. Currently, the most widely used biomarkers of skeletal muscle damage are creatine kinase, aspartate aminotransferase, lactate dehydrogenase, and myoglobin [35]. However, it should be noted that all the abovementioned proteins lack specificity: in addition to skeletal muscle, they are expressed in other tissues, that may reduce accuracy of the diagnosis. Therefore, special studies have been conducted to seek and identify new and more specific biomarkers. These included fatty acid binding protein 3, myosin light chain 3, MM isoform of creatine kinase, and skeletal troponin I (TnI) isoforms [36]. Although all the above biomarkers are comparable or superior in sensitivity to conventional biomarkers, only the skeletal TnI isoforms are specifically expressed in skeletal muscle [9, 36, 37]. TnI, together with troponin C (TnC) and troponin T (TnT), constitutes the troponin complex that regulates muscle contraction [38, 39]. TnT isoforms are also specific to skeletal muscle, but we were unable to find information on their use as biomarkers of skeletal muscle damage. The utilization of skeletal muscle TnT as a biomarker requires further investigation.

For a long time, skeletal troponins have attracted attention of the scientists, primarily due to the studies reporting their role in regulation of muscle contraction. In this review, we describe role of these proteins in the development of certain skeletal muscle diseases and discuss applications of troponins for diagnosis and therapy of skeletal muscle disorders.

BIOCHEMICAL PROPERTIES OF THE TROPONINS

Troponin I. In humans, TnI is represented by three isoforms: cardiac TnI (cTnI) and two skeletal isoforms, namely fast skeletal (fsTnI) and slow skeletal (ssTnI). fsTnI and ssTnI are expressed in fast-twitch and slow-twitch muscle fibers, respectively (Table 1) [23, 40][1]. ssTnI is also expressed in cardiac muscle during embryonic development and is replaced by cTnI in the postnatal period [41]. cTnI is only expressed in cardiac muscle after birth [42, 43].

Table 1. Some properties of human skeletal TnI isoforms

Isoforms	Fast skeletal TnI (UniProt P48788)	Slow skeletal TnI (UniProt P19237)
Gene	*TNNI2*	*TNNI1*
Chromosomal locus	11p15.5	1q32.1
Number of exons	8	9
Number of amino acid residues	182	187
Molecular mass (kDa)	21.3	21.7
Isoelectric point	8.9	9.6
Tissue specificity	fast-twitch muscle fibers	slow-twitch muscle fibers, embryonic cardiomyocytes

TnI inhibits interaction between myosin and actin in the absence of Ca^{2+}. In the presence of Ca^{2+}, the inhibitory region of TnI dissociates from actin and this facilitates actin-myosin interaction [44, 45]. The major difference between the skeletal TnI isoforms and cTnI is the presence of a unique 31 amino acid N-terminal sequence (residues 2-32) in cTnI (Fig. 1). Phosphorylation of the Ser23 and Ser24 residues is considered as a mechanism regulating muscle contraction [48]. For the skeletal TnI isoforms, the three-dimensional (3D) structure has been resolved for fsTnI only. The highest resolution 3D structure was obtained using X-ray crystallography for the chicken fsTnI in complex with TnC and for the portion of TnT in both Ca^{2+}-activated state (3.00 Å resolution) and Ca^{2+}-free state (7.00 Å resolution) [46]. The 3D structure has been resolved for the full-length fsTnI with the exception of C-terminal part of the molecule. Several functional sites could be distinguished in the TnI structure: the IT arm, inhibitory, switch, and C-terminal regions. Similar sites for ssTnI have been denoted based on their sequence similarity with fsTnI and cTnI [46, 47].

[1] Numbering in all proteins of this review includes N-terminal Met.

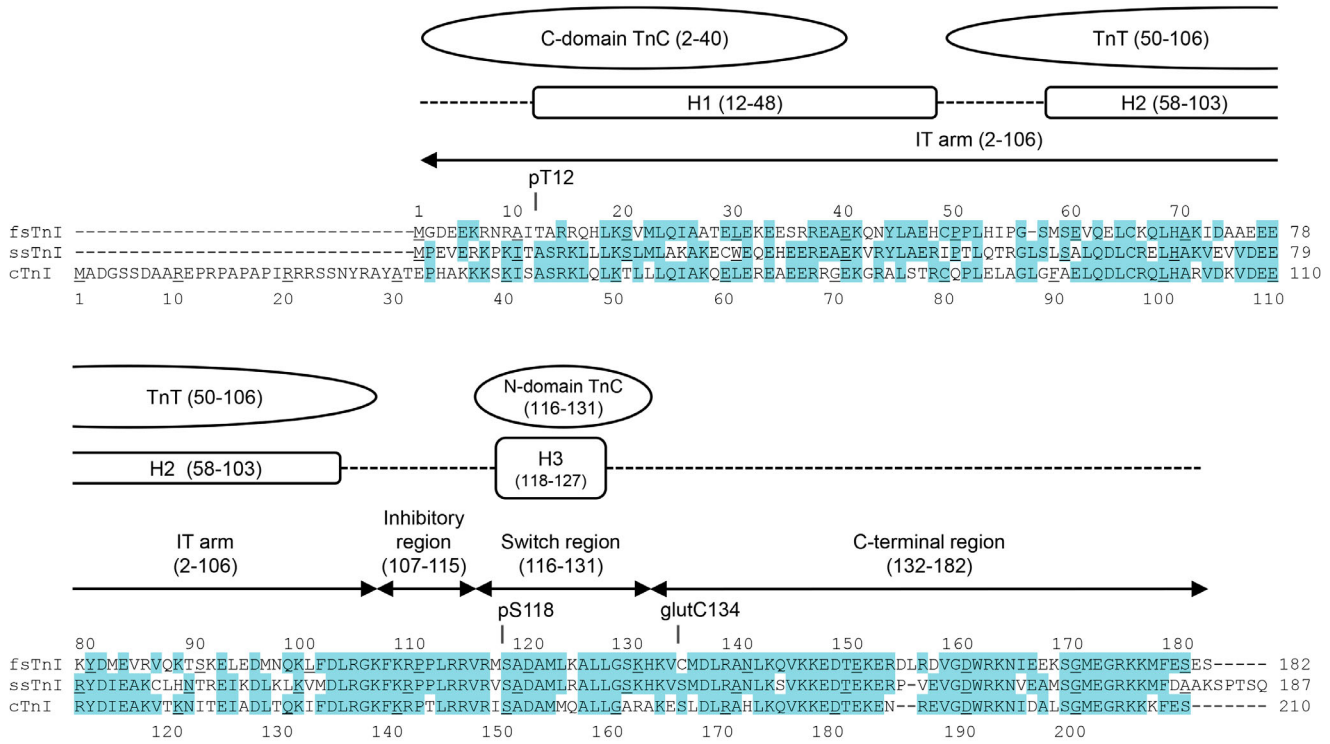

Fig. 1. Protein sequence alignment of three TnI isoforms: fsTnI, ssTnI and cTnI. Amino acid sequences are derived from the Uniprot database: fsTnI (TNNI2_HUMAN, P48788), ssTnI (TNNI1_HUMAN, P19237), cTnI (TNNI3_HUMAN, P19429). Clustal Omega online software was used to make protein sequence alignments. Identical amino acid residues in the aligned sequences are colored in blue. Boxes show the borders of α-helices (numbering is for fsTnI sequence). Arrows indicate regions and TnC- and TnT-binding sites, putative fsTnI and ssTnI phosphorylation (p) and fsTnI glutathionylation (glut) sites are marked above the sequence [44-47].

