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DEVELOPMENT OF THE IMMUNOCHEMICAL SYSTEMS FOR SPECIFIC DETECTION OF DIFFERENT FORMS OF HUMAN SKELETAL TROPONIN I

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INTRODUCTION

Troponin I (TnI) together with troponin T (TnT) and troponin C (TnC) forms a troponin complex that regulates Ca^{2+}-dependent muscle contraction. In humans, TnI is presented by cardiac (cTnI) isoform that is expressed only in the myocardium, and fast skeletal (fsTnI) and slow skeletal (ssTnI) isoforms that are expressed in fast-twitch and slow-twitch muscle fibers, respectively. Skeletal TnIs could be used as markers of various skeletal muscle pathologies: mechanical traumas, inherited and secondary myopathies, muscle atrophy, and rhabdomyolysis. In contrast to commonly used biomarkers (such as creatine kinase and myoglobin), which are also expressed in various tissues in addition to skeletal muscle, skeletal TnIs are specific for skeletal muscles. The goal of this study was to design methods of specific and sensitive immunochemical detection of skeletal TnIs in various applications.

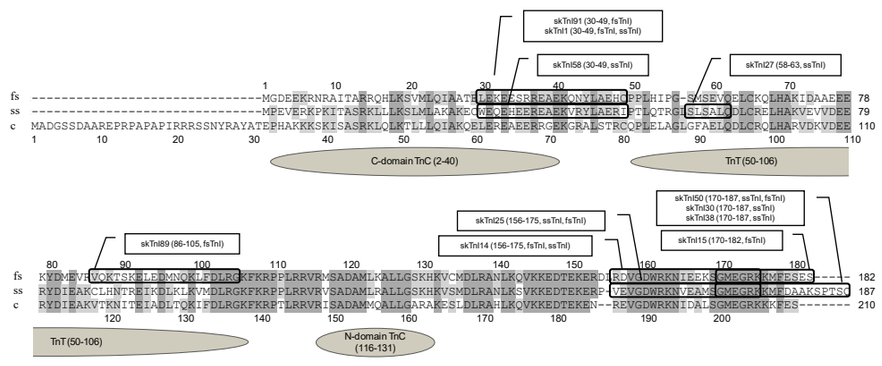


Figure 1. Alignment of three isoforms of human TnI.
Identical amino acid residues are marked in gray. Epitopes of the antibodies are marked with rectangles on the sequence, while the names of the antibodies are indicated in footnotes. Potential TnC-binding sites and TnT-binding sites are marked as ovals.

cTnI exists in the blood of patients not as a free protein but in association with other troponins: TnC (binary IC complex) and with TnT and TnC (ternary ITC complex). It is still unclear as to whether skeletal TnIs exist in the blood of patients as free proteins or in complexes.

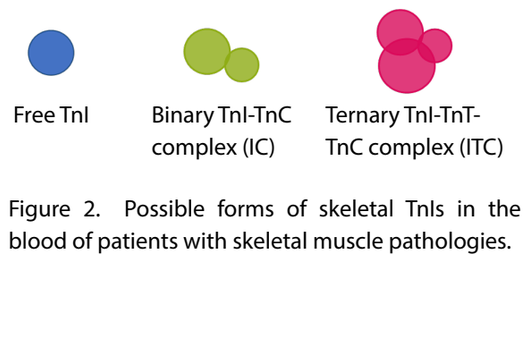


Figure 2. Possible forms of skeletal TnIs in the blood of patients with skeletal muscle pathologies.

