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Characterising a novel biomarker of early myocardial injury

Thomas Edward Kaier

MD MRCP(UK) MBA

A Dissertation submitted for the Degree of

Doctor of Philosophy

To the

University of London

Cardiovascular Division, The Rayne Institute

British Heart Foundation Centre of Research Excellence

Faculty of Life Sciences and Medicine

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'We are all products of our environment; every person we meet, every new experience or adventure, every book we read, touches and changes us, making us the unique being we are.' (C.J. Heck)

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Declaration

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Chapters 2, 3, 4 and 5 have been published in peer-reviewed journals and were reproduced with inline figures, language- and style-adjustments only. The thesis has not been accepted in any previous applications for a degree and all sources of information have been acknowledged. All studies were undertaken in accordance with the regulations of the local ethics boards and with the Declaration of Helsinki.

Thomas Edward Kaier

London, 4th May 2018

Abstract

Background: We previously identified cardiac myosin-binding protein C (cMyC) in coronary venous effluent and developed a high-sensitivity assay by producing an array of monoclonal antibodies and choosing an ideal pair based on affinity and epitope maps. Compared to high-sensitivity cardiac Troponin (hs-cTn), we demonstrated that cMyC appears earlier and rises faster following myocardial necrosis and is also more abundant. Contemporarily, we investigated (i) analytic sensitivity, (ii) whether cMyC can aid in the diagnosis of Acute Myocardial Infarction (AMI) (a) amongst unselected patients presenting to the emergency department (ED) and (b) presenting in a pre-hospital setting, (iii) derived and validated optimal cut-offs for the use of cMyC in a 0/1h algorithm for the rule-out/rule-in of AMI.

Methods: We compared abundance of cMyC to hs-cTn by spiking cardiomyocytes/cardiac tissue into aliquots of human serum. We evaluated the clinical utility of cMyC by calculating the area under the receiver-operating characteristics curve (AUC) in 1,954 patients (17% AMI), for presentation and 1h-change values. Cut-offs were derived using a derivation/validation split, determining optimal thresholds based on NPV/PPV/triage efficiency for >390,000 combinations. Net Reclassification Improvement (NRI) determined immediate triage effectiveness. In 776 patients (22% AMI) sensitivity & specificity were calculated from in-ambulance blood draws using a real and feasible Limit of Detection (LoD) on a point-of-care testing (POCT) device for cTnT and cMyC, respectively.

Results: cTnT, cTnI, and cMyC increased by 3.9ng/L (3.6-4.3), 4.3ng/L (3.8-4.7), and 41.0ng/L (38.0-44.0) per μg of human myocardium. In the ED, the diagnostic accuracy for AMI was comparable between the three biomarkers in baseline blood samples. cMyC increased the diagnostic performance of hs-cTnI but not hs-cTnT 0&1h samples. NRI was up

to 30% better when comparing cMyC to hs-cTnT/I, translating into a more effective triage into rule-out and rule-in of AMI using a single blood test at presentation. The best performing cMyC 0/1h rule-out/rule-in algorithm matched the ESC hs-cTnT/I algorithms in terms of safety, and specificity in comparison to hs-cTnI, but not hs-cTnT. cMyC increased triage-efficiency by 3.9-10.6%. Further, cMyC significantly increases the number of patients eligible for direct rule-out or rule-in based on a single blood test at presentation to the ED.

In the pre-hospital setting, the diagnostic accuracy of cMyC was significantly higher than hs-cTnT (0.839 vs 0.813, p=0.005). The POCT threshold of cTnT (50 ng/L, 10-fold LoD of laboratory assay) achieved a sensitivity of 40.5% [33.6-47.6%]; cMyC (12 ng/L, 30-fold LoD) achieved a sensitivity of 94.8% [91.2-97.7%]. Risk prediction was superior for cMyC at the POCT-detection limit.

Conclusions: hs-cTnT/I and cMyC are exquisitely sensitive biological signals – all assays are able to detect the equivalent of necrosis of a single cardiomyocyte in spiked human serum. cMyC is more abundant than cTnT/I and provides discriminatory power comparable to hs-cTnT/I for the diagnosis of AMI in all-comers, but identifies a greater proportion of patients with AMI in very early presenters. A standout feature is cMyC's ability to more effectively triage patients into rule-out and rule-in categories, with comparable safety endpoints (as with hs-cTnT/I). This distinction is likely related to the documented greater abundance and more rapid release profile of cMyC. If used on a POCT platform, cMyC could significantly improve the early triage of patients with suspected AMI.

