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Research paper

Novel clearance of muscle proteins by muscle cells

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ABSTRACT

Blood levels of cardiac troponins (cTn) and myoglobin are analysed when myocardial infarction (MI) is suspected. Here we describe a novel clearance mechanism for muscle proteins by muscle cells. The complete plasma clearance profile of cTn and myoglobin was followed in rats after intravenous or intermuscular injections and analysed by PET and fluorescence microscopy of muscle biopsies and muscle cells. Compared with intravenous injections, only 5 % of cTnT, 0.6 % of cTnI and 8 % of myoglobin were recovered in the circulation following intramuscular injection. In contrast, 47 % of the renal filtration marker FITC-sinistrin and 81 % of cTn fragments from MI-patients were recovered after intramuscular injection. In addition, PET and biopsy analysis revealed that cTn was taken up by the quadriceps muscle and both cTn and myoglobin were endocytosed by cultured muscle cells. This local clearance mechanism could possibly be the dominant clearance mechanism for cTn, myoglobin and other muscle damage biomarkers released by muscle cells.

1. Introduction

Cardiac troponins (cTn), troponin T (cTnT) and troponin I (cTnI), are cardiac-specific proteins that bind to thin filaments within the cardiomyocyte sarcomere and cooperate with troponin C (TnC) to make muscular contraction dependent on calcium (Filatov et al., 1999). cTnT and cTnI are released into the circulation following cardiac damage (Starnberg et al., 2014) and are now the preferred diagnostic biomarkers when myocardial infarction (MI) is suspected (Thygesen et al., 2019).

Diagnosing a chest pain patient as having MI often relies on the patients' cTn levels. If the cTn level is elevated, further testing is needed to examine whether the elevation is stable or dynamic (Bjurman et al., 2017). Stable cTn levels are often used to exclude MI since it is thought that stable cTn elevations are not due to acute cardiac damage (Chapman et al., 2018, 2017b; Sandoval et al., 2017).

Over a third of older patients in emergency departments have stable cTn elevations without an obvious connection to MI or other cardiac damage (Hammarsten et al., 2012; Shah et al., 2018). These patients constitute a significant health care problem since stable elevation of cTn is one of the strongest known risk factors for death and heart disease (Carlsson et al., 2017; Chapman et al., 2017a; Roos et al., 2017a).

After MI has been excluded, patients with stable cTn elevations are often left with information of future risks but without treatment (Roos et al., 2017b), as we still do not understand the pathophysiological mechanisms that link stable cTn elevations to mortality (Hammarsten et al., 2018).

One possibility is that a stable cTn elevation is due to decreased clearance (Friden et al., 2017). Surprisingly, little is known about how cTn, myoglobin and other muscle damage biomarkers are cleared from the circulation. cTn and myoglobin are cleared, in part, by the kidneys

Abbreviations: cTnI, Cardiac Troponin I; cTnT, Cardiac Troponin T; hs-cTnI, high-sensitivity Troponin I; hs-cTnT, high-sensitivity cardiac Troponin T; Myo, Myoglobin.

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(Friden et al., 2017), and are often elevated in patients with low kidney function (Bjurman et al., 2015; Hallgren et al., 1978; Wodzig et al., 1997; Ziebig et al., 2003). However, circumstantial evidence suggests that other clearance systems, like receptor-mediated endocytosis and degradation in the liver (Muslimovic et al., 2020), are involved (Friden et al., 2017; Smit et al., 1988, 1987).

During our studies of cTn clearance we found that very little cTn and other muscle proteins emerged in the circulation after injection into the rat quadriceps muscle. Here, we have examined the local clearance of cTn and myoglobin in muscles in greater detail.

2. Material and methods

2.1. Animals

Male Sprague Dawley rats (Taconic, Denmark) kept on standard fodder and with free access to water, were used, unless otherwise stated. Anesthesia was induced and maintained by inhalation of isoflurane (Isobavet, Schering-Plough Animal Health, Buckinghamshire, UK; 4.5 % induction and 3.2–3.9 % maintenance), using the Univentor 400 anesthesia unit (AgnTho's AB, Lidingö, Sweden).

2.2. Ethical standards

All protocols and procedures involving animal experiments were in accordance with the guidelines in Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes and approved by the Regional Board for Ethical Review of research projects using animals in Gothenburg, appointed by the Swedish Ministry of Agriculture, (Ethical Approval # 282-2012).

2.3. Cell maintenance

Mouse muscle C2C12 cells (ATCC) were cultured in a Dulbecco's Modified Eagle's Medium (DMEM) with High Glucose (Thermo Fisher Scientific) supplemented with 10 % fetal bovine serum or 10 % horse serum, 1 % penicillin/streptomycin. The cells were detached with trypsin-EDTA 0.25 % for five minutes and passaged twice a week. For confocal microscopy imaging, the cells were plated on 8-well chamber slides (ibi-treat 8-well μ-slides from ibidi, LRI instruments Inc.) with 5.0×10^3 viable cells/cm^2 and cultured for 5−10 days prior to the experiment with medium change twice a week.

2.4. Preparation of cardiac extract

Serum extract of rat cardiac tissue: Frozen small pieces of rat cardiac tissue free from connective tissue and fat were homogenized in a 1:2 vol ratio in rat serum using a glass Dounce homogenizer at room temperature. The resulting homogenate was centrifuged at 10 000 RFC for 30 min. The clear supernatant was supplemented with 10 μl/mL sterile 0.2 M CoCl3 0.4 M EDTA solution before being filtered through a 2 μm filter (Millex®GS) and stored at −20 °C. The cardiac troponin T (cTnT) concentration was 1.53–4.9 mg/L in different extracts, as measured by the Roche hs-cTnT assay.

2.5. Purification of a cTn complex from pig heart

The cTn complex was prepared essentially as described, using 700 g of frozen left ventricular cardiac pig tissue or 8 g of rat left ventricular cardiac tissue. The method below pertains to the cTn preparation of left ventricular cardiac pig tissue that was used in the animal experiments. The clearance kinetics of rat cTn and pig cTn were similar after an intravenous (i.v.) injection, data not shown. The diethyl ether powder was extracted twice, for 1 h at a time, with 400 mL phosphate-buffered saline, PBS complemented with 1 M NaCl, 1 mM CaCl2 and 1 mM Dithiothreitol, DTT and protease inhibitors (1x cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche). The extract was fractionated by ammonium sulphate precipitation as described. The 43.5 % ammonium sulphate pellet was dissolved in a total of 110 mL of Buffer P (0.1 M Tris-HCl pH 7.5, 0.1 M NaCl, 0.1 m M CaCl2), cleared by centrifugation and loaded on a 10 mL MMC column (GE Health Care) equilibrated with Buffer P1, washed with 5 column volumes of PBS supplemented with 1 M NaCl, followed by 5 column volumes of MQ water and finally developed with a 100 mL gradient using 50 mL 1 M NaCl, 0.1 M NaOH, collecting 2 mL fractions. The fractions were rapidly supplemented with 200 μL Tris-HCl, pH 7.5, 1 mM DTT to normalize the pH level, and stored at −20 °C. The final yield from 700 g heart tissue was 23 mg cTn, measured with the Bicinchoninic Acid Kit for Protein Determination (Sigma) with bovine serum albumin as standard. The presence of cTnT and cTnI was confirmed using the Abbott STAT high-sensitivity cTnI assay and the Roche high-sensitivity cTnT assay. The presence of troponin C was inferred from overloaded SDS gels (Supplementary Fig. 1), but not directly detected or measured. The undiluted peak fractions with a protein concentration of 2 mg/mL had a tendency to precipitate at low ionic strength. For that reason, the cTn fractions were stored and labeled with NHS Alexa dye in buffers containing 1 M NaCl, as described in the next section. Prior to injection in the animals, the cTn preparations were diluted to 100-fold lower concentrations (< 0.1 mg/mL, see below) and centrifuged at 13 000 G for 5 min to remove any precipitate. However, at these lower concentrations, both the Alexa-labeled and unlabeled cTn preparations remained in solution with no evidence of precipitation.