The IT arm of TnI (~2-106 amino acid residue of fsTnI; ~2-107 amino acid positions of ssTnI; numbering varies in different animal species) has a structural function: it consists of the N-terminal region that binds the C-terminal domain of TnC (~2-40 residues of fsTnI; ~2-40 residues of ssTnI) and the TnT-binding site (~50-106 amino acid residues of fsTnI; ~50-107 residues of ssTnI). The IT-arm is formed by two oppositely directed α-helices, H1 (~12-48 amino acids of fsTnI; ~12-48 residues of ssTnI) and H2 (~58-103 residues of fsTnI; ~59-104 residues of ssTnI), connected by a linker (~49-57 residues of fsTnI; ~49-58 residues of ssTnI). The inhibitory region (~107-115 residues of fsTnI; ~108-116 residues of ssTnI) interacts with actin in the absence of Ca^{2+} ions, moving tropomyosin molecule to the position where it blocks the interaction of myosin with actin thus preventing muscle contraction. The switch region (~116-131 residues of fsTnI; ~117-132 residues of ssTnI) includes the H3 α-helix (~118-127 residues of fsTnI; ~119-128 residues of ssTnI), it binds to the N-terminal domain of TnC when Ca^{2+} concentration increases, which leads to dissociation of the inhibitory region of TnI from actin, displacement of tropomyosin, and interaction of myosin with actin.

The C-terminal portion of fsTnI could not be resolved by X-ray crystallography due to its mobility. The nuclear magnetic resonance (NMR) data for the chicken fast skeletal troponin complex shows that the C-terminal region lacks secondary structure and is in a disordered state, both in the absence and presence of Ca^{2+} [49, 50]. According to the results of small-angle neutron scattering of the chicken fast skeletal troponin complex, the C-terminal part of fsTnI is an elongated structure at low Ca^{2+} concentrations that may represent a supercoiled helix or β-layers, while in the presence of Ca^{2+} the site adopts a compact structure [51]. Other NMR data suggest that the C-terminal region of the chicken fsTnI has a secondary structure consisting of an α-helix, two β-layers, and two more α-helices [52-54]. The C-terminal region interacts with actin in the absence of Ca^{2+} and thus participates in inhibition of myosin binding to actin [52, 55, 56].

Troponin T. Like TnI, TnT is represented in the human body by three isoforms: a cardiac (cTnT) and two TnT skeletal isoforms, namely fast skeletal (fsTnT) and slow skeletal (ssTnT) variants (Table 2). fsTnT and ssTnT are only expressed in skeletal muscle, while cTnT is expressed in both heart and skeletal muscle during embryonic development and neonatal period [57, 58].

Table 2. Some properties of human TnT isoforms

Isoforms	Fast skeletal TnT (UniProt P45378)	Slow skeletal TnT (UniProt P13805)
Gene	*TNNT3*	*TNNT1*
Chromosomal locus	11p15.5	19q13.42
Number of exons	19	14
Number of amino acid residue	245-269	251-278
Molecular mass (kDa)	29.1-31.8	30-33
Isoelectric point	5.7-9	5.6-6.1
Tissue specificity	skeletal muscles	skeletal muscles

The function of TnT is to attach the troponin complex to the actin and regulate the interaction of the complex with thin filament proteins [59]. The TnT molecule includes an N-terminal variable region and conserved central and C-terminal regions (Fig. 2). The N-terminal domain of TnT is variable not only in amino acid composition but also in length, and diversity of the forms is the result of alternative splicing. The conserved portion has been shown to contain two α-helices, H1 (~162-188 residues of fsTnT) and H2 (~196-240 residues of fsTnT). The H2 TnT α-helix forms a supercoiled helix with H2 of TnI and interacts with TnC via its C-terminus [46, 47, 60-62]. The conserved portion also includes two tropomyosin binding sites, Tm1 and Tm2. While localization of Tm1 is determined with sufficient accuracy (~61-99 residues of fsTnT; ~65-103 residues of ssTnT), the data on localization of the Tm2 are controversial. Jin and Chong [60] presumed that Tm2 is located within the ~161-200 residues of fsTnT and ~165-204 residues of ssTnT, whereas it was suggested in the other works that Tm2 is formed by the last C-terminal residues of TnT [63-66]. Studies describing structure of the cardiac thin filament obtained with cryo-electron microscopy (cryo-EM) and cryo-electron tomography (cryo-ET) have suggested that cTnT is capable of binding two Tm strands simultaneously. With the Tm2 site TnT interacts with the same strand as TnI does, while with the Tm1 region it binds to the neighboring thread of tropomyosin [67, 68]. Investigation of the structure of cardiac [67, 68] and skeletal muscle thin filaments [69] by cryo-EM and cryo-ET also suggested that fsTnT is likely to interact with the skeletal muscle protein nebulin, which, like tropomyosin, binds to actin along the entire thin filament. Although the exact structure of skeletal TnT and nebulin has still not been resolved, it is assumed that the R134-R179 portion of the mouse fsTnT (R123-R169 in human fsTnT) has two nebulin binding sites [69].

The *TNNT3* gene encoding fsTnT consists of 19 exons. Eight exons among them can undergo alternative splicing: namely exons 4, 5, 6, 7, 8, and the fetal exon in the N-terminal portion of the molecule, as well as exons 16 and 17 (or exons α and β) encoding a part of the C-terminus [71]. The fetal exon, located between the exons 8 and 9, is expressed in embryonic skeletal muscle only [70, 72]. Expression of the exons 16 and 17 is mutually exclusive: exon 16 is expressed predominantly in adulthood, while exon 17 is expressed primarily in embryonic and neonatal muscles [73, 74]. During embryonic development and in postnatal period, expression of the fsTnT splice variants is changing: high molecular weight variants are replaced with low molecular weight splice forms, low isoelectric point (acidic) variants are replaced with high isoelectric point (basic) splice forms (see below). The differences in size and charge of these forms are due to alternative splicing of the N-terminal exons that encode predominantly acidic residues [75].

In the adult rabbit muscle, expression of five splice variants of fsTnT has been demonstrated by Western blotting: dominant TnT1f (the longest splice form, containing all exons except the fetal one), TnT2f, TnT3f, and to a lesser extent TnT2fa and TnT4f [72, 76, 77]. It is important to note, however, that the differences in the splice variant composition have been observed in various muscle types. In the adult rat, six splice variants of fsTnT have been identified by mass spectrometry, and their composition also differed in various muscle types [78]. The same method detected six splice variants of fsTnT in the rhesus macaque [79]. In the adult humans, only three fsTnT splice variants (fsTnT III, VI, and VII) have been identified by the muscle top-down mass spectrometry to date, but in that study, the authors analyzed only two types of muscles – *tibialis anterior* and *vastus lateralis* – the former of which contains more slow-twitch fibers, and so some of the fsTnT splice variants may not have been detected [80].