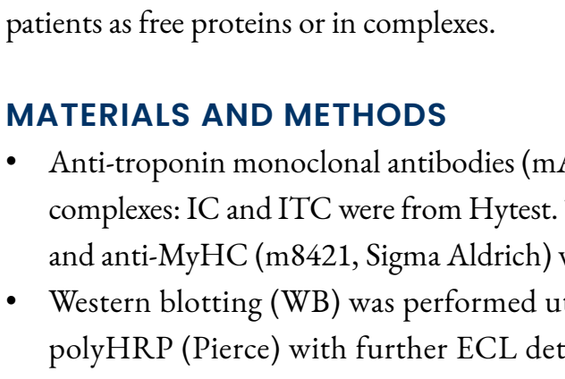
MATERIALS AND METHODS

- Anti-troponin monoclonal antibodies (mAbs), recombinant fsTnI, ssTnI, and troponin complexes: IC and ITC were from Hytest. The mAbs Anti-MyHC1 (ab127539, Abcam) and anti-MyHC (m8421, Sigma Aldrich) were used.
- Western blotting (WB) was performed utilizing biotinylated mAbs and streptavidin-polyHRP (Pierce) with further ECL detection (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Fisher).
- Immunohistochemistry (IHC) was performed according to a standard protocol. Human tissue samples were collected in agreement with the local ethics committee.
- Two-step sandwich fluoroimmunoassays (FIAs) with Eu-labeled specific mAbs for detection were used.
- Gel filtration (GF) studies were performed on the AKTA pure chromatography system (GE Healthcare) with HiLoad Superdex 200 PG 16/60 column (GE Healthcare).

RESULTS

Detection of skeletal TnIs by WB

We selected mAbs, specific to fsTnI (skTnI89), ssTnI (skTnI38) and to both skeletal TnIs (skTnI50) with high specificity and sensitivity.



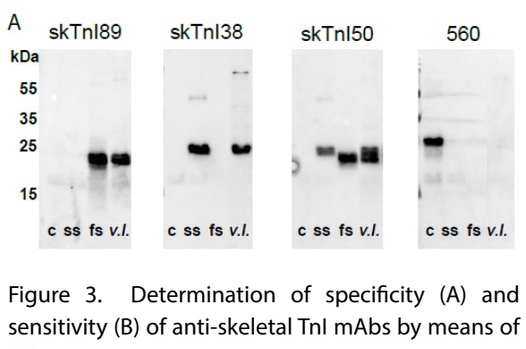


Figure 3. Determination of specificity (A) and sensitivity (B) of anti-skeletal TnI mAbs by means of WB.
mAbs: *skTnI89; skTnI38; skTnI50; 560 (anti-cTnI)*
Antigens: *cITC (c), ssITC (ss), fsITC (fs), human m. vastus lateralis extract (v.l.).*

Detection of skeletal TnIs by IHC

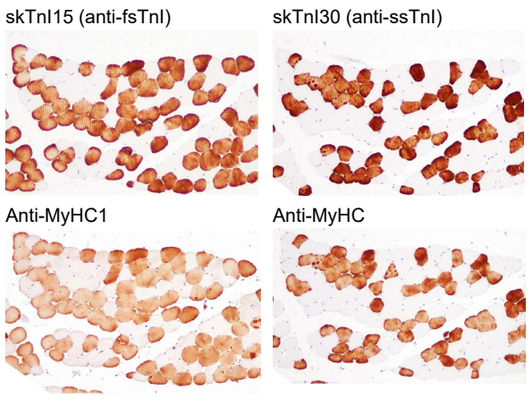
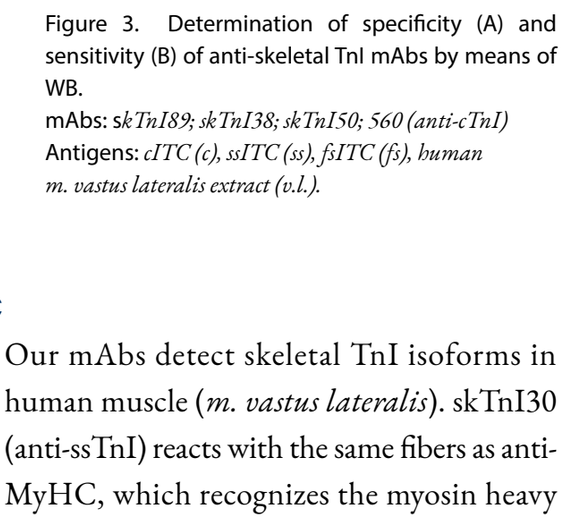


Figure 4. Skeletal TnIs and MHC detection in human *m. vastus lateralis*.
mAbs: *skTnI15; anti-MyHC1; skTnI30; anti-MyHC.*

ssTnI is synthesized in the human heart during the prenatal period and is replaced by cTnI in the postnatal period. While skTnI30 detects cardiomyocytes in fetal hearts, it is unable to do so in adult hearts.