2.6. Enrichment of cTnT fragments from patients with myocardial infarction

Serum samples were pooled from 50 patients with myocardial infarction and diluted twofold with 2 M NaCl to reach a final concentration of 1 M NaCl. The diluted serum was centrifuged for 10 min at 14 000 rpm and then filtered through a 0.22 μm filter before it was loaded on a Biorad 40 mL open column packed with 10 mL of Capto MMC resin (GE Healthcare), equilibrated with 150 mmol/L KPO4, pH 7.0. The 10 mL Capto MMC resin was washed with 50 mL 1 M NaCl, 1 mM $CaCl_2$ and eluted in 1 mL fractions with 100 mmol/L NaOH with the addition of EDTA-free Protease Inhibitor Cocktail (Roche) in plastic tubes prefilled with 200 μL of KPO_4 pH 4.5, to normalize pH to 7.0 quickly. The peak cTnT activity measured with the Roche high-sensitivity cTnT assay was found in fraction 8–12 and was pooled. The final preparation had cTnT levels of 4900 ng/L and were composed of cTnT degradation products with a molecular weight < 20 kDa, according to SDS-PAGE analysis, as previously described.

2.7. Labeling of proteins with Alexa-N-hydroxysuccinimide (Alexa-NHS)

Pig cTn preparations, horse myoglobin (Myo) and pig lactate dehydrogenase (LDH) (Sigma) were desalted on a G50 column in Buffer L (0.2 M $NaCO_3$ pH 8.3, 1 M NaCl) to remove all possible traces of Tris or other amine contaminants that otherwise could interfere with the N-hydroxysuccinimide (NHS) labeling. The proteins were concentrated on ultracentrifugation columns (Amicon) with the exclusion limit of 3 K to a protein concentration of 5−10 g/L. If lower protein concentrations were used, labeling with the NHS-Alexa reagents was inefficient. Alexa-NHS 488, Alexa-NHS 305 or Alexa-NHS 700 (Thermo Fisher Scientific) were added in fivefold molar excess and allowed to react in Buffer L for 3−24 h at room temperature. The reaction mixture was then supplemented with an equal volume of 2 M Tris-HCl pH 7.5 to inactivate the NHS group, and purified away from the unreacted Alexa dye on a G50 column in Buffer L. High molecular weight peak fractions containing the labeled proteins were concentrated on ultracentrifugation columns (Amicon) with the exclusion limit of 3 K and stored at −20 °C. The molar ratio of Alexa label to protein was 1–3 molecules per protein. When higher levels of labeling were used, the Alexa-labeled cTn preparations

had a tendency to precipitate.

2.8. Intramuscular injection of ground rat cardiac tissue

The rats were anesthetized and a reference blood sample (150 μL) was collected by tail snip. A tissue adhesive (Histoacryl®, B. Braun, Germany) was used for wound closure. Intramuscular injections were performed in the quadriceps muscle, located on the cranial aspect of the femur. Two rats were injected with 75 μL of ground rat cardiac tissue and one rat was injected with physiological saline (0.9 % NaCl). The weight of the syringe was measured before and after injections in order to quantify the exact amount of injected substance. Blood samples (150 μL) were collected via the tail vein in conscious animals 1 h, 3 h, 6 h, and 24 h post injection. Whole blood samples were left to stand for 60 min at room temperature prior to centrifugation (Heraeus Fresco 17, Thermo Scientific GmbH, Germany) at 9600 rcf for 10 min at 4 °C. Serum was removed from the red blood cells and stored at −20 °C.

2.9. Intravenous and intramuscular injection of cardiac extract and biomarkers

The rats were anesthetized and reference blood samples (150 μL) were collected from all rats by tail snip, and a tissue adhesive (Histoacryl®, B. Braun, Germany) was applied for wound closure. The intravenous injections of 200 μL of cardiac extract or biomarkers including purified cTn complex, FITC-Dextran and FITC-Sinestrin were administered via the tail vein in three rats, while another group of three rats was given intramuscular injections of 100 μL of the cardiac extract or biomarkers administered in the biceps femoris (hind limb). Blood samples (150 μL) were collected via the tail vein in conscious animals 5 min, 10 min, 30 min, 60 min, 90 min, 3 h, 6 h and 24 h after the injection. The maximum measured cTnT activity during the experiments described below was 10 000–12 000 ng/L, 19 000–22 000 ng/L for cTnI and 40–90 u g/L for Myo. As the analysis of cTnT and cTnI was performed with clinical assays calibrated using human cTnT or cTnI, the relative concentration was calculated where the highest measured concentration in each rat was set to 100. Whole blood samples were left to stand for 60 min at room temperature prior to centrifugation (Heraeus Fresco 17, Thermo Scientific GmbH, Germany) at 9600 RCF for 10 min at 4 °C. Serum was removed from the red blood cells and stored at −20 °C.

2.10. Intramuscular injection of cTn and histology

Pig cTn was fluorescently labeled with Alexa 488 and injected in to the left quadriceps muscle in rats. Muscle biopsies were collected after 30 min or 24 h and fresh frozen using isopentane chilled in liquid nitrogen. The frozen tissue was sectioned using a cryotome into 7 μm sections. For analysis of histology sections were stained with Hematoxylin-Eosin. The frozen tissue sections were fixed in −20 °C acetone for 10 min, washed in Phosphate Buffer Saline (PBS) and mounted with prolong gold antifade reagent with DAPI (Invitrogen).

The results were visualized using an ECLIPSE Ti microscope (Nikon Corporation, Tokyo, Japan) using brightfield microscopy for histology and fluorescence for analyses of the cTn content. Images were acquired at 3 Z-levels to capture all the nuclei in focus. For brightfield images Nikon DS-2 Mv camera was used. Fluorescence images were acquired for two channels; DAPI and FITC using an Andor Zyla camera. All images were exported to Image J software (v. 1.47 h, Fiji distribution) (Schindelin et al., 2012) for further analysis. For each channel, displayed pixel ranges were set so that most of the background was extinguished. The composite photos were used for analyses of the content of Alexa 488-cTn in large areas, provided by the photos.

2.11. PET analysis of i.v. ^{18}F-cTn injections in rat

Pig cTn (2 mg/mL) was labeled with ^{18}F for PET-imaging by using a similar approach as described by Olberg et al. (Olberg et al., 2010). ^{18}F-cTn was then purified on a PD MidiTrap G-10 gravity column (GE Healthcare Bio-Sciences) using saline as eluent. The integrity of ^{18}F-cTn was confirmed by size-exclusion chromatography using a TSKgel G2000SWXL column (Tosoh Bioscience LLC) and a Thermo Scientific UltiMate 3000 Rapid Separation system. The elution pattern of ^{18}F-cTn, as detected by an inline 35 μL gamma D flow cell and a Radiomatic 625TR Flow Scintillation Analyzer (PerkinElmer Inc.), was similar as unlabeled cTn complex as detected by the UltiMate™ 3000 Diode Array Detector Flow Cell (2.5 μm). 30 min dynamic PET scan (small-animal PET/CT, NanoScan PC PET/CT; Mediso Ltd, Budapest, Hungary) was obtained during sevoflurane anesthesia after bolus injection of 1 mL saline containing 5–8 MbQ 18 F-cTn in into a lateral tail vein. The rat was then allowed to wake up and a final 5 min static PET-scan was performed under sevoflurane anesthesia about 3 h after tracer injection. PET scan acquisitions parameters were as follows: Field of view 9.6 cm in the axial direction and 10 cm in the transaxial direction, 1:5 coincidence mode and normal count rate mode. The body temperature was maintained at 37 degrees throughout the whole procedure. Reconstruction of the PET data was performed with 3D OSEM and 1:5 coincidence mode, no filtering, attenuation correction (from a helical CT-scan, 50 kVp, 300 ms, 360 projections reconstructed using Ram-Lak filter), decay correction and normalization of detectors. The images were analysed using Interview fusion software (version 3.01.021.0000)".