The *TNNT1* gene encoding ssTnT consists of 14 exons. Unlike fsTnT, diversity of the splice forms resulting from alternative splicing of ssTnT is low. Presence of high molecular weight and low molecular weight splice variants of ssTnT has been shown by Western blotting for mouse and sheep [81]. Cloning of the mouse cDNA demonstrated that the low molecular weight ssTnT form appears due to alternative splicing deletion of 11 residues of the N-terminal exon 5.

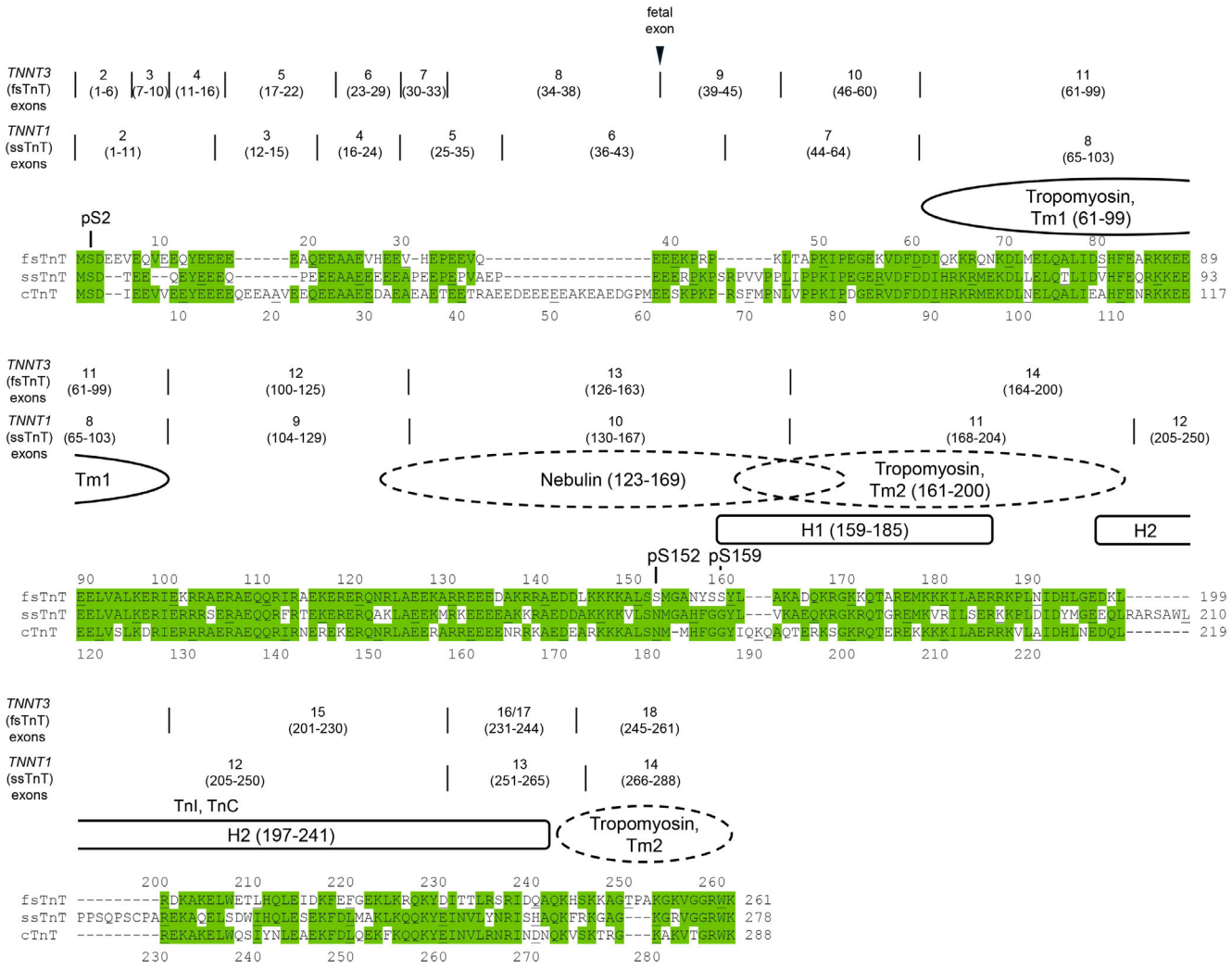

Fig. 2. Protein sequence alignment of three TnT isoforms: fsTnT, ssTnT and cTnT. Amino acid sequences are derived from the Uniprot database: fsTnT (TNNT3_HUMAN, P45378-3), ssTnT (TNNT1_HUMAN, P13805-1), cTnT (TNNT2_HUMAN, P45379-6). The longest adult human splice variants were chosen; for fsTnT and cTnT, we selected those variants that do not contain the fetal exon. Clustal Omega online software was used to make sequence alignments. The identically positioned amino acid residues are marked with green. fsTnT and ssTnT exon structures are represented above. Boxes show borders of α-helices (residues numeration is for fsTnT sequence), TnI and TnC binding site is marked above. Solid line oval indicates tropomyosin Tm1 binding site, dashed line ovals indicate putative tropomyosin Tm2 binding sites and potential nebulin binding site. Possible fsTnT phosphorylation sites are marked above the sequence [46, 47, 60, 63-66, 69, 70].

Two high molecular weight ssTnT splice forms are distinguished by a single residue deletion in the exon 6 [81, 82]. Cloning of the human cDNA also revealed three splice variants of ssTnT: a full-length splice form, a splice form with a C-terminal deletion of 16 residues derived from alternative splicing of the exon 12, and a splice form with two deletions, the aforementioned C-terminal deletion of the exon 12 and an N-terminal deletion of 11 residues (exon 5), as in the mouse ssTnT [82-84]. At the protein level, two splice variants of ssTnT (ssTnT II and III) with deletions were identified in the human skeletal muscle (*vastus lateralis* and *tibialis anterior*) by mass spectrometry [80].

Troponin C. In contrast to TnI and TnT, TnC is represented in humans by two isoforms: the slow skeletal/cardiac isoform ss/cTnC and the fast skeletal isoform fsTnC, which are expressed in the cardiac/slow-twitch and fast-twitch muscle fibers, respectively (Table 3) [85].

TnC provides the sensitivity of thin filament to Ca^{2+}. It changes conformation of the troponin complex after binding of Ca^{2+}, whose concentration increases due to propagation of the nerve impulse along the muscle fiber. TnC consists of a short N-terminal region and four EF-hands (Ca^{2+}-binding domains) (Fig. 3). These four EF-hands are joined in pairs to form N-terminal and C-terminal domains connected by a linker.

Table 3. Some properties of human TnC isoforms

Isoforms	Fast skeletal TnC (UniProt P02585)	Slow skeletal/ cardiac TnC (UniProt P63316)
Gene	*TNNC2*	*TNNC1*
Chromosomal locus	20q13.12	3p21.1
Number of exons	6	6
Number of amino acid residue	160	161
Molecular mass (kDa)	18.1	18.4
Isoelectric point	4.1	4.0
Tissue specificity	fast-twitch muscle fibers	slow-twitch muscle fibers, cardiomyocytes

The N-terminal EF-hands (EF-hands I and II) have low affinity, while the C-terminal hands (EF-hands III and IV) have high affinity for Ca^{2+}. The C-terminal EF-hands are constantly saturated with Ca^{2+} or Mg^{2+} ions, whereas the N-terminal ones bind Ca^{2+} with high selectivity only when its concentration increases during propagation of action potential along the length of the muscle fiber. In the fsTnC, all four EF-hands can bind ions whereas in the ss/cTnC, the EF-hand I has lost this ability due to a single residue insertion and substitution of two residues [47, 62, 89].