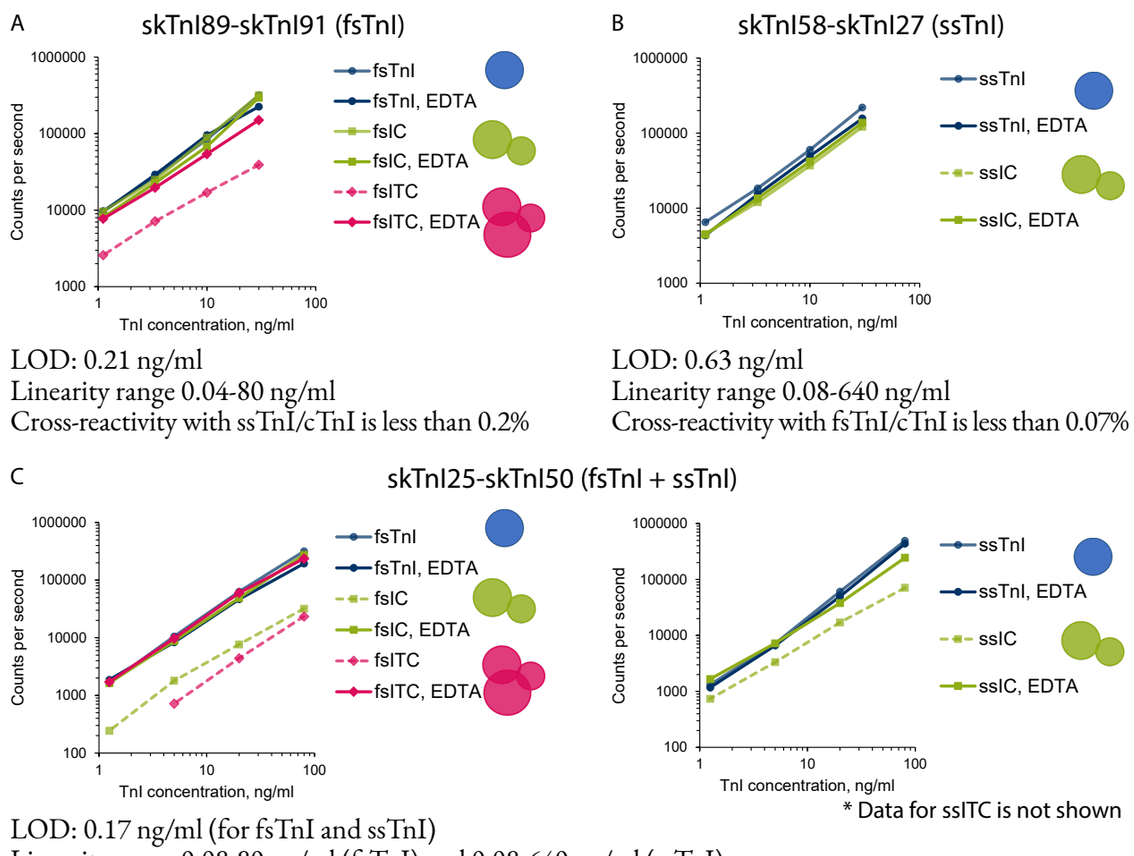
Figure 5. sTnIs detection in skeletal muscle and fetal/adult human hearts.
mAbs: *skTnI30; 4C2 (anti-cTnI)*
Tissues: *adult m. vastus lateralis; 28-week fetal heart; 41/42-week fetal heart; adult heart.*

Our mAbs detect skeletal TnI isoforms in human muscle (*m. vastus lateralis*). skTnI30 (anti-ssTnI) reacts with the same fibers as anti-MyHC, which recognizes the myosin heavy chain (MHC) isoform specific to slow-twitch muscle fibers. skTnI15 (anti-fsTnI) reacts with the same fibers as anti-MyHC1, which recognizes the MHC isoform specific to fast-twitch muscle fibers. The mAbs skTnI15 and skTnI30 could be used to specifically detect fast-twitch and slow-twitch muscle fibers, respectively.