2.12. Endocytosis experiments and confocal microscopy

Mouse muscle C2C12 cells were washed twice with 37 °C preheated serum-free medium and incubated at 37 °C for 30 min prior to protein addition. The proteins Alexa488-cTn (4.6ug/L), Alexa488-transferrin (0.1 g/L), Alexa488-myoglobin (1.3ug/L), and Alexa488-BSA (1.7ug/L) were diluted in 37 °C preheated medium and added to the cells for 2 h at 37 °C. The endocytosis inhibitor Dynasore hydrate (Sigmaaldrich) (50 μM) was added to the cells 30 min before addition of the proteins. As recommended, Dynasore hydrate treatment was performed in DMEM, High-Glucose without serum. Following incubation, the cells were washed twice with PBS, fixed with 4 % paraformaldehyde (PFA) in PBS for 15 min at room temperature and washed once again with PBS. The nuclei were stained with 1 μM NucBlue Live ReadyProbes Reagent containing Hoechts 33342 (Thermo Fisher Scientific) in PBS for 5 min, washed 1X with PBS and 2X with MQ water, and mounted with ibidi mounting medium (LRI Instruments Inc.). Confocal imaging was performed on a Nikon Eclipse Ti confocal microscope using a Nikon Apo 20X and Apo 60 × 1.40 oil immersion objective with 405 nm and 488 nm lasers for Hoecht 33342 and Alexa Fluor 488, respectively, and fluorescence was detected using filter/bandpass of 450 nm/50 nm and 525 nm/50 nm for the blue and green channel, respectively. All images were exported to Image J software (v. 1.47 h, Fiji distribution) (Schindelin et al., 2012) for further analysis.

2.13. Laboratory analyses

Serum samples were stored at −20 °C before dilution in PBS supplemented with 1 g/L bovine serum albumin and analysis at the Clinical Chemistry Laboratory at Sahlgrenska University Hospital. Analysis of cTnT was performed using the latest versions of Roche hs-cTnT method on Cobas (Hammarsten et al., 2013). Analysis of cTnI was performed using the latest versions of the Abbott hs-cTnI method on Architect. All methods had a CV < 5 % within the range measured in the study.

The Abbott hs-cTnI method is specific for cardiac cTnI and does not cross-react with skeletal muscle proteins. The Roche hs-cTnT method cross-reacts with something in skeletal muscle and extensive skeletal muscle damage or myositis is known to generate cTnT elevations in patients (Schmid et al., 2018). However, the amount of cTnT measured in skeletal muscle extracts are several orders of magnitude lower

compared to cardiac muscle extracts from humans (Schmid et al., 2018) and rats (Hammarsten, unpublished observations) and this possible interference is likely of no significance in our studies.

Myoglobin was measured using a high-sensitive rat Myoglobin ELISA with a CV of 14.9 % at 13.5 ug/L (LIFE diagnostics) The same form of myoglobin is expressed in both skeletal and cardiac muscle and we can therefore not exclude that myoglobin released after i.m. injection interfered with our measurements.

2.14. Statistical analysis

The statistical significance was tested using the two-sided non-paired *t*-test using Excel 2011. P values < 0.05 were considered significant.

3. Results

3.1. Clearance following tail vein or quadriceps muscle injection

We followed the serum concentration for 24 h following a tail vein injection (i.v.) or injection into the left quadriceps muscle (i.m.) in rats (Fig. 1). Almost 50 % of the renal filtration marker FITC-sinistrin, not expected to be taken up by muscle cells and with a free passage over the muscular capillaries, was recovered in the circulation after an i.m. injection. In contrast, less than 10 % of FITC-dextran, known to be taken up through endocytosis by muscle cells (Lawoko and Tagerud, 1995), was recovered after an i.m. injection (Fig. 2). We then compared the recovery of cTnT, cTnI and myoglobin after i.v. or i.m. injections. We decided to use cardiac troponins and not skeletal muscle troponins, as we have excellent clinical assays for cardiac troponins capable of measuring also small degradation products (Friden et al., 2017; Streng et al., 2017) in plasma samples. The source of the cTnT, cTnI and myoglobin was either plasma extract of *in vitro* crushed rat cardiac tissue to simulate the cTnT, cTnI and myoglobin release following muscle damage (Fig. 3), or a purified pig cTn complex (Fig. 4) (Supplemental Fig. 1). Only small amounts of the cTnT and cTnI injected by the i.m. route reached the circulation during 24 h (Table 1). Myoglobin, a small protein (17 kDa) with expected free passage over muscular capillaries (Sarin, 2010) also seemed to be catabolized by the muscular tissue (Table 1) (Fig. 5A) Supplementary Figs. 5–9). In contrast, over 80 % of serum cTnT fragments enriched from patients with myocardial infarction (Friden et al., 2017) was recovered in the circulation after i.m. injection in one rat (Fig. 5B, Table 1, Supplementary Fig. 10).

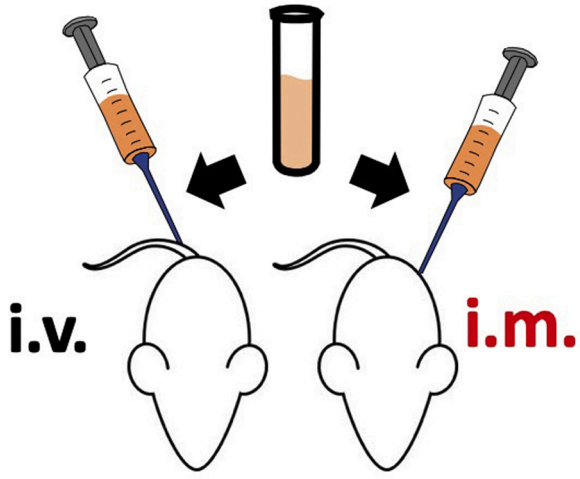


Fig. 1. Outline of the experimental system. Rats were injected with different preparations in pairs through the tail vein (i.v.) or into the quadriceps muscle (i.m.), and the clearance of cTnT, cTnI and other molecules was followed by blood sampling or by positron emission tomography (PET) analysis.

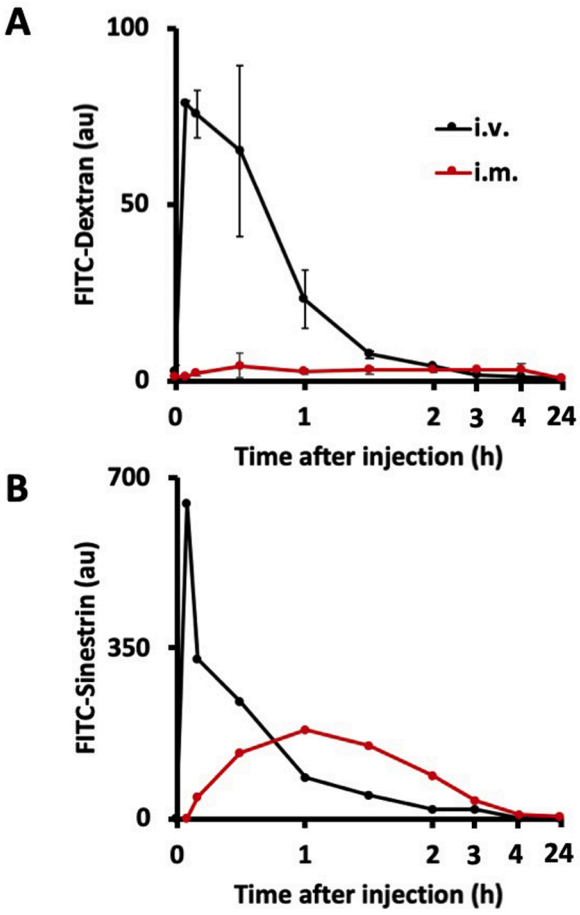


Fig. 2. Kinetics of FITC-sinestrin (1 kDa) (A) or FITC-Dextran (40 kDa) (B) clearance following an i.v. (black line) or i.m. (red line) injection. Mean values from three rats. Error bars represent ± 1SD.

3.2. Fluorescence microscopic examination after intramuscular injection of cTn

To examine if cTn was retained in muscle cells after an i.m. injection, fluorescently labeled pig cTn was injected into the left quadriceps muscle in rats (Fig. 6). Muscle biopsies were collected after 30 min or after 24 h, flash frozen and examined by fluorescence microscopy. Within 30 min of injection, essentially all of the fluorescence signal was found within the myocytes. Green fluorescence was still detected but with weaker intensity in the injection area after 24 h.