The 3D protein structure of fsTnC had been deciphered by X-ray crystallography for rabbit fsTnC [86], for chicken fsTnC [46] alone and as a part of the troponin complex for four Ca^{2+}-binding and two Ca^{2+}-binding states. Although the ss/cTnC isoform is expressed in slow-twitch muscle fibers, most of the studies on its 3D structure have been focused on its place and role in the human cardiac troponin complex [47].

The C-terminal globular domain of TnC is a part of the IT arm that interacts with other troponins in the supercoiled helix region (H2 TnI and H2 TnT) and H1 helix of TnI. The N-terminal and C-terminal

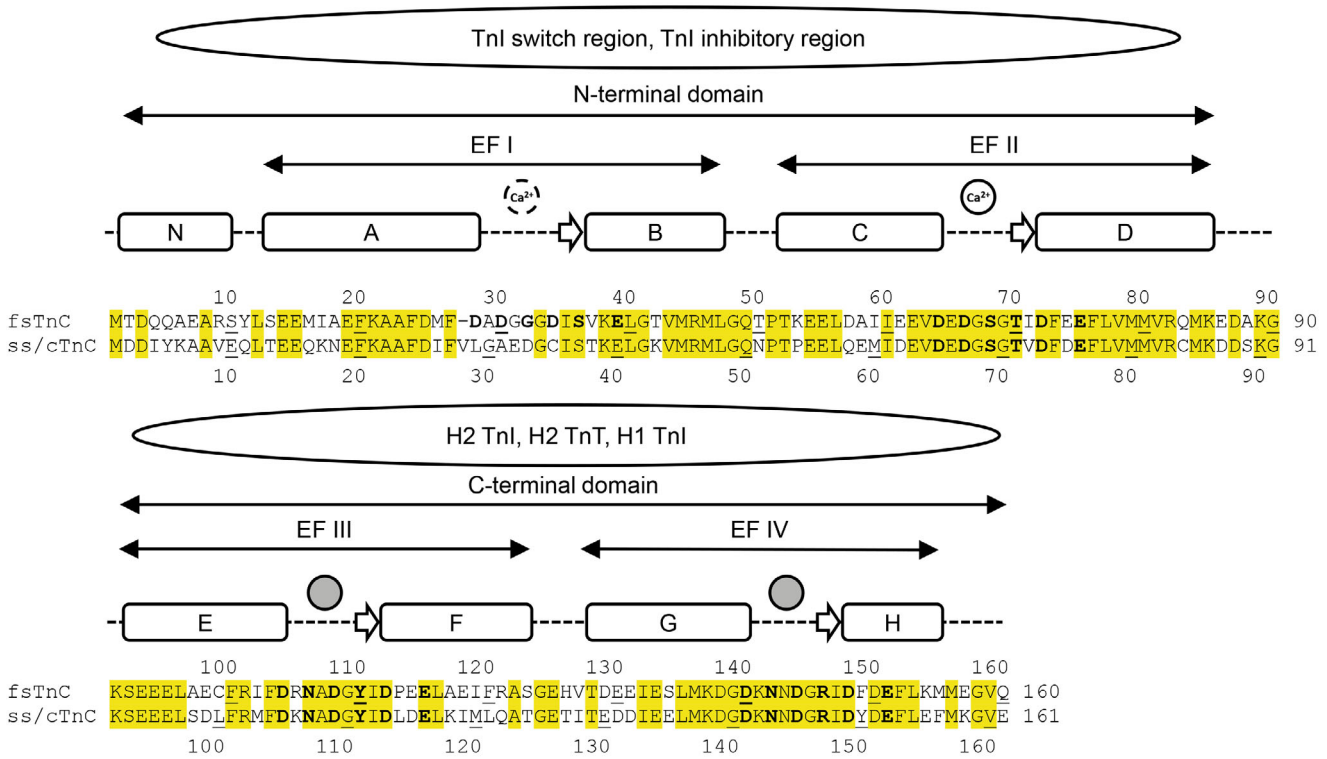

Fig. 3. Protein sequence alignment of two TnC isoforms: fsTnC and ss/cTnC. Amino acid sequences are derived from the Uniprot database: fsTnC (TNNC2_HUMAN, P02585), ss/cTnC (TNNC1_HUMAN, P63316). The identically positioned amino acid residues are colored yellow. Amino acid residues that participate in binding of Ca^{2+} and Mg^{2+} ions are marked with bold. Boxes show the borders of α-helices (N, A-H). Arrows indicate N-terminal and C-terminal domains and EF-hands. Grey circles indicate the sites of Ca^{2+} and Mg^{2+} binding, solid line circles stand for a site that is specific to binding Ca^{2+} ions in both TnC isoforms, dashed circle stands for the site of Ca^{2+} binding in fsTnC isoform (the first EF hand in ss/cTnC does not bind ions) [46, 47, 62, 86-89].

globular domains are connected by the central linker [46, 47]. Although the TnC protein C-domain is always occupied by ions, its N-domain can switch between two states: closed conformation in the absence of Ca^{2+} or open conformation initiated by Ca^{2+} binding to its EF-hand(s). Binding of Ca^{2+} ions induces formation of a hydrophobic cleft that binds to the switch region of TnI and moves the adjacent inhibitory region of TnI away from actin [87, 88].

FUNCTIONAL DIFFERENCES BETWEEN THE TROPONIN ISOFORMS

Different fibers of striated muscle – fast-twitch, slow-twitch, and cardiac – vary in their response to elevated Ca^{2+} concentrations. The slow-twitch fibers have the highest Ca^{2+} sensitivity (threshold of Ca^{2+} concentration for activation is lower), while the fast-twitch fibers have the lowest Ca^{2+} sensitivity [21, 90, 91]. The highest cooperativity of contraction (Hill coefficient) is observed in the fast-twitch fibers and the lowest in the slow-twitch fibers. Evidence suggests that these differences could be mediated by the troponin isoforms expressed in different fiber types. Replacement of ss/cTnC with fsTnC in the cardiac fibers decreases Ca^{2+} sensitivity, while substitution of fsTnC with ss/cTnC in the fast skeletal fibers increases it [90]. Replacement of the whole troponin complex in the fast-twitch skeletal fibers with cardiac troponins results in the increased sensitivity to Ca^{2+} and decreased cooperativity of contraction [92].