List of Abbreviations

Acute Coronary Syndrome (ACS)

Acute Myocardial Infarction (AMI)

Advantageous Predictors of Acute Coronary Syndrome Evaluation (APACE), XIII

American College of Cardiology (ACC)

American Heart Association (AHA)

area under the receiver-operating characteristics curve (AUC)

basic local alignment search tool (BLAST)

British Heart Foundation (BHF), XIII

bundle branch block (BBB)

calmodulin kinase (CAMK)

cardiac magnetic resonance imaging (cMRI)

cardiac myosin-binding protein C (cMyBP-C, cMyC)

cardiac Troponin (cTn)

cardiac Troponin I (cTnI)

cardiac Troponin T (cTnT)

coefficient of variation – analytical imprecision (CVA)

coefficient of variation (CV)

coefficient of variation within-subject (CVI)

Confidence Interval (CI)

coronary artery bypass surgery (CABG)

European Society of Cardiology (ESC)

high-sensitivity cardiac Troponin (hs-cTn)

High-sensitivity Troponin in the Evaluation of Patients with Acute Coronary Syndrome (HighSTEACS), XIII

hypertrophic cardiomyopathy (HCM)

Integrated Discrimination Improvement (IDI)

Interquartile Range (IQR)

kilo-Dalton (kDa)

Limit of Detection (LoD)

lower limit of quantification (LLoQ)

MesoScale Discovery (MSD),

Myocardial Infarction (MI)

negative predictive value (NPV)

Net Reclassification Improvement (NRI)

Non-ST elevation myocardial infarction (NSTEMI)

Percutaneous Coronary Intervention (PCI)

point-of-care testing (POCT)

positive predictive value (PPV)

protein kinase A (PKA)

protein kinase C (PKC)

septal hypertrophy (TASH)

sodium dodecyl sulphate (SDS)

ST elevation myocardial infarction (STEMI)

standard operating procedure (SOP)

surface plasmon resonance (SPR)

upper reference limit (URL)

Related Material

Publications

1. *__Kaier TE__, *Twerenbold R, Puelacher C, Marjot J, Imambaccus N, Boeddinghaus J, Nestelberger T, Badertscher P, Sabti Z, Gimenez MR, Wildi K, Hillinger P, Grimm K, Loeffel S, Shrestha S, Widmer DF, Cupa J, Kozhuharov N, Miró Ò, Martin-Sanchez FJ, Morawiec B, Rentsch K, Lohrmann J, Kloos W, Osswald S, Reichlin T, Weber E, Marber M, Mueller C. Direct Comparison of Cardiac Myosin-Binding Protein C With Cardiac Troponins for the Early Diagnosis of Acute Myocardial Infarction. *Circulation*. 2017;136:1495–1508.
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9. Maznyczka A, **Kaier T**, Marber M. Troponins and other biomarkers in the early diagnosis of acute myocardial infarction. *Postgrad Med J*. 2015 May 22;91(1076):1–10.

Investigator Awards & Prizes

- Finalist, Young Investigator Award, British Cardiovascular Society: From Bench To Improved Diagnosis Of AMI – Cardiac Myosin-binding Protein C. Manchester, 06/2018
- Finalist, Royal Society of Medicine, Research & Innovation Prize, Emergency Medicine Section: Improving chest pain triage by using established and novel markers of myocardial injury; London, 05/2018
- Finalist, Young Investigator Award: American College of Cardiology, Basic & Translation Science section; Orlando, 03/2018
- Finalist at British Heart Foundation – Journal of Clinical Investigation International Symposium: Best scoring posters; London, 01/2018
- Winner of the 2017 EFLM-HyTest Cardiac Marker Award for remarkable scientific work in the field of cardiovascular diseases (European Federation of Clinical Chemistry and Laboratory Medicine); Athens, 06/2017
- European Society of Cardiology (ESC) Congress 2017 – Educational Grant