3.3. PET analysis of cTn after intramuscular injection

PET analysis of ^{18}F-labeled cTn revealed that most ^{18}F-activity accumulated in the liver and kidneys after an i.v. injection (Fig. 7A). In contrast, after an i.m. injection, most of the ^{18}F-activity remained in the quadriceps muscle during the first 30 min and was then lost in the urine during the following three hours (Fig. 7B). Some of the ^{18}F-activity was found in a local point-like structure, most likely a lymph gland (Supplemental Fig. 2). Very small amounts of ^{18}F -activity were found in the liver or spleen after an i.m. injection as opposed to after an i.v. injection, indicating that most of the ^{18}F-activity that escaped from the muscle were degradation products or free ^{18}F that was cleared by the kidneys.

3.4. Endocytosis of cTn by myotubes

Mouse C2C12 myotubes (Supplemental Fig. 3) internalized fluorescently labeled cTn, myoglobin and transferrin (Fig. 8A) that was

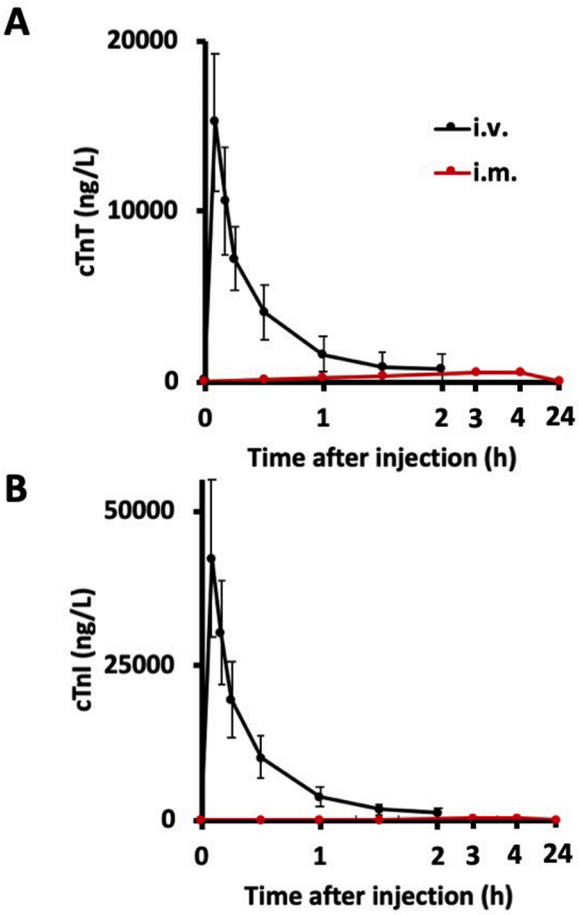


Fig. 3. Kinetics of cTnT and cTnI clearance following an i.v. (black line) or i.m. (red line) injection of rat cardiac serum extract. Mean values from three rats. Error bars represent ± 1SD.

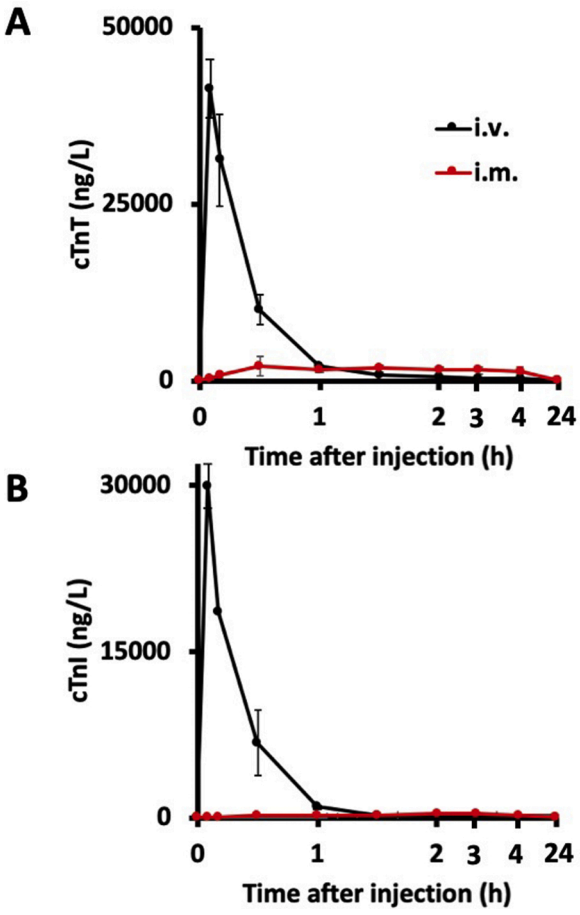


Fig. 4. Kinetics of cTnT and cTnI clearance following an i.v. (black line) or i.m. (red line) injection of partially purified cardiac troponin complex from pig heart. Mean values from three rats. Error bars represent ± 1SD.
Source: Reproduced with permission from SciRep, http://creativecommons.org/licenses/by/4.0/.

blocked by the endocytosis inhibitor Dynasore (Fig. 8B). No appreciable internalization of fluorescently labeled bovine serum albumin was observed (Supplemental Fig. 4). This indicates that myocytes are able to take up cTn and Myoglobin, likely by endocytosis.

4. Discussion

We show by using cardiac troponins and myoglobin that muscle proteins are cleared by muscle tissue. The system prevented over 90 % of the injected cTn and myoglobin from reaching the circulation and could therefore be the dominant clearance mechanism for released cTn and myoglobin. Thus, a troponin or a myoglobin molecule in between rat skeletal muscle cells is unlikely to reach the circulation despite myoglobin having free passage over muscular capillaries (Sarin, 2010). The retention of cTn after i.m. injection was similar when we used purified cTn and serum extract of cardiac tissue. Therefore, the local clearance of cTn was not inhibited by serum proteins and therefore displayed some form of specificity.

We used clinical immunoassays capable of measuring degradation products of cTnT, cTnI and myoglobin, making it unlikely that degradation between cells was the only reason for poor recovery in the circulation after i.m. injection.

Fluorescently labeled cTn was rapidly taken up by skeletal muscle after an i.m. injection. This is in contrast to albumin that only occupies the interstitial space between muscle cells shown by injection of the albumin-specific (Huggins et al., 1963) dye Evans blue (Hamer et al., 2002).

Long term retention of ^{18}F labeled cTn after i.m. injection was also observed by PET analysis. Very little of the ^{18}F activity, was recovered in the rat liver or spleen in the PET analysis after an i.m. injection, indicating that cTn was catabolized locally in the muscle tissue, possibly in the lysosomes after uptake via endocytosis. The possibility that local

Table 1
Comparison of recovery in blood samples after i.v. or i.m. injection.

Substance measured in blood samples at different times after i.v. or i.m. injection	Area under time concentration curve after i.v. injection (reference)	Area under time concentration curve after i.m. injection[†] (SEM)
FITC-Sinestrin	100	47 (n.a.)
FITC-Dextran	100	8.1 (1.7)
Myoglobin (rat)[*]	100	7.8 (1.4)
cTnT (rat)[*]	100	4.8 (1)
cTnI (rat)[*]	100	0.6 (0.1)
cTnT (pig)[#]	100	11.4 (2.4)
cTnI (pig)[#]	100	2.4 (0.2)
cTnT (patient serum)[§]	100	81.1 (n.a.)

[†] Relative area under the time-concentration curve (AUC) using the AUC after an i.v. injection as reference; that is, the area under the time-concentration curve (AUC) from an i.v. injection (black line in plots) divided by the AUC after an i.m. injection (red line in plots).
[*] Serum extract of rat heart.
[#] Pig cardiac troponin complex.
[§] cTnT activity from pooled serum samples from patients with myocardial infarction.