Detection of skeletal TnIs by FIA

We developed FIAs to measure skeletal TnIs in the blood samples of patients: fsTnI, ssTnI, or both skeletal TnIs simultaneously.
FIAs were designed to measure all possible forms of skeletal TnIs: free form and complexed forms (IC and ITC). As some epitopes could be shielded by TnC/TnT, EDTA was added. Complexes dissociated in the presence of EDTA and epitopes became opened to ensure the better recognition by mAbs.



LOD: 0.21 ng/ml
Linearity range 0.04-80 ng/ml
Cross-reactivity with ssTnI/cTnI is less than 0.2%

LOD: 0.63 ng/ml
Linearity range 0.08-640 ng/ml
Cross-reactivity with fsTnI/cTnI is less than 0.07%

* Data for ssITC is not shown

LOD: 0.17 ng/ml (for fsTnI and ssTnI)
Linearity range 0.08-80 ng/ml (fsTnI) and 0.08-640 ng/ml (ssTnI)
Cross-reactivity with cTnI is less than 0.02%

Figure 6. Sandwich FIAs for the detection of skeletal TnIs: fsTnI (A), ssTnI (B), and both skeletal TnIs (C).

Development of skeletal TnI assays for the differential detection of TnI forms

skTnI89-skTnI1 recognizes fsTnI and fsIC, cross-reactivity with fsITC was ~4%; skTnI14-TnC99A5 (TnC99A5 is specific to TnC in fsITC) predominantly recognizes fsITC, cross-reactivity with fsTnI was ~4%, with fsIC was ~37%.