Presentations & Posters

- **From Bench To Improved Diagnosis Of AMI - Cardiac Myosin-binding Protein C. Kaier TE.** Young Investigator Award Finalist - oral presentation, British Cardiovascular Society, Manchester, 06/2018
- **Improving chest pain triage by using established and novel markers of myocardial injury. Kaier TE.** Finalist - oral presentation, Royal Society of Medicine, Research & Innovation Prize, Emergency Medicine Section, London, 05/2018
- **cMyC - how a novel biomarker could transform chest pain triage. Kaier TE.** Presentation at World Precision Medicine Congress, London, 05/2018
- **Cardiac Myosin-Binding Protein C in the pre-hospital setting – identifying the high-risk patient. Kaier TE, Stengaard C, Williams L, Sørensen JT, Marjot J, Terkelsen CJ, Thygesen K, Marber MS, Bøtker HE.** Young Investigator Award finalist - oral presentation, Annual meeting of American College of Cardiology (ACC.18), Orlando, 03/2018
- **Cardiac Myosin-binding Protein C in the Pre-hospital Setting - Identifying the High-risk Patient. Kaier TE.** Presentation at International Symposium of the British Heart Foundation - Journal of Clinical Investigation Meeting, London, 01/2018
- **Cardiac Myosin-binding Protein C for rule-out and rule-in of AMI. Kaier TE.** Presentation at Danish Biomarker Meeting, Båstad, 11/2017
- **Cardiac Myosin-binding Protein C - a new biomarker for myocardial necrosis. Kaier TE.** Presentation at Association for Clinical Biochemistry and Laboratory Medicine (ACB) Meeting, London, 10/2017

- **Cardiac Myosin-binding Protein C in the Pre-hospital Setting - Identifying the High-risk Patient. Kaier TE, Stengaard C, Williams L, Sørensen JT, Marjot J, Terkelsen CJ, Thygesen K, Marber MS, Bøtker HE.** Rapid Fire Abstract Presentation, European Society of Cardiology, Barcelona, 08/2017
- **Performance of Cardiac Myosin-binding Protein C in the Rule-Out/Rule-In of Acute Myocardial Infarction. Kaier TE, Marber MS, Mayr M.** King's BHF Center of Research Excellence Postgraduate Symposium, 05/2017
- **Direct Comparison of Cardiac Myosin-binding Protein C to Cardiac Troponins for the Early Diagnosis of Acute Myocardial Infarction. Kaier TE, Twerenbold R, Marjot J, Imambaccus N, Boeddinghaus J, Nestelberger T, Wildi K, Rubini Gimenez M, Reichlin T, Marber MS, Mueller C.** Poster presentation, Annual meeting of American College of Cardiology (ACC.17), Washington, 03/2017
- **Cardiac Myosin-Binding Protein C as Alternative to Cardiac Troponin T for the Diagnosis of Acute Myocardial Infarction in the Very Early Phase. Kaier TE, Stengaard C, Marjot J, Sorensen J, Stravropoulou-Tatla S, Terkelsen C, Thygesen K, Marber MS, Bøtker H.** Poster presentation, Annual meeting of American College of Cardiology (ACC.17), Washington, 03/2017
- **Direct comparison of Cardiac Myosin Binding Protein C to Cardiac Troponins for the Early Diagnosis of Acute Myocardial Infarction; Kaier TE, Twerenbold R, Reichlin T, Marjot J, Boeddinghaus J, Nestelberger T, Wildi K, Rubini Gimenez M, Marber MS, Mueller C.** Poster presentation, Annual meeting of American Heart Association (AHA), New Orleans, Nov 2016

- **Impact of a High-Sensitivity Troponin Rapid Rule-in / Rule-out Algorithm on Routine Clinical Practice within the Emergency Department; Kaier TE, Marjot J, Henderson K, Hunter L, Marber MS, Perera D.** Poster presentation at the Annual Conference of the Royal Society of Emergency Medicine, Bournemouth, Oct 2016

Accepted for presentation

- Derivation and Validation of a 0/1h-algorithm to diagnose Myocardial Infarction using Cardiac Myosin-binding Protein C – direct Comparison to hs-cTnI ; Kaier TE. European Society of Cardiology (ESC) Conference 2018 – Rapid Fire Abstract presentation

Teaching/Supervision/Extracurricular

- Supervision of Academic Foundation Trainees: Dr Jack Marjot, Dr Shuai (Huza) Zhang, Dr Luke Williams
- Completion of distance-learning Master of Business Administration (MBA) 03/2017
- Data Science Specialisation using R, John Hopkins University; 2017-2018
- Weekly ECG teaching for 3rd year Medical Students, 2015-2018
- Lecture at 'HeadStart in Cardiology', Royal Free London – Autumn meeting 2016 arranged by the British Junior Cardiologists' Association (BJCA), >100 attendees
- CPD teaching on the use of cardiac biomarkers for Viapath Pathology (audience: biomedical scientists, clinical biochemists), St Thomas' Hospital, 02/2017
- Launch of www.MyosinC.com website
- Completion of ski challenge as part of Coeur Blanc Charity event 2018 (skiing all 52 lifts in Meribel France in a single day: >65 km distance, 15,000 m descend), personally fundraising >£1,800; see http://www.coeurblanc.eu