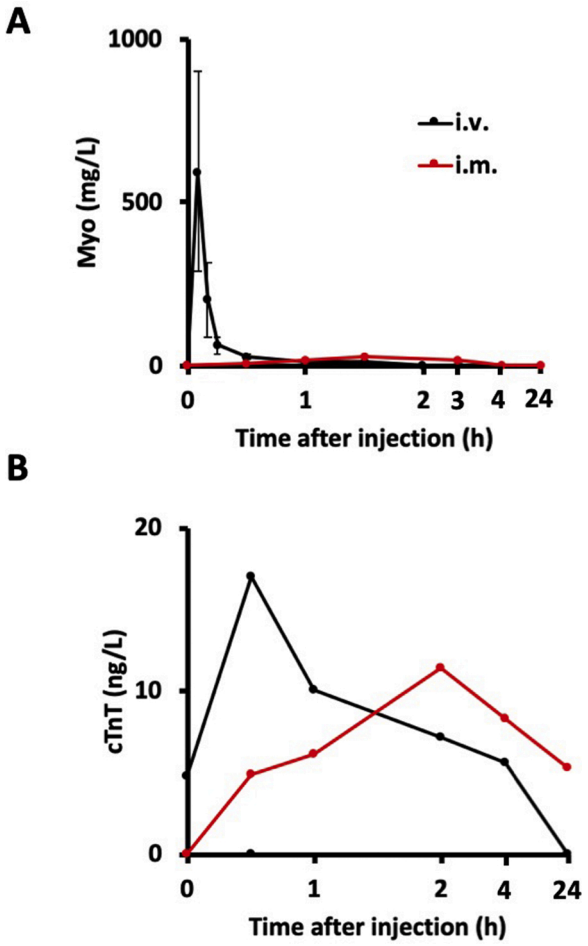


Fig. 5. (A) Kinetics of myoglobin clearance (A) following an i.v. (black line) or i.m. (red line) injection. Mean values from three rats. Error bars represent ± 1SD. (B) Clearance of cTnT following an i.v. (black line) or i.m. injection (red line) of enriched cTnT fragments from patients with myocardial infarction in one rat.

clearance was due to endocytosis was supported by the ability of the endocytosis inhibitor Dynasore to decrease myotube uptake of fluorescent cTn and myoglobin.

In general, despite its obvious importance, not much is known about the plasma clearance of muscle damage biomarkers. Early studies indicate that myoglobin is predominantly cleared in the liver after an MI (Hallgren et al., 1978; Roxin et al., 1979; Sylven, 1978) and rhabdomyolysis (Amako et al., 1963), although myoglobin is small enough to be cleared by the kidneys. Studies in dogs indicate that creatine kinase is cleared from the circulation by the reticuloendothelial system (Guzy, 1977). Studies in rats and mice show that intracellular proteins like lactate dehydrogenase and creatine kinase that are too large to pass through the glomerular membrane are cleared by receptor-mediated endocytosis in the liver and the spleen (Ashwell and Harford, 1982; Smit et al., 1987). Although the responsible receptors were not identified, scavenger receptors are likely involved. Scavenger receptors, like the LDL-receptor(Schneider et al., 1982), are a loosely defined group of receptors that bind a vast array of ligands and often destine them for endocytosis and degradation in lysosomes (Prabhudas et al., 2014).

Mice with a compromised reticuloendothelial system (Hayashi and Notkins, 1994; Hayashi et al., 1988) or with mutations in scavenger receptors (Radi et al., 2011) develop stable elevations of lactate dehydrogenase, creatine kinase and aspartate aminotransferase. Common sequence variants in scavenger receptors are linked to high serum levels of lactate dehydrogenase and creatine kinase in humans (Kristjansson et al., 2016), indicating that inefficient scavenger receptor-mediated clearance could contribute to stable elevations of muscle damage biomarkers also in humans.

Most cells, including skeletal muscle cells, express scavenger receptors that are involved in endocytosis and the uptake of various molecules (Ezzat et al., 2015; Miyatake et al., 2019). Indeed, it is well established that skeletal muscle cells have a very active receptor-mediated endocytosis system capable of removing dextran, hemoglobin (Janeczko et al., 1985) and other macromolecules from their surroundings (Elmquist et al., 1992; Garfield et al., 1975; Lawoko and Tagerud, 1995; Lawoko et al., 1992; Libelius et al., 1981; Libelius and Tagerud, 1984; Satkauskas et al., 2001; Tagerud and Libelius, 1985; Tagerud et al., 1990; Vult von Steyern et al., 1994). It is therefore possible that receptor-mediated endocytosis was responsible for the local clearance of cTn and myoglobin that we observed in rat muscular tissue.

One might speculate on why this clearance system has not been found before. Early studies report results similar to ours following paired i.v. and i.m. injections of cytoplasmic proteins like creatine phosphokinase (Hsu and Watanabe, 1984). However, the strong clearance of creatine phosphokinase activity after an i.m. injection was interpreted as local inactivation of the enzymatic activity. The possibility that creatine phosphokinase was absorbed by the muscle tissue was never explored. Indeed, another muscle enzyme, creatine kinase, is rapidly inactivated if incubated in dog cardiac lymph (Clark et al., 1978). The theory that the poor recovery of muscle proteins in the circulation after muscle damage is due to local inactivation has prevailed (Clark et al., 1978) and possibly

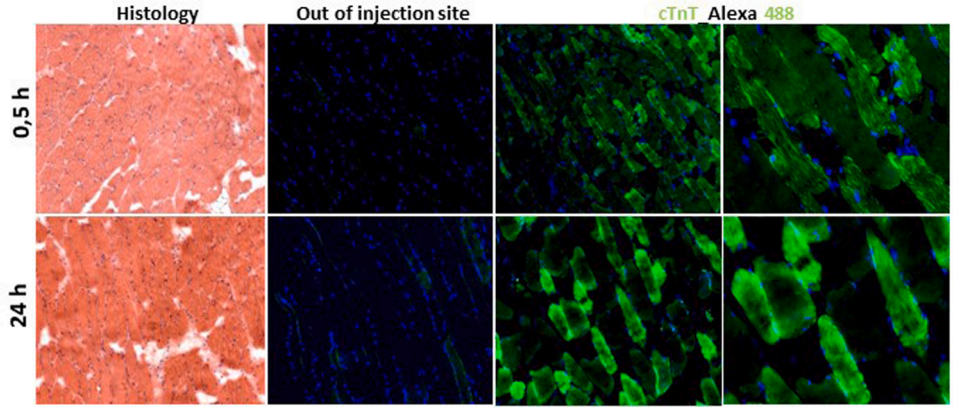
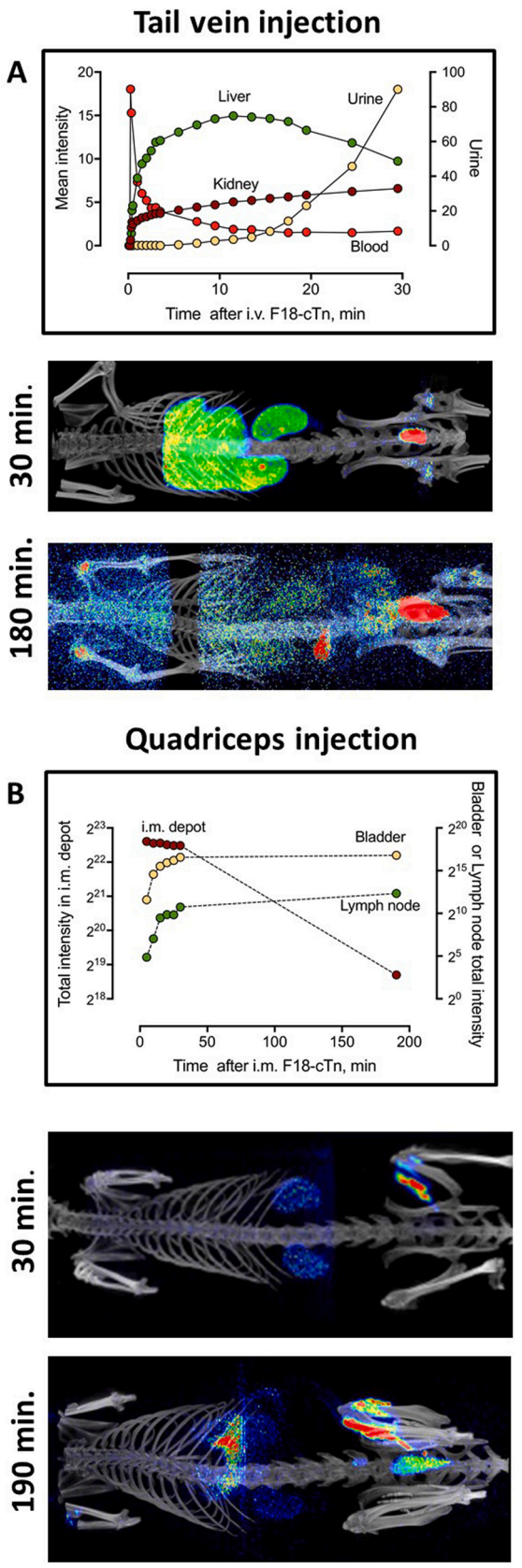


Fig. 6. Distribution of Alexa488 fluorescence in cryosections of muscle biopsies collected 0.5 h or 24 h after an injection of Alexa488-labeled pig cTn complex in the quadriceps muscle in rat. "Out of injection site" is located far away from the injection site and represents the background signal of the muscle biopsy. Hematoxylin and eosin-stained sections are included as a reference to muscle cell morphology.