Decrease of intracellular pH (acidosis) leads to contractile dysfunction in all types of striated muscle and involves reduced Ca^{2+} sensitivity. In the heart muscle, reduction in intracellular pH to 6.5 and below is observed during prolonged ischemia and is caused by accumulation of metabolic products, including lactate [93, 94]. In the skeletal muscle, decrease of pH is observed during exhaustive exercise, and although it does affect skeletal muscle, the effect is not as significant as in the heart muscle. In the human skeletal muscle, pH drops down to ~6.5 after high-intensity exercise, with typical average values of ~6.8-6.9 [95-97]. As with the response to Ca^{2+}, the extent of pH decrease varies between the muscle fiber types, being most pronounced in the cardiac muscle and least prominent in the slow-twitch fibers [90, 98]. Experiments with isolated muscle fibers have shown that when pH drops from 7.0 to 6.5, the smallest decrease in Ca^{2+} sensitivity is observed in the slow-twitch muscle fibers: change in Ca^{2+} sensitivity is greater in the fast-twitch and cardiac fibers, and it is approximately equal in amplitude [90]. Moreover, further reduction in pH to 6.2 results in the strongest drop in Ca^{2+} sensitivity in the cardiac fibers, but not in the skeletal muscle.

Sensitivity of the contractile apparatus to acidosis is largely determined by the troponin complex, and magnitude of this effect is determined, among other things, by different troponin isoforms [99]. In particular, experiments performed with isolated muscle fibers have shown that replacing of fsTnC with ss/cTnC in the fast-twitch fibers leads to an increase in pH sensitivity, and, conversely, replacing of ss/cTnC with fsTnC in the slow-twitch fibers leads to a slight decrease in pH sensitivity [100]. The latter effect is even more pronounced when ss/cTnC is replaced with fsTnC in the cardiac fibers [99]. Thus, the ss/cTnC increases pH-dependence and fsTnC decreases it. It was shown in another study that combinations of TnC and TnI affect pH sensitivity [90]. The authors isolated cardiac fibers and made substitutions of TnI and TnC together and separately. In the fibers with cTnI or fsTnI, ss/cTnC accounted for the increased pH sensitivity compared to fsTnC. Moreover, replacement of cTnI with ssTnI resulted in the reduced pH sensitivity regardless of which TnC isoform was present. Thus, the authors concluded that TnC is responsible for pH sensitivity in the fast-twitch or cardiac fibers, while TnI performs that function in the slow-twitch fibers [90]. These findings are in good agreement with the fact that the neonatal cardiomyocytes expressing ssTnI are less sensitive to acidosis than the adult cardiomyocytes expressing cTnI [101]. This effect has been reproduced when cTnI was replaced with ssTnI in the adult transgenic mice: sensitivity to acidosis was reduced [102]. More recent studies have shown that the effect could be associated with the residue His131 (His131 in fsTnI, His132 in ssTnI) that is present in ssTnI and fsTnI, but is replaced with Ala163 in cTnI. This residue is localized in the switch region of TnI and participates in the interaction with TnC. It has been shown that at lower pH, electrostatic interaction between the His131 and Glu15 and Glu19 of TnC stabilizes conformation of the troponin complex, leading to activation of the muscle contraction [103-106].

Three isoforms of TnT differ in their isoelectric points, with the adult fsTnT being the most basic isoform. Moreover, there is an acidic-to-basic fsTnT splice variant shift that ensures expression of the basic splice forms in the adult muscle. These differences are due to the sequence variation in the TnT N-terminal region. There is a unique acidic fsTnT splice variant that is synthesized in some of the adult chicken muscle types [107]. These muscles show lower sensitivity to Ca^{2+} as well as higher tolerance to acidosis compared to the other muscle types that express basic fsTnT splice forms [108]. These findings are consistent with the fact that expression of fsTnT in the transgenic mouse hearts lowers tolerance of the cardiac muscle to acidosis [109].

SKELETAL TROPONINS AS BIOMARKERS OF SKELETAL MUSCLE DAMAGE

One of the most sensitive and specific approaches to detect and measure concentration of a biomarker is the sandwich immunoassay [110]. In most cases, this method is based on the use of two antibodies that recognize epitopes on the antigen surface, one of which is an immobilized capture antibody and the other is a detection antibody, conjugated with a label. To our knowledge, there have been no registered immunoassays to detect skeletal TnI isoforms and all existing studies have employed "research use only" assays.

Concentrations of the skeletal TnI isoforms in the healthy individuals have been determined in a number of studies. The mean values of basal TnI concentrations have ranged between 1.74 ± 0.27 ng/mL and 2.5 ± 0.9 ng/mL when measured by the same method in different studies [111-114]. In another study, concentrations of the skeletal TnI isoforms in the healthy individuals were below the limit of detection (LOD) (LOD = 1.2 ng/mL) [115]. In the studies where fsTnI concentration was determined, its basal concentration was below the LOD of the detection method (2.4 ng/mL) [9, 116]. Although further clarification is needed, these data suggest that the median blood levels of skeletal TnI isoforms in the healthy cohort are below 1-2 ng/mL.

In cases of skeletal muscle damage, integrity of the muscle fiber membrane is compromised, and intracellular proteins are released into the bloodstream. It has been shown that the circulating levels of skeletal TnI isoforms increase during skeletal muscle impairment of various etiologies. In particular, the skeletal TnI isoform concentrations rise in the patients with rhabdomyolysis [117, 118]; as well as after intensive physical exercises: for example, after triathlon [113], running [119], eccentric contractions [26, 120-122]; and in orthopedic and soft tissue injuries, including those resulting from surgical operations [114, 119]. Elevated concentrations of the skeletal TnI isoforms have also been observed in the patients with inflammatory myopathies such as polymyositis and dermatomyositis [115, 119, 123]. Concentration of the skeletal TnI isoforms correlates with the clinical picture in muscular dystrophies (Duchenne, Becker, and limb-girdle muscular dystrophy), and therefore it is possible to use this parameter to assess severity of the disease and monitor response to the therapy [9].

One of the potential applications of skeletal TnI isoforms as a biomarker is in determination of myotoxicity of drugs, where muscle fibers are destroyed and troponin is released into the bloodstream [36, 37, 124, 125]. Feasibility of this application has been demonstrated in rats as well as using 3D human muscle tissue *in vitro* [126, 127].

Presence of the TnI isoforms specific for different types of skeletal fibers provides additional diagnostic possibilities. As described in the previous section, a number of skeletal muscle injuries/diseases could affect a particular type of muscle fiber. For example, eccentric muscle contractions damage predominantly fast-twitch type fibers, and it is fsTnI rather than ssTnI that is released into the bloodstream [26, 121, 122]. The increased level of fsTnI, but not the level of ssTnI concentrations have also been shown for some muscular dystrophies [128].

Maximum concentration of the skeletal TnI isoforms reached values of 500 ng/mL 6 h after completing a triathlon (4 km of swimming, 120 km bike-riding, 30 km running), with a median concentration of 62.2 ± 139 ng/mL [113]. Also, high skeletal TnI isoform concentrations were reached 6 h after downhill running: the median value was 27.3 ng/mL (8.5-43 ng/mL interquartile range), while lower concentrations were found 6 h after level running: 6.6 ng/ml (3.7-9 ng/mL) and after eccentric contractions of the *quadriceps femoris* muscle: 6.8 ng/ml (3.1-14.9 ng/mL) [26]. Mean concentration levels of the skeletal TnI isoforms within 24 h of injury were: 15.3 ± 2.4 ng/mL after orthopedic injury and 10.4 ± 1.8 ng/mL after soft tissue injury [114]. Maximum concentration of the skeletal TnI isoforms in the patients with inflammatory myopathies was 516 ng/mL and the median value was 8.6 ng/mL (3.2-33.5 ng/mL interquartile range) [115].