Funding Bodies

- British Heart Foundation, Fellowship Grant FS/15/13/31320 (£229,175)
- British Heart Foundation, Project Grant Award: joint applicant with Prof Mike Marber (£50,000), awarded 08/2017
- King's College London, Health Accelerator Award 2017: joint applicant with Prof Mike Marber for King's College London Commercial Development Plan award (£200,000), awarded 08/2017
- Charitable Funding – Coeur Blanc Challenge Recipients 2018: successful joint applicant with Prof Mike Marber for support from the charity 'Coeur Blanc' to exclusively raise charitable donations for the cMyC project at the annual ski challenge in March 2018 (funding: approx. £290,000)

Chapter 1. Background to the project and pilot data

Chest pain is a common symptom – according to recent literature, it is responsible for at least 6% of presentations to emergency departments[1–4], as well as 1% of visits to General Practitioners[5] in England and Wales. This amounts to approximately 700,000 emergency department attendances of which approximately one-third are admitted (253,765 in 2011[6]) but only 10% have a final diagnosis of Myocardial Infarction (MI).[7] The financial burden caused by this inability to make a rapid and accurate diagnosis of Acute Myocardial Infarction (AMI) is substantial and is compounded by National Directives such as the 4-hour wait limit in A&E that mandate further assessment as an in-patient. In addition to this financial price, there is also a medical, psychological and social burden as some patients are exposed to antiplatelet and anticoagulant drugs, the psychological strain of acute admission and lasting impacts on employment, mortgage and life assurance, despite eventually having the diagnosis of AMI ruled out. The main reason why this situation has arisen is that fewer patients now have the diagnostic ECG changes of ST-elevation or depression that allow triage at presentation[8,9] – in fact, 68% of all patients eventually diagnosed with an acute coronary syndrome (ACS) present with Non-ST elevation myocardial infarction (NSTEMI)[10]. Consequently, triage has become reliant on the elevation in the blood of the biomarker cardiac Troponin (cTn). This is enshrined in the Universal Definition of Myocardial Infarction[11] by mandating the detection of a cardiac biomarker rise and/or fall for the diagnosis of AMI. This carries over to the European guidelines, which mandate the 'measurement of a biomarker of cardiomyocyte injury, preferably high-sensitivity cardiac Troponin' in all patients with suspected NSTEMI[12]. By the ESC's own admission, the clinical implications of using high-sensitivity (hs) cTn assays include a 2-fold increase of detection of type 2 AMI, ~20% relative increase in detection of

type 1 AMI and 'elevations up to 3-fold the upper reference limit (URL)…may be associated with a broad spectrum of conditions'. The very definition of a hs-cTn assay – according to the International Federation of Clinical Chemistry and Laboratory Medicine Task Force on Clinical Applications of cardiac Bio-Markers (IFCC TF-CB) – includes 1) a CV ≤10% at the 99th centile value and 2) the ability to measure at least 50% of healthy individuals with concentrations above the assay's Limit of Detection (LoD).[13,14] The clinical reality of this advance in assay-technology is that many more patients test 'Troponin-positive', but not necessarily 'AMI-positive' – all in an attempt to overcome the limitations which made cTn inherently unsuited for early diagnosis of acute myocardial injury.

1.1. Historical Background to the Development of hs-cTn assays

Two limitations laid the foundation for the development of increasingly sensitive assay platforms to quantify cardiac Troponin concentrations: 1) In 1999/2000, the American Association for Clinical Chemistry and then the ESC embedded troponin as the biomarker of choice for the definition of MI.[15,16] From a laboratory perspective, this shift was challenging: the 99th centile was used as a binary decision aid to discriminate between AMI and no AMI, and as such the proposed (im-)precision goal was a coefficient of variation ≤10% at the 99th centile. Unfortunately, none of the available assays at the time achieved this performance criterion. 2) The cardiac-restricted troponin isoforms (cTnT and cTnI) are only released slowly after myocardial injury reaching their peak concentration after about 18 hours.[17,18] Clinically, more sensitive assay support earlier decision making in chest pain triage (as significant myocardial injury can be ruled out earlier), and as such provided an additional incentive to increase the analytic performance of cTn platforms.