(caption on next column)

Fig. 7. (A) Positron emission tomography (PET) analysis of cTn distribution following an i.v. (A) or i.m. (B) injection of partially purified pig troponin complex labeled with ^{18}F. *The graphs* are obtained from a dynamic 0-30 min PET scan showing ^{18}F activity in blood, liver, kidney and bladder urine (A) and in leg muscles, bladder urine and a possible abdominal lymph node (B). *PET-CT images*: A; Maximum intensity projection (MIP) 3D volume displaying uptake in both kidneys, liver and bladder (See supplemental Fig. 2C for a 2D coronal view displaying the uptake in liver and left kidney). Both images are reconstructed by summing the 30 min uptake (static). The MIP image after 180 min was acquired by a 5 min static scan, showing most of the i.v. injected ^{18}F activity had been excreted in the urine or intestines. B MIP reconstructions 30- and 190 min after i.m. injection showing slow removal and some expansion of ^{18}F activity at the injection site, low uptake in kidney, but nothing in liver (30 min) and low overall abdominal uptake and urinary excretion (190 min) (see Supplemental Fig. 2D for a 2D coronal view and more details). The i.v. and i.m. experiments were conducted in two different rats allowing to wake up from anesthesia and move freely in the cage between the 30 min dynamic scan and the 3 h static scan.

prevented anyone to pursue the possibility that muscle cells are capable of clearing proteins from their surroundings by endocytosis. The only report we have found that discusses this possibility is a study of myoglobin pharmacokinetics, indicating that most myoglobin released from skeletal muscle in humans is catabolized within the skeletal muscle itself (Hallgren et al., 1978).

In contrast to full length cTnT, cTnT fragments prepared from plasma from patients with myocardial infarction were able to reach the circulation after i.m. injection suggesting cTnT fragments residing in the circulation were able to escape local clearance. We (Friden et al., 2017; Starnberg et al., 2020) and others (Streng et al., 2017) have shown that most cTnT in patient plasma measured by our clinical assays are degradation products and very little full-length cTnT reside in the circulation a few hours after myocardial infarction. We have also shown that most full-length cTnT injected i.v. is taken up by the liver, possibly by scavenger receptors (Muslimovic et al., 2020). One possible explanation for the escape is that the cTnT degradation fragments found in patient plasma bind less well to scavenger receptors and therefore "survive" both local clearance and liver clearance and accumulate in the circulation. This finding points to the possibility that circulating forms of cell damage markers is a subset of molecules that are inefficiently cleared by scavenger receptors. However, this possibility can only be resolved by identification of the receptors involved.

Finally, we have not been able to do intramyocardial injections to study possible local clearance in the heart. We do, however, find that cardiac cell cultures take up troponin and myoglobin, but not albumin (Muslimovic, unpublished observations). As endocytosis is blocked in cardiac cells by limited ischemia (Khaidakov et al., 2014) it is possible that troponin elevations in patients with events that are sknown to induce limited ischemia such as atrial flutter or strenuous exercise could be due to impaired local clearance.

There are several limitations to this study. First, the number of animals included in each experiment were low. Second, our data is for the most part phenomenological. The data suggest the presence of an endocytosis-driven local clearance of muscle proteins between muscle cells, but we have not undertaken any attempts to identify putative receptors at this stage.

In summary, we report that muscle proteins between muscle cells are cleared by muscle cells.

5. Author contributions

A.M., V.F., and O.H. designed research, implemented acquisition and analysis of data. A.M., VF, K.S., S.N. K.V. K.G. performed research. E.W. and E.K.E. supported endocytosis experiments and confocal imaging. O.

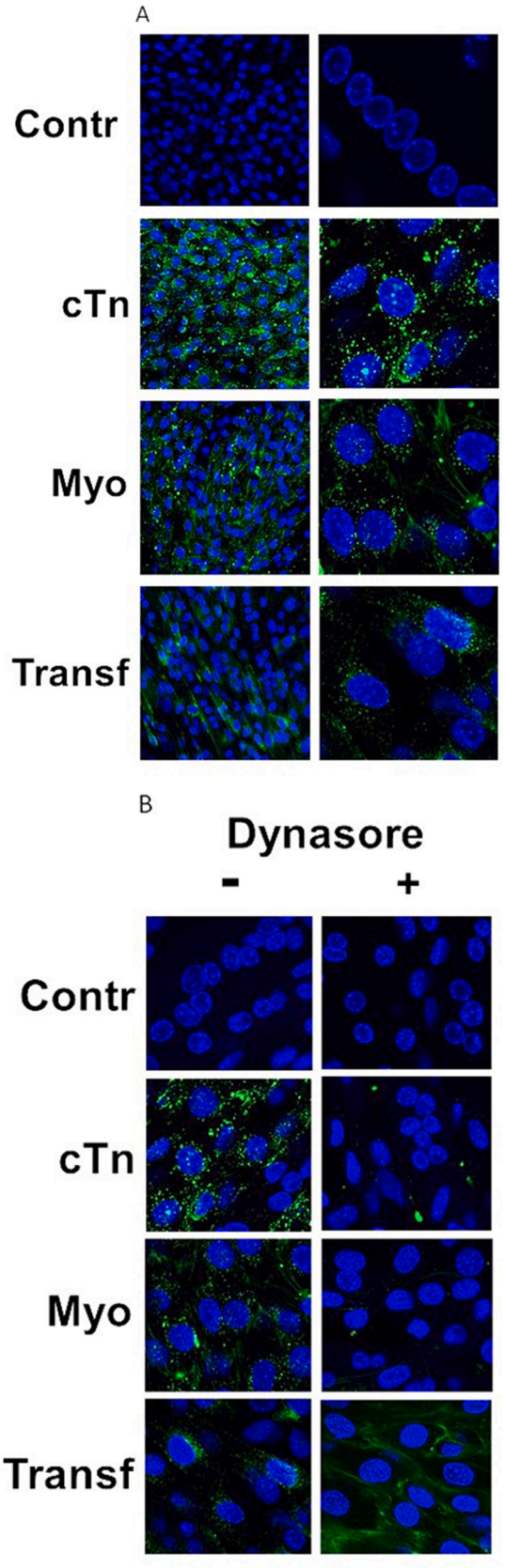


Fig. 8. (A) Internalization of Alexa488-labeled cTn complex, Myoglobin or transferrin in cultured mouse myotubes. (B) Inhibition of internalization in the presence of the Dynasore endocytosis inhibitor.

T. and H.E. performed PET experiments and analysis. B.L. contributed to the study conception, participated in discussions and provided helpful comments. O.H. and A.M. prepared the first version of the manuscript with all authors contributing to the final version.

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Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ejcb.2020.151127.