We were not able to find any studies devoted to the utilization of skeletal TnT isoforms as possible biomarkers of muscle damage. Nevertheless, we assume that, like cardiac troponins I and T, which are markers of cardiac muscle damage, the skeletal TnT isoforms, along with the skeletal TnI isoforms, may also be a potential marker for diagnosing skeletal muscle injury.

fsTnC has also been proposed as a biomarker of skeletal muscle deterioration [129]. However, TnC recognition would have less specificity than detection of the skeletal TnI or TnT isoforms because fsTnC is expressed in the fast-twitch muscle fibers only, while c/ssTnC is expressed in the slow-twitch fibers and cardiomyocytes.

FACTORS INFLUENCING IMMUNOCHEMICAL DETECTION OF SKELETAL TROPONINS

Various factors could affect interaction of antibodies with skeletal troponins. These include homology with other troponin isoforms, presence of splice variants, post-translational modifications, proteolysis, autoantibodies binding, and shielding of epitopes by proteins of the troponin complex. Such effects could significantly distort the results of the measurements and should be taken into account when studying

the biochemical properties of the troponins and developing immunochemical systems.

Homology of isoforms. The degree of similarity between the isoforms of human TnI and TnT is ~60% for the both TnI and TnT isoforms (when comparing the longest splice variants expressed in the adults: P45378-3 for fsTnT, P13805-1 for ssTnT, and P45379-6 for cTnT) (Figs. 1 and 2). Therefore, one of the intricacies of immunochemical detection of skeletal troponins is cross-reactivity of antibodies with the cardiac isoform of the protein or with the second skeletal isoform (in the case of differential detection of slow- or fast-twitch skeletal troponin), and this could reduce specificity of detection of the target biomarker. High degree of troponin homology significantly reduces the number of epitopes to which antibodies could be generated for the specific detection of individual protein isoforms. The most conserved sites for both TnI and TnT are located in the C-terminal parts of these proteins, while the most variable sites are in their N-terminal portions.

Alternative splicing of TnT. As described above, the skeletal TnT isoforms, especially fsTnT, are represented in the human muscle by several splice variants. To develop immunodetection test systems that would recognize all possible splice forms of fsTnT, antibodies specific to the domains encoded by exons 1-3 and 9-15 should be utilized. For the fsTnT, antibodies specific to the N-terminal residues 11-38 as well as the C-terminal region (residues 231-244 that may differ between splice variants) should be avoided (residues numbering is indicated for the sequence accession number P45378-3). In the case of ssTnT, antibodies recognizing residues 25-35 and 205-220 (numbering is for the longest splice variant; sequence accession number P13805-1), should be avoided because these sequences may differ between the splice variants.

TnI and TnT phosphorylation. Phosphorylation is a modification that changes charge of a protein and could have significant effect on the antibody-antigen interaction.

TnI phosphorylation. The rabbit fsTnI could be phosphorylated by phosphorylase kinase, 3′,5′-cAMP-dependent protein kinase, Ca^{2+}-phospholipid-dependent protein kinase, and the human fsTnI by AMP-activated protein kinase *in vitro* [130-134]. The residues that undergo phosphorylation have been identified as Thr12 and Ser118 for the rabbit fsTnI and Ser118 for the human protein [131, 132, 134]. Presence of the Thr12 residue in the human fsTnI sequence as well as in the rabbit fsTnI provides the possibility of its phosphorylation in the human protein as well. It has been shown that phosphorylation *in vitro* is inhibited by interaction with TnC, and this is consistent with the data on the structure of troponin complex: both of these residues are located in the sites of interaction with TnC [46, 130]. Phosphorylation of ssTnI has not been described *in vitro*, but there is one residue homologous to the fsTnI phosphorylation site in the ssTnI sequence, namely Ser118 (ssTnI) (Fig. 1).

The fsTnI isolated from the rabbit skeletal muscle is present in the partially phosphorylated form, and the degree of phosphorylation could depend on the method of protein isolation [130, 131, 135]. The fsTnI from the human muscle extract (*vastus lateralis* and *tibialis anterior*) and rat muscle extract (7 muscle types) analyzed by mass spectrometry were found to be in the non-phosphorylated form, whereas the ssTnI was present in both phosphorylated and non-phosphorylated forms [78, 80]. No phosphorylated forms of the skeletal TnI isoforms were detected by mass spectrometry of the rhesus macaque muscle extract [79]. Based on the above data, it could be expected that the proportion of phosphorylated forms of skeletal TnI isoforms in blood would be negligible and, therefore, would not affect their recognition by antibodies.

TnT phosphorylation. Using *in vitro* experimentation, the fsTnT isolated from the rabbit skeletal muscle has been shown to be a substrate for various kinases including phosphorylase kinase, 3′,5′-cAMP-dependent protein kinase, casein kinase 2, and Ca^{2+}-phospholipid-dependent protein kinase [59, 130, 132, 133, 136-140]. Three phosphorylation sites have been identified for the rabbit fsTnT: Ser2, Ser152, and Ser159 (numbers of residues are indicated for Uniprot accession number P45372-3). Ser2 is also represented in the human fsTnT, while the Ser152 and Ser159 residues are in composition of the Ala-Leu-Ser-Ser-SerP-Met-Gly-Ala-Asn-Tyr-Ser-SerP-Tyr sequence, which in the rabbit fsTnT is completely consistent with the human protein. This suggests the possibility of phosphorylation of these residues in the human fsTnT as well [132].

Meanwhile, in a series of *in vivo* studies, fsTnT isolated from the rabbit skeletal muscle has been shown to be present in a solely monophosphorylated form [130, 132]. Moreover, phosphorylation occurred at only one of the three residues identified *in vitro*: Ser2. These results are consistent with the mass spectrometry data for the proteins extracted from the muscle tissue of various species. For human, rat, and rhesus macaque fsTnT samples isolated from several muscle types, unphosphorylated and monophosphorylated forms (present in different splice variants) of fsTnT were identified by mass spectrometry [78-80] and Ser2 was the only site of phosphorylation determined *in vivo*.

There are no data on phosphorylation of the ssTnT *in vitro*. However, ssTnT contains Ser2 homologous to the Ser2 in the fsTnT and cTnT, for which phosphorylation has been shown both *in vitro* and *in vivo* [141, 142]. High probability of the ssTnT phosphorylation

at Ser2 in the human muscle tissue was confirmed by mass spectrometry [80]. Using this method, it was found that in the human and rat skeletal muscle, ssTnT was presented in a predominantly phosphorylated form, and in the rhesus macaque muscle in both phosphorylated and non-phosphorylated forms [78-80].

It could be assumed that fsTnT and ssTnT are released into the bloodstream in a partially phosphorylated form, but phosphorylation site is located in the terminal part of the protein and, therefore, could be presumed not to affect immunochemical detection by antibodies.

TnI glutathionylation. Lamb et al. showed in a series of studies on isolated rat and human muscle fibers that fsTnI, but not ssTnI, could undergo glutathionylation [143-145]. This modification occurred at one of the cysteine residues, Cys134. This is consistent with the mass spectrometry data for the protein: two proteoforms of fsTnI, with and without glutathione, were detected in the skeletal muscles of rhesus macaque and rat, although, abundance of the glutathionylated form was quite low [78, 79].