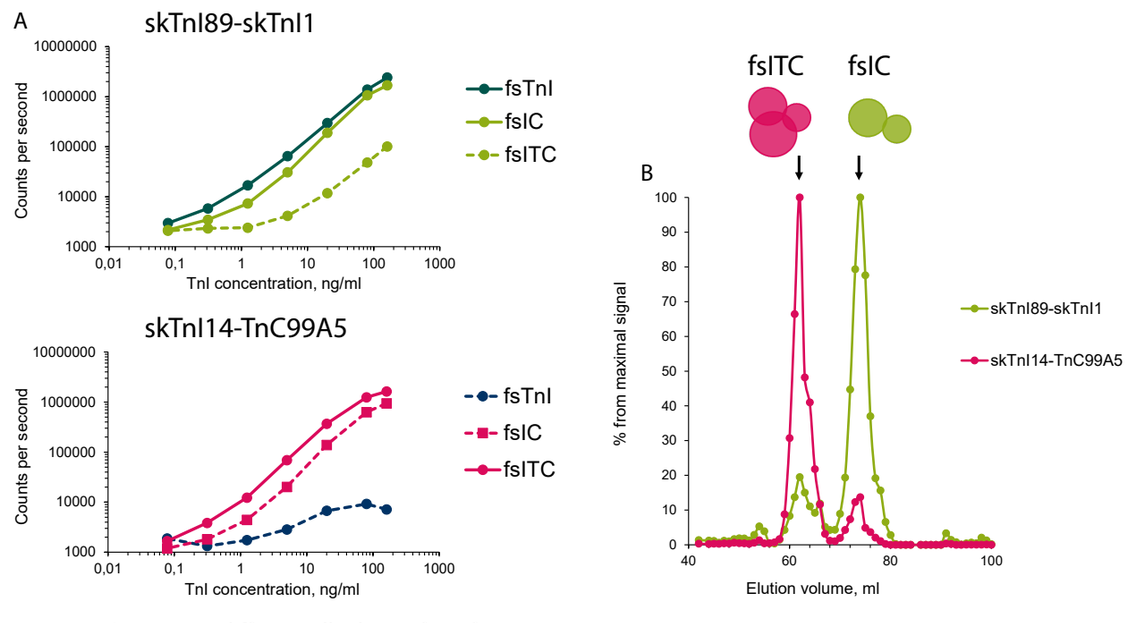


Figure 7. Systems to differentially detect fsTnI forms.
A – sandwich FIAs, B – GF profile of m. vastus lateralis extract, analyzed by FIAs.

TnT111-skTnI38 (TnT111 is specific to TnT) that recognizes ssITC only. skTnI58-skTnI27 detects all ssTnI forms.

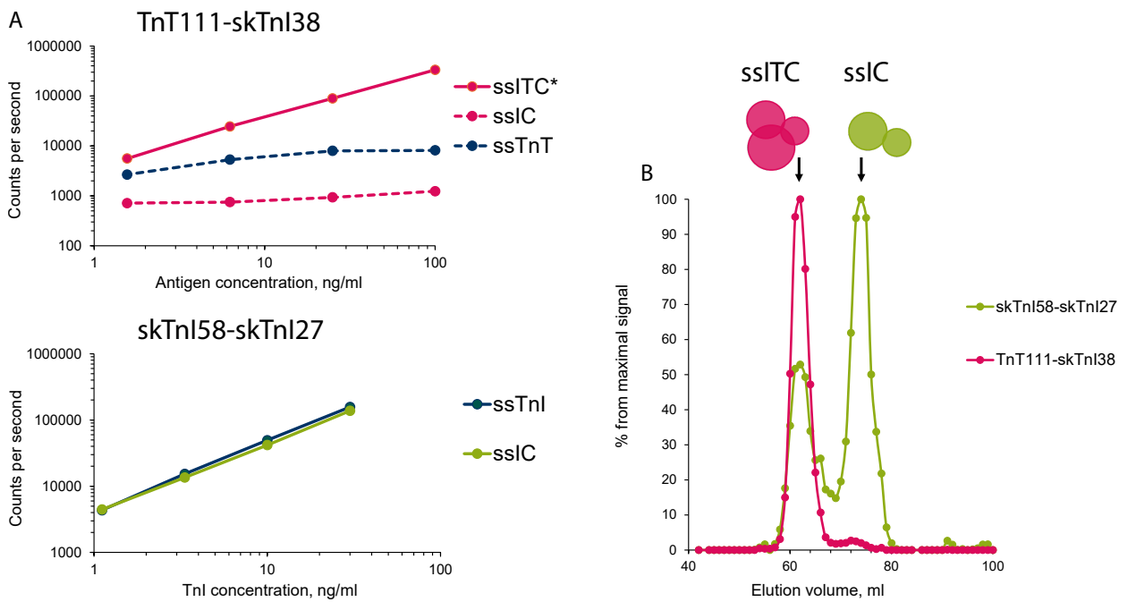


Figure 8. Systems to differentially detect ssTnI forms.
A – sandwich FIAs, B – GF profile of m. vastus lateralis extract, analyzed by FIAs.
**ssITC is from m. vastus lateralis.*

CONCLUSIONS

- mAbs could be used in Western blotting for the specific and sensitive detection of fsTnI, ssTnI, and both skeletal TnIs.
- mAbs specific to fsTnI and ssTnI could be used in immuhistochemistry to differentiate fast-twitch and slow-twitch muscle fibers.
- We developed fluoroimmunoassays that detect both isoforms of skeletal TnIs with no cross-reactivity with cTnI and assays that only detect ssTnI or fsTnI. These systems recognize skeletal TnIs in both free and complexed forms and are suitable for the detection of skeletal TnIs in the blood of patients.
- We developed methods to differentially detect forms of fsTnI/ssTnI for the further investigation of forms of skeletal TnIs present in human blood.