References

Amako, T., Koga, J., Kobayashi, A., Urakado, S., Tokunaga, J., 1963. Experimental investigation of the metabolism of myoglobin. Kyushu J. Med. Sci. 14, 277–287.
Ashwell, G., Harford, J., 1982. Carbohydrate-specific receptors of the liver. Annu. Rev. Biochem. 51, 531–554.
Bjurman, C., Petzold, M., Venge, P., Farbemo, J., Fu, M.L., Hammarsten, O., 2015. High-sensitive cardiac troponin, NT-proBNP, hFABP and copeptin levels in relation to glomerular filtration rates and a medical record of cardiovascular disease. Clin. Biochem. 48, 302–307.
Bjurman, C., Zywczyk, M., Lindahl, B., Carlsson, T., Johanson, P., Petzold, M., Holzmann, M., Lx Fu, M., Hammarsten, O., 2017. Decreased admissions and hospital costs with a neutral effect on mortality following lowering of the troponin T cutoff point to the 99th percentile. Cardiol. J.
Carlsson, A.C., Bandstein, N., Roos, A., Hammarsten, O., Holzmann, M.J., 2017. High-sensitivity cardiac troponin T levels in the emergency department in patients with chest pain but no myocardial infarction. Int. J. Cardiol. 228, 253–259.
Chapman, A.R., Lee, K.K., McAllister, D.A., Cullen, L., Greenslade, J.H., Parsonage, W., Worster, A., Kavsak, P.A., Blankenberg, S., Neumann, J., Sorensen, N.A., Westermann, D., Buijs, M.M., Verdel, G.J.E., Pickering, J.W., Than, M.P., Twerenbold, R., Badertscher, P., Sabti, Z., Mueller, C., Anand, A., Adamson, P., Strachan, F.E., Ferry, A., Sandeman, D., Gray, A., Body, R., Keevil, B., Carlton, E., Greaves, K., Korley, F.K., Metkus, T.S., Sandoval, Y., Apple, F.S., Newby, D.E., Shah, A.S.V., Mills, N.L., 2017a. Association of high-sensitivity cardiac troponin I concentration with cardiac outcomes in patients with suspected acute coronary syndrome. JAMA 318, 1913–1924.
Chapman, A.R., Shah, A.S., Mills, N.L., 2017b. Appropriate use of high-sensitivity cardiac troponin levels in patients with suspected acute myocardial infarction. JAMA Cardiol. 2, 228.
Chapman, A.R., Hesse, K., Andrews, J., Ken Lee, K., Anand, A., Shah, A.S.V., Sandeman, D., Ferry, A.V., Jameson, J., Piya, S., Stewart, S., Marshall, L., Strachan, F.E., Gray, A., Newby, D.E., Mills, N.L., 2018. High-sensitivity cardiac troponin I and clinical risk scores in patients with suspected acute coronary syndrome. Circulation 138, 1654–1665.
Clark, G.L., Robison, A.K., Gnepp, D.R., Roberts, R., Sobel, B.E., 1978. Effects of lymphatic transport of enzyme on plasma creatine kinase time-activity curves after myocardial infarction in dogs. Circ. Res. 43, 162–169.
Elmquist, S., Libelius, R., Lawoko, G., Tagerud, S., 1992. Dextrans as markers for endocytosis in innervated and denervated skeletal muscle. Muscle Nerve 15, 876–884.
Ezzat, K., Aoki, Y., Koo, T., McClorey, G., Benner, L., Coenen-Stass, A., O'Donovan, L., Lehto, T., Garcia-Guerra, A., Nordin, J., Saleh, A.F., Behlke, M., Morris, J., Goyenvalle, A., Dugovic, B., Leumann, C., Gordon, S., Gait, M.J., El-Andaloussi, S., Wood, M.J., 2015. Self-assembly into nanoparticles is essential for receptor mediated uptake of therapeutic antisense oligonucleotides. Nano Lett. 15, 4364–4373.
Filatov, V.L., Katrukha, A.G., Bulargina, T.V., Gusev, N.B., 1999. Troponin: structure, properties, and mechanism of functioning. Biochemistry (Mosc) 64, 969–985.
Friden, V., Starnberg, K., Muslimovic, A., Ricksten, S.E., Bjurman, C., Forsgard, N., Wickman, A., Hammarsten, O., 2017. Clearance of cardiac troponin T with and without kidney function. Clin. Biochem. 50, 468–474.