With the advent of hs-cTn it has become clear that many patients with cardiovascular risk factors and/or underlying cardiac disease have cTn concentrations above the 99th centile in the absence of an acute event. This may, in part, be because – somewhat counterintuitively – the 99th centile shifted as the assays became more sensitive too. Worryingly, these 'chronic' elevations in cTn have a prevalence as high as 50% in those with underlying chronic heart disease.[19] The problem is that these are also the very patients who are at increased risk of AMI. It would therefore seem fairly obvious that when cTn concentration cut-offs are defined by the 99th centile of a healthy population, specificity for AMI will suffer when using a healthy-population-derived 99th centile as the 'upper limit of normal'.[20] This is indeed the case since when the assays are used in this way, as recommended by the American Heart Association (AHA), American College of Cardiology (ACC) and European Society of Cardiology (ESC), specificity for AMI is below 50%.[21] This conundrum has been nicely summarised by Robert Jesse by commenting that 'when troponin was a lousy assay it was a great test, but now that it's becoming a great assay, it's getting to be a lousy test'.[22] As we continue to learn more about the biomarker, it becomes evident that these cTn-elevations do not represent 'false-positive' tests in the traditional sense, but form part of a spectrum of disease, which can lead to acute as well as chronic 'elevations' above the reference limit. (Analytic) False-positive cTn results are thus rather rare, usually due to interference (haemolysis, bilirubin, biotin), antibodies or skeletal muscle disease[23,24] and it is more the perceived lack of 'clinical specificity', leading to confusion when using a 99th centile decision threshold, which can impact effective patient care.

This lack of 'clinical specificity' for a particular disease entity adds to the conundrum, as hs-cTn assays are also not as sensitive as initially hoped since they remain limited by the slow release of troponin. Only the latest ultra-sensitive Troponin assays (and one high-sensitivity

assay) reaches a detection limit that can reliably quantify >97% of (healthy) individuals, but impact on acute care provision has yet to be seen (and, to date, there are few random-access analysers available that can achieve that level of sensitivity).[25,26]

Consequently, the ESC advocates the use of its 0/1hr rule-out/rule-in algorithm only in patients presenting >3 hours after chest pain onset. Further, the algorithm relies on the measurement of tiny delta-change values (<3 ng/L for hs-cTnT, <2 ng/L for hs-cTnI) to determine whether a patient has sustained acute myocardial injury or not. Such delta changes are time and vendor specific, complicating the matter further. This is problematic for two reasons: 1) At least hs-cTnT is subject to marked diurnal variation, characterised by a mean difference of 4 ng/L between morning and evening samples in healthy volunteers;[27] 2) within-subject coefficient of variation values (CV_I) appear to range from 3.4-24% for hs-cTnI and 1.2-48.2% for hs-cTnT even during short-term repeats.[28–30] This calls into question how many patients can actually benefit from 0/1hr rule-out pathways, given that most hs-cTn assays yield a CV_A of around 20% below the 99th centile – which is precisely the concentration range where the delta-change values are used to triage the individual with chest pain (see also Kavsak et al.[31]).

Several publications have reported on the variable effectiveness of the ESC algorithm in clinical practice – many patients have to undergo a second blood draw for correct triage, and only 20-30% of patients benefit from immediate rule-out/-in using the cut-offs published.[32–34] This poses logistical challenges for the Emergency Department. In addition, 30-50% of patients remain in an indeterminate risk zone, labelled 'observe' zone by the ESC, after the second blood draw. This might be related to variable prevalence of AMI, which is fluctuating between 4% in US and >17% in European populations[35]: Prevalence can influence the size of

the observe zone through its impact on sample distribution – most patients with AMI would be ruled-in (as the ESC used thresholds with high positive predictive value (PPV; ≥70%) as cut-off – i.e. patients, who then do not contribute to the observe zone). The smaller the rule-in cohort, the more patients have to be either assigned to rule-out or observe categories. As a significant proportion of patients with cardiovascular comorbidities has quantifiable levels of hs-cTn[19], it is likely that a proportion of these contributes to a large observe zone. To date, very few studies exist that have tested the 0/1h-algorithm prospectively in a real-life clinical environment – most published evidence is derived and validated in retrospective cohorts, which are carefully adjudicated but frequently built on the clinical interpretation of results from contemporary (sensitive) cTn assays.