TnI and TnT proteolysis. N-terminal and C-terminal proteolysis has been demonstrated for cTnI: various proteolyzed forms of cTnI were observed in both necrotic cardiac tissue and in blood [146, 147]. cTnI was found to be a substrate of a number of proteases: metalloproteinase-2, calpain 1 (μ-calpain) and calpain 2 (m-calpain) [148-151]. Studies of the cTnI proteolysis in blood of the patients with myocardial infarction showed presence of at least 11 proteolytic fragments of different lengths [147, 152]. Due to the sequence homology to cTnI, proteolysis of skeletal TnI isoforms is also possible. The proteolyzed fsTnI and ssTnI and their fragments have been identified in the blood of patients, and amount of these fragments in the blood varies depending on the type of injury/disease [118]. Based on these results, the authors suggested that proteolysis occurs directly in the tissue and that such proteolyzed forms are released into the bloodstream. Further studies on proteolysis of the skeletal TnI isoforms and localization of the sites of proteolysis are needed.

cTnT also undergoes proteolysis. In the apoptotic cardiomyocytes, N-terminal cleavage of cTnT by caspase-3 with formation of a 25-kDa fragment has been demonstrated [153]. cTnT is also prone to limited N-terminal proteolysis by calpain 1 (μ-calpain) during myocardial ischemia-reperfusion [148, 154]. In the blood of patients with myocardial infarction, at least 23 cTnT fragments of different lengths and carrying several sites of proteolysis in the N-terminal, central, and C-terminal parts of the molecule were found [155]. Similar studies have not yet been performed for the skeletal TnT isoforms. For fsTnT, proteolysis has been demonstrated only in the muscles after vigorous exercise [156]. Further investigations are required to identify the forms of skeletal TnT isoforms present in the bloodstream and to localize their sites of proteolysis.

Shielding of epitopes in the troponin complex. cTnI and cTnT are present in the blood of patients with myocardial infarction as part of a ternary troponin complex (TnI–TnT–TnC), a binary troponin complex (TnI–TnC), and as proteolytic fragments of TnT [152, 157, 158]. Although there is no experimental data describing the form in which skeletal troponins are released into the blood, it can be assumed that they would also be present in the samples as complexes. For immunochemical detection, it is important to use antibodies that recognize troponin epitopes that are present in all forms that exist in blood. The skeletal TnT isoforms could be covered and shielded from detection by TnI and TnC at their binding sites, limited to approximately 197-241 residues for fsTnT. In the case of the skeletal TnI isoforms, a significant portion of the molecules could be shielded from detection at binding sites located at approximately 2-40, 50-106, and 116-131 residues for fsTnI, and 2-40, 51-107, and 117-132 residues for ssTnI. Thus, small regions in the central and C-terminal part of TnI molecule could remain accessible to antibodies. In order to uncover the epitopes shielded by other troponins in the central part of TnI, an alternative method could be to dissociate the troponin complex using EDTA [115, 116, 157].

Autoantibodies binding. One of the factors influencing the immunochemical detection of cardiac troponins is the presence of autoantibodies that interact with the analyzed protein in the blood. They might interfere with the antibodies in composition of the immunochemical test systems for detection of the protein and lead to false negative results. It has been demonstrated that approximately 10% of the healthy individuals have autoantibodies to cTnI in their blood [159]. These immunoglobulins have been shown to be primarily specific for the conformational epitopes that are formed when TnI and TnT combine into a complex [160]. There are no studies on the presence of autoantibodies interacting with the skeletal isoforms of TnI; however, considering high degree of homology, it can be assumed that they could also be present in the blood of some patients.

Finally, we examined the sites that could be most susceptible to various factors interfering with recognition of troponins by antibodies. The C-terminal regions of TnI could be associated with the cross-reactivity displayed by antibodies to other TnI isoforms, while the central parts of the molecule could be shielded by TnC or TnT. The effect of posttranslational modifications such as phosphorylation and glutathionylation is considered to be negligible, while the role and extent of proteolysis and influence of autoantibodies needs

further investigation. Taking into account the above factors, the most promising sites for the detection of skeletal TnI isoforms are central parts of the molecule, especially those that are not shielded by other troponins.

In the case of TnT, the N-terminal (11-38 residues for fsTnT, 25-35 residues for ssTnT) and C-terminal (231-244 residues for fsTnT and 205-220 residues for ssTnT) parts of the molecule are alternatively spliced, while the C-terminal parts (~197-241 residues for fsTnT) could be screened by TnI and TnC. The effect of phosphorylation is considered to be negligible, while the role and extent of N- and C-terminal proteolysis needs further investigation. Therefore, central parts of the skeletal TnT isoforms could be considered as the most promising sites for their immunochemical detection.

MUTATIONS IN TROPONINS AS CAUSES OF SKELETAL MUSCLE PATHOLOGIES

The troponin complex is of high importance for regulation of muscle contraction. Mutations could lead to the changes in protein structure and functionality. To date, a number of mutations have been identified that are associated with the development of such skeletal muscle diseases as arthrogryposis and congenital myopathies.

Mutations in the skeletal TnI genes. Mutations in the *TNNI2* gene (fsTnI) have been demonstrated to be one of the causes of distal arthrogryposis. This is an autosomal dominant disease, in which joints are affected and mobility of distal limbs is reduced. The *TNNI2* mutations associated with distal arthrogryposis are present in the following locations within the C-terminal region of the molecule: R156X [leading to expression of a truncated form of fsTnI (1-156)], R162G, R162K, I165F, E167X, K168E, R174Q, R174W, K175N, K175X, K176X, F178C, and F178L [161-172].

For ssTnI (*TNNI1*), two pathogenic mutations have been discovered to date [170, 173]. One is located in the same region as mutations in the fsTnI and is associated with the development of proximal arthrogryposis, an autosomal dominant disease in which proximal joints are affected. This mutation, K175X, results in the expression of a truncated form of ssTnI [170]. Mutation in the N-terminal region of ssTnI, R37C, is associated with expression of ssTnI in the heart for up to two years after birth [173] that increases the risk of sudden infant death.

Mutations in the skeletal TnT genes. Mutations in the *TNNT3* gene (fsTnT) lead to the development of distal arthrogryposis. For *TNNT3*, several substitutions affecting the same residue have been found: R66C, R66H, and R66S [171, 174-177][2]. It is also worth highlighting the *TNNT3* (fsTnT) mutation, which causes two diseases simultaneously: the aforementioned distal arthrogryposis and the nemaline myopathy that affects fast-twitch muscle fibers [178]. It is a mutation in an intron that leads to the impaired splicing and decreased expression of fsTnT, with compensatory hypertrophy of slow-twitch muscle fibers being observed.

Deletions in the *TNNT1* gene (ssTnT) are associated with nemaline myopathy inherited in an autosomal recessive pattern. This disease is manifested in early childhood as a respiratory failure, slow-twitch muscle fiber atrophy, and compensatory hypertrophy of fast-twitch muscle fibers. Ultimately, nemaline myopathy leads to death in childhood from the respiratory failure. E180X (expression of the truncated form of ssTnT 1-179), S108X (expression of the truncated form of ssTnT 1-107), deletion of the exons 8 and 14, and expression of the truncated form of ssTnT (1-203) have been found [179-182]. These deletions affect tropomyosin binding sites as well as TnI and TnC binding sites.