Garfield, R.E., Chacko, S., Blose, S., 1975. Phagocytosis by muscle cells. Lab. Invest. 33, 418–427.
Guzy, P.M., 1977. Creatine phosphokinase-MB (CPK-MB) and the diagnosis of myocardial infarction. West. J. Med. 127, 455–460.
Hallgren, R., Karlsson, F.A., Roxin, L.E., Venge, P., 1978. Myoglobin turnover-influence of renal and extrarenal factors. J. Lab. Clin. Med. 91, 246–254.
Hamer, P.W., McGeachie, J.M., Davies, M.J., Grounds, M.D., 2002. Evans Blue Dye as an in vivo marker of myofibre damage: optimising parameters for detecting initial myofibre membrane permeability. J. Anat. 200, 69–79.
Hammarsten, O., Fu, M.L., Sigurjonsdottir, R., Petzold, M., Said, L., Landin-Wilhelmsen, K., Widgren, B., Larsson, M., Johanson, P., 2012. Troponin T percentiles from a random population sample, emergency room patients and patients with myocardial infarction. Clin. Chem. 58, 628–637.
Hammarsten, O., Jacobsson, C.-E., Widegren, M., Danylchenko, T., Jaffe, A.S., 2013. Long-time quality assessment of the Elecsys Troponin T hs assay. Clin. Biochem. 46, 1055–1057.
Hammarsten, O., Mair, J., Mockel, M., Lindahl, B., Jaffe, A.S., 2018. Possible mechanisms behind cardiac troponin elevations. Biomarkers 1–10.
Hayashi, T., Notkins, A.L., 1994. Clearance of LDH-5 from the circulation of inbred mice correlates with binding to macrophages. Int. J. Exp. Pathol. 75, 165–168.
Hayashi, T., Salata, K., Kingman, A., Notkins, A.L., 1988. Regulation of enzyme levels in the blood. Influence of environmental and genetic factors on enzyme clearance. Am. J. Pathol. 132, 503–511.
Hsu, H., Watanabe, J., 1984. The absolute bioavailability of rabbit muscle creatine phosphokinase after intramuscular administration. Chem. Pharm. Bull. (Tokyo) 32, 1922–1928.
Huggins, R.A., Smith, E.L., Deavers, S., 1963. Volume distribution of evans blue dye and iodinated albumin in the dog. Am. J. Physiol. 205, 351–356.
Janeczko, R.A., Carriere, R.M., Etlinger, J.D., 1985. Endocytosis, proteolysis, and exocytosis of exogenous proteins by cultured myotubes. J. Biol. Chem. 260, 7051–7058.
Khaidakov, M., Mercanti, F., Wang, X., Ding, Z., Dai, Y., Romeo, F., Sawamura, T., Mehta, J.L., 2014. Prevention of export of anoxia/reoxygenation injury from ischemic to nonischemic cardiomyocytes via inhibition of endocytosis. Am. J. Physiol. Heart Circ. Physiol. 306. H1700-1707.
Kristjansson, R.P., Oddsson, A., Helgason, H., Sveinbjornsson, G., Arnadottir, G.A., Jensson, B.O., Jonasdottir, A., Jonasdottir, A., Bragi Walters, G., Sulem, G., Oskarsdottir, A., Benonisdottir, S., Davidsson, O.B., Masson, G., Magnusson, O.T., Holm, H., Sigurdardottir, O., Jonsdottir, I., Eyjolfsson, G.I., Olafsson, I., Gudbjartsson, D.F., Thorsteinsdottir, U., Sulem, P., Stefansson, K., 2016. Common and rare variants associating with serum levels of creatine kinase and lactate dehydrogenase. Nat. Commun. 7, 10572.
Lawoko, G., Tagerud, S., 1995. High endocytotic activity occurs periodically in the endplate region of denervated mouse striated muscle fibers. Exp. Cell Res. 219, 598–603.
Lawoko, G., Tagerud, S., Libelius, R., 1992. Increased endocytotic and lysosomal activities in denervated type I and type II muscle fibres. Histochemistry 97, 221–224.
Libelius, R., Tagerud, S., 1984. Uptake of horseradish peroxidase in denervated skeletal muscle occurs primarily at the endplate region. J. Neurol. Sci. 66, 273–281.
Libelius, R., Lundquist, I., Tagerud, S., Thesleff, S., 1981. Endocytosis and lysosomal enzyme activities in dystrophic muscle: the effect of denervation. Acta Physiol. Scand. 113, 259–261.
Miyatake, S., Mizobe, Y., Tsoumpra, M.K., Lim, K.R.Q., Hara, Y., Shabanpoor, F., Yokota, T., Takeda, S., Aoki, Y., 2019. Scavenger receptor class A1 mediates uptake of morpholino antisense oligonucleotide into dystrophic skeletal muscle. Mol. Ther. Nucleic Acids 14, 520–535.
Muslimovic, A., Friden, V., Tenstad, O., Starnberg, K., Nystrom, S., Wesen, E., Esbjorner, E.K., Granholm, K., Lindahl, B., Hammarsten, O., 2020. The Liver and Kidneys mediate clearance of cardiac troponin in the rat. Sci. Rep. 10, 6791.
Olberg, D.E., Arukwe, J.M., Grace, D., Hjelstuen, O.K., Solbakken, M., Kindberg, G.M., Cuthbertson, A., 2010. One step radiosynthesis of 6-[(18)F]fluoronicotinic acid 2,3,5,6-tetrafluorophenyl ester ([(18)F]F-Py-TFP): a new prosthetic group for efficient labeling of biomolecules with fluorine-18. J. Med. Chem. 53, 1732–1740.
Prabhudas, M., Bowdish, D., Drickamer, K., Febbraio, M., Herz, J., Kobzik, L., Krieger, M., Loike, J., Means, T.K., Moestrup, S.K., Post, S., Sawamura, T., Silverstein, S., Wang, X.Y., El Khoury, J., 2014. Standardizing scavenger receptor nomenclature. J. Immunol. 192, 1997–2006.
Radi, Z.A., Koza-Taylor, P.H., Bell, R.R., Obert, L.A., Runnels, H.A., Beebe, J.S., Lawton, M.P., Sadis, S., 2011. Increased serum enzyme levels associated with kupffer cell reduction with no signs of hepatic or skeletal muscle injury. Am. J. Pathol. 179, 240–247.
Roos, A., Bandstein, N., Lundback, M., Hammarsten, O., Ljung, R., Holzmann, M.J., 2017a. Stable high-sensitivity cardiac troponin t levels and outcomes in patients with chest pain. J. Am. Coll. Cardiol. 70, 2226–2236.
Roos, A., Hellgren, A., Rafatnia, F., Hammarsten, O., Ljung, R., Carlsson, A.C., Holzmann, M.J., 2017b. Investigations, findings, and follow-up in patients with chest pain and elevated high-sensitivity cardiac troponin T levels but no myocardial infarction. Int. J. Cardiol. 232, 111–116.
Roxin, L.E., Venge, P., Friman, G., Hallgren, R., 1979. Radioimmunoassays of human myoglobin in serum and urine. Scand. J. Clin. Lab. Invest. 39, 37–46.
Sandoval, Y., Smith, S.W., Shah, A.S., Anand, A., Chapman, A.R., Love, S.A., Schulz, K., Cao, J., Mills, N.L., Apple, F.S., 2017. Rapid rule-out of acute myocardial injury using a single high-sensitivity cardiac troponin I measurement. Clin. Chem. 63, 369–376.
Sarin, H., 2010. Physiologic upper limits of pore size of different blood capillary types and another perspective on the dual pore theory of microvascular permeability. J. Angiogenes. Res. 2, 14.
Satkauskas, S., Bureau, M.F., Mahfoudi, A., Mir, L.M., 2001. Slow accumulation of plasmid in muscle cells: supporting evidence for a mechanism of DNA uptake by receptor-mediated endocytosis. Mol. Ther. 4, 317–323.
Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682.
Schmid, J., Liesinger, L., Birner-Gruenberger, R., Stojakovic, T., Scharnagl, H., Dieplinger, B., Asslaber, M., Radl, R., Beer, M., Mair, J., Szolar, D., Berghold, A., Quasthoff, S., Binder, J.S., Rainer, P.P., 2018. Elevated cardiac troponin t in skeletal myopathies: new evidence for a skeletal muscle origin. J. Am. Coll. Cardiol. 71, 1540–1549.
Schneider, W.J., Beisiegel, U., Goldstein, J.L., Brown, M.S., 1982. Purification of the low density lipoprotein receptor, an acidic glycoprotein of 164,000 molecular weight. J. Biol. Chem. 257, 2664–2673.
Shah, A.S.V., Anand, A., Strachan, F.E., Ferry, A.V., Lee, K.K., Chapman, A.R., Sandeman, D., Stables, C.L., Adamson, P.D., Andrews, J.P.M., Anwar, M.S., Hung, J., Moss, A.J., O'Brien, R., Berry, C., Findlay, I., Walker, S., Cruickshank, A., Reid, A., Gray, A., Collinson, P.O., Apple, F.S., McAllister, D.A., Maguire, D., Fox, K.A.A., Newby, D.E., Tuck, C., Harkess, R., Parker, R.A., Keerie, C., Weir, C.J., Mills, N.L., High, S.I., 2018. High-sensitivity troponin in the evaluation of patients with suspected acute coronary syndrome: a stepped-wedge, cluster-randomised controlled trial. Lancet 392, 919–928.
Smit, M.J., Duursma, A.M., Bouma, J.M., Gruber, M., 1987. Receptor-mediated endocytosis of lactate dehydrogenase M4 by liver macrophages: a mechanism for elimination of enzymes from plasma. Evidence for competition by creatine kinase MM, adenylate kinase, malate, and alcohol dehydrogenase. J. Biol. Chem. 262, 13020–13026.
Smit, M.J., Beekhuis, H., Duursma, A.M., Bouma, J.M., Gruber, M., 1988. Catabolism of circulating enzymes: plasma clearance, endocytosis, and breakdown of lactate dehydrogenase-1 in rabbits. Clin. Chem. 34, 2475–2480.
Starnberg, K., Jeppsson, A., Lindahl, B., Berggren, K., Hammarsten, O., 2014. Revision of the troponin T release mechanism from a damaged human myocardium. Clin. Chem. 60, 1098–1104.
Starnberg, K., Friden, V., Muslimovic, A., Ricksten, S.E., Nystrom, S., Forsgard, N., Lindahl, B., Vukusic, K., Sandstedt, J., Dellgren, G., Hammarsten, O., 2020. A possible mechanism behind faster clearance and higher peak concentrations of cardiac troponin I compared with troponin t in acute myocardial infarction. Clin. Chem. 66, 333–341.
Streng, A.S., de Boer, D., van Doorn, W.P., Bouwman, F.G., Mariman, E.C., Bekers, O., van Dieijen-Visser, M.P., Wodzig, W.K., 2017. Identification and characterization of cardiac troponin t fragments in serum of patients suffering from acute myocardial infarction. Clin. Chem. 63, 563–572.
Sylven, C., 1978. The kinetics of myoglobin in old volunteers and in patients with acute myocardial infarction. Scand. J. Clin. Lab. Invest. 38, 561–565.
Tagerud, S., Libelius, R., 1985. Receptor-mediated uptake of horseradish peroxidase in innervated and denervated skeletal muscle. Exp. Cell Res. 160, 95–105.
Tagerud, S., Libelius, R., Shainberg, A., 1990. High endocytotic and lysosomal activities in segments of rat myotubes differentiated in vitro. Cell Tissue Res. 259, 225–232.
Thygesen, K., Alpert, J.S., Jaffe, A.S., Chaitman, B.R., Bax, J.J., Morrow, D.A., White, H. D., Group, E.S.C.S.D., 2019. Fourth universal definition of myocardial infarction (2018). Eur. Heart J. 40, 237–269.
Vult von Steyern, F., Libelius, R., Lawoko, G., Tagerud, S., 1994. Endocytotic activity of mouse skeletal muscle fibres after long-term denervation. J. Neurol. Sci. 125, 147–152.
Wodzig, K.W., Kragten, J.A., Hermens, W.T., Glatz, J.F., van Dieijen-Visser, M.P., 1997. Estimation of myocardial infarct size from plasma myoglobin or fatty acid-binding protein. Influence of renal function. Eur. J. Clin. Chem. Clin. Biochem. 35, 191–198.
Ziebig, R., Lun, A., Hocher, B., Priem, F., Altermann, C., Asmus, G., Kern, H., Krause, R., Lorenz, B., Mobes, R., Sinha, P., 2003. Renal elimination of troponin T and troponin I. Clin. Chem. 49, 1191–1193.