From the synopsis above it is clear that new biomarkers are needed but the only way they can improve the triage process is if they possesses equivalent cardiac selectivity to cTn but (1) rise more rapidly after acute myocardial injury (advances sensitivity) and/or (2) have a lower 'background' concentration in those with vascular risk factors or underlying chronic heart disease (advances 'clinical specificity', when used for chest pain triage and – in particular – the diagnosis of Acute Myocardial Infarction).

1.2. Cardiac testing on point-of-care – can it be done?

Significant research & development has been undertaken to migrate cardiac troponin assays to point-of-care testing (POCT) analysers – with, to date, limited success owing to inferior analytical sensitivity, precluding the use for rapid rule-out of AMI. Commercially available are e.g. the Roche Cobas h323 handheld instrument or the Abbott i-STAT device. Both have in common that the analytic sensitivity does not reach the laboratory equivalent: The Cobas h323 can detect a laboratory-equivalent value of 50 ng/L (POCT LoD, correct at date of

submission) – about 3-fold the LoQ or 10-fold the LoD of the laboratory assay.[36] The result is reported as 'negative' <50 ng/L, 'positive' at 50-100 ng/L, and quantitatively positive with a numerical value >100 ng/L. The i-STAT is not harmonised with the latest Abbott Architect hs-cTnI assay, and reaches a sensitivity equivalent to the contemporary Abbott cTnI assay, but with discordance in 16% of results.[37]

Other biomarkers have been evaluated: Copeptin, the C terminal part of pro-vasopressin, has been successfully migrated as a biomarker onto a POCT device and evaluated by collaborators in the pre-hospital setting before.[38] An early increase after severe stress aids its use in the early rule-out of AMI, with NPVs ranging from 92.4% to 99.7%, depending on whether it was used in conjunction with (contemporary) troponin or not.[39,40] It appears to add little to hs-cTn assays[38,41], probably owing to a lack of specificity for myocardial injury.

A maybe more suitable, cytoplasmic protein for the diagnosis of acute myocardial injury is heart fatty acid binding protein (H-FABP) – involved in fatty acid transport for mitochondrial oxidation, it was considered more specific to the myocardium than copeptin. Unfortunately, it has since been shown that it is expressed in other tissues too, albeit in smaller concentrations.[42,43] The biomarker peaks early after injury (within 6 hours), however, in several previous publications the marker has been found to be inferior to high-sensitivity cardiac troponin alone and yields a small incremental benefit when used in addition to hs-cTn.[44–47] What might be required for successful migration and translation into clinical practice, either on laboratory or point-of-care testing platforms, is a protein that is as specific to myocardial injury as cardiac Troponin, but exhibits a similar release profile as cytosolic proteins, and is more abundant – both, in the cardiac muscle and the circulation following significant injury. This might overcome the limitations of analytical sensitivity and signal loss, naturally expected from

a miniaturisation of advanced biochemical reactions used in sandwich immunoassays – to be used on handheld, or near-patient testing devices.

1.3. Cardiac Myosin-binding Protein C (cMyBP-C, cMyC)

The ideal biomarker for early diagnosis of an acute coronary syndrome would have a release profile that is temporally analogous to cytosolic proteins (such as creatine kinase, fatty-acid binding protein and myoglobin) but possesses the cardiac-restricted expression of cardiac Troponins. Our group has identified cardiac myosin-binding protein C (cMyBP-C, cMyC; UniProtKB – Q14896) as a candidate marker[48], a cardiac sarcomeric protein which is at least twice as abundant in the heart as cTnI or cTnT[49]. We have shown it is released into the serum after myocardial infarction in the mouse[48] and in patients[50], findings which have been confirmed by others[51].

1.4. Discovery and first description of Cardiac Myosin-binding Protein C (cMyC)

Originally described as the C-protein by Offer et al. in 1973[52], its discovery relied on the characterisation of 'impurities' detected alongside myosin in sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (Figure 1). The resulting bands were labelled alphabetically, the third heaviest being correctly identified at the band corresponding to a molecular weight of 140 kilo-Dalton (kDa). Offer et al. hypothesised that the protein's main function might be that of a core protein, it might control or modify the movement of cross-bridges, or 'serve a purely mechanical function' – preserving integrity and stabilising the filaments.