Absence of the troponin and tropomyosin binding sites in ssTnT after E180X deletion impedes formation of the troponin complex and its incorporation into thin filaments [183]. While the ssTnT (1-179) mRNA is still detectable in muscle fibers, the ssTnT (1-179) protein molecule degrades rapidly and can no longer be detected [184]. This fact explains recessive nature of the mutation: one copy of the gene is sufficient for expression of the full-length ssTnT and its incorporation into the troponin complex, while expression of the mutant ssTnT (1-179) protein is not cytotoxic, as the truncated ssTnT is immediately degraded [185].

Mutations in the TnC genes. Mutations in the *TNNC2* gene (fsTnC) are associated with congenital myopathy inherited in an autosomal dominant pattern. These mutations comprise the D34Y and M79Y substitutions, which are located in the EF-hand I and near EF-hand II, respectively. They affect Ca^{2+} binding and interaction with TnI switch region, so there is reduced Ca^{2+} sensitivity in the presence of these mutations [186, 187].

Since ss/cTnC is expressed in the slow-twitch skeletal muscle as well as in the cardiac muscle, mutations in TNNC1 (ss/cTnS) are associated with cardiomy-

[2] To specify this mutation, the authors of the studies used the fsTnT sequence corresponding to UniProt accession number P45378-2, which is three residues shorter than the P45378-3 sequence used in this review. Therefore, this substitution appears in the articles as R63.

opathies and cardiac dysfunction. These genetic alterations include missense mutations A8V, L29Q, A31S, C84Y, E134D, and D145E, and frameshift mutations Q122AfsX30 [188-191]. Along with cardiomyopathies, some of the patients carrying these mutations also exhibit skeletal muscle abnormalities, but more research is needed to understand which mutations affect skeletal muscle and exact mechanism involved in the process [187, 192]. It was shown in the *in vitro* experiments that all of the abovementioned mutations except for C84Y had no effect on phenotype and function of the slow-twitch skeletal muscle fibers, while the C84Y mutation increased Ca^{2+} sensitivity, as it does in the cardiac muscle fibers. These differences in the effects of the ss/cTnC mutations on slow-twitch and cardiac muscle fibers could be due to the fact that in the slow-twitch muscle fibers ss/cTnC forms complex with the slow-twitch skeletal but not cardiac isoforms of TnT and TnI [193].

TROPONINS AS DRUG TARGETS

In addition to being the source of pathologies, skeletal troponins could also serve as drug targets used in treatment of skeletal muscle diseases. These include peripheral motor neuropathies – amyotrophic lateral sclerosis (ALS), spinal muscle atrophy, Charcot–Marie–Tooth disease – in which the motor neurons are damaged, and nerve impulse transmission is impaired, and also myasthenia gravis, in which the neuromuscular junction is impaired. Disruption of the skeletal muscle innervation ultimately leads to muscle weakness, disability, and high mortality. One of the therapeutic approaches for these pathologies is to increase sensitivity of the muscle fibers to Ca^{2+}, so that sufficient muscle activation is achieved even at low Ca^{2+} concentrations. A molecule from the group of first fast skeletal troponin activators, CK-2017357 (tirasemtiv), selectively interacts with the fast skeletal troponin complex: binding occurs in the hydrophobic pocket formed by the N-terminal domain of fsTnC and the switch region of fsTnI [194]. Tirasemtiv binding has been shown to increase the degree of affinity of low-affinity Ca^{2+} binding sites on fsTnC, thereby increasing sensitivity of the fsTnC to Ca^{2+} [195]. Tirasemtiv has shown promising results in the patients with ALS, patients with myasthenia gravis, and studies even reached phase III clinical trials, but were halted due to the discovery of side effects [196-202]. Today, other drugs from the group of fast skeletal muscle troponin activators, namely CK-2127107, CK-2066260, and reldesemtiv are under development [203-206]. Reldesemtiv, a second-generation molecule that was derived by optimization of tirasemtiv and has similar mechanism of action, has been most successful [207, 208]. Reldesemtiv has reached phase II clinical trials in spinal muscular atrophy and phase III in ALS, but, unfortunately, trials on the patients with ALS were stopped due to the lack of therapeutic success [206, 209, 210]. Although the ss/cTnC is expressed in both slow-twitch skeletal and cardiac muscles, regulators of the ss/cTnC Ca^{2+} sensitivity have been studied primarily in the context of modulating heart function. These drug molecules regulate Ca^{2+} sensitivity of muscle contraction by adjusting the Ca^{2+}-binding affinity of the N-terminal domain of ss/cTnC. One of the most widely studied calcium sensitizers, levosimendan, binds to the hydrophobic pocket formed by the N-terminal domain of ss/cTnC and the switch region of cTnI – in a manner similar to that of tirasemtiv [211]. Levosimendan is approved for use in some countries, and its analogs are intended to be used for treatment of the heart diseases associated with depressed cardiac function.

In contrast to the above drug molecules, the troponin inhibitor W7 acts by decreasing Ca^{2+} sensitivity, and it could be used to treat contractile dysfunction arising from the alkalosis-induced and inherited cardiomyopathies [212, 213]. W7 also binds to the hydrophobic pocket between the N-terminal domain of ss/cTnC and the cTnI switch region, but, unlike levosimendan, it shifts the cTnI switch region from its binding site, which leads to the reduced Ca^{2+} sensitivity [213, 214].

CONCLUSION

Skeletal muscle dysfunction has a negative impact on the whole-body functioning and could be caused by mechanical muscle injury, myopathies, and other diseases accompanied by muscle atrophy, as well as by rhabdomyolysis. Determination of the concentration of protein biomarkers released into the bloodstream when muscle fibers are damaged is a convenient method of diagnosing and monitoring such pathologies. Skeletal troponins are potential candidates to be used as biomarkers of skeletal muscle dysfunction including various myopathies, traumas, and injuries caused by vigorous physical exercise, as well as for assessing severity of muscular dystrophies and determining myotoxicity of the drugs.

Structure and properties of troponins could affect accuracy of their antibody-based detection in human blood. Main factors influencing immunochemical detection of these proteins in clinical samples are isoform homology, alternative splicing of TnT, post-translational modifications (phosphorylation and glutathionylation), proteolysis, shielding of TnI and TnT epitopes within the complex, and autoantibodies binding. Therefore, a careful selection of monoclonal

antibodies is required for the development of immunoassays capable of reliable quantitative detection of these proteins.

Certain mutations in skeletal troponins are associated with the development of arthrogryposis and congenital myopathies. Furthermore, skeletal troponins could be used as drug targets for treatment of muscle diseases such as peripheral motor neuropathies and myasthenia gravis.

Contributions. A.P.B. and I.A.K. collected and analyzed the data, prepared illustrations, wrote the manuscript, edited the manuscript; I.A.K. conceived and supervised the study.

Funding. This work was supported by ongoing institutional funding. No additional grants to carry out or direct this particular research were obtained.

Ethics declarations. This work does not contain any studies involving human and animal subjects. The authors of this work declare that they have no conflicts of interest.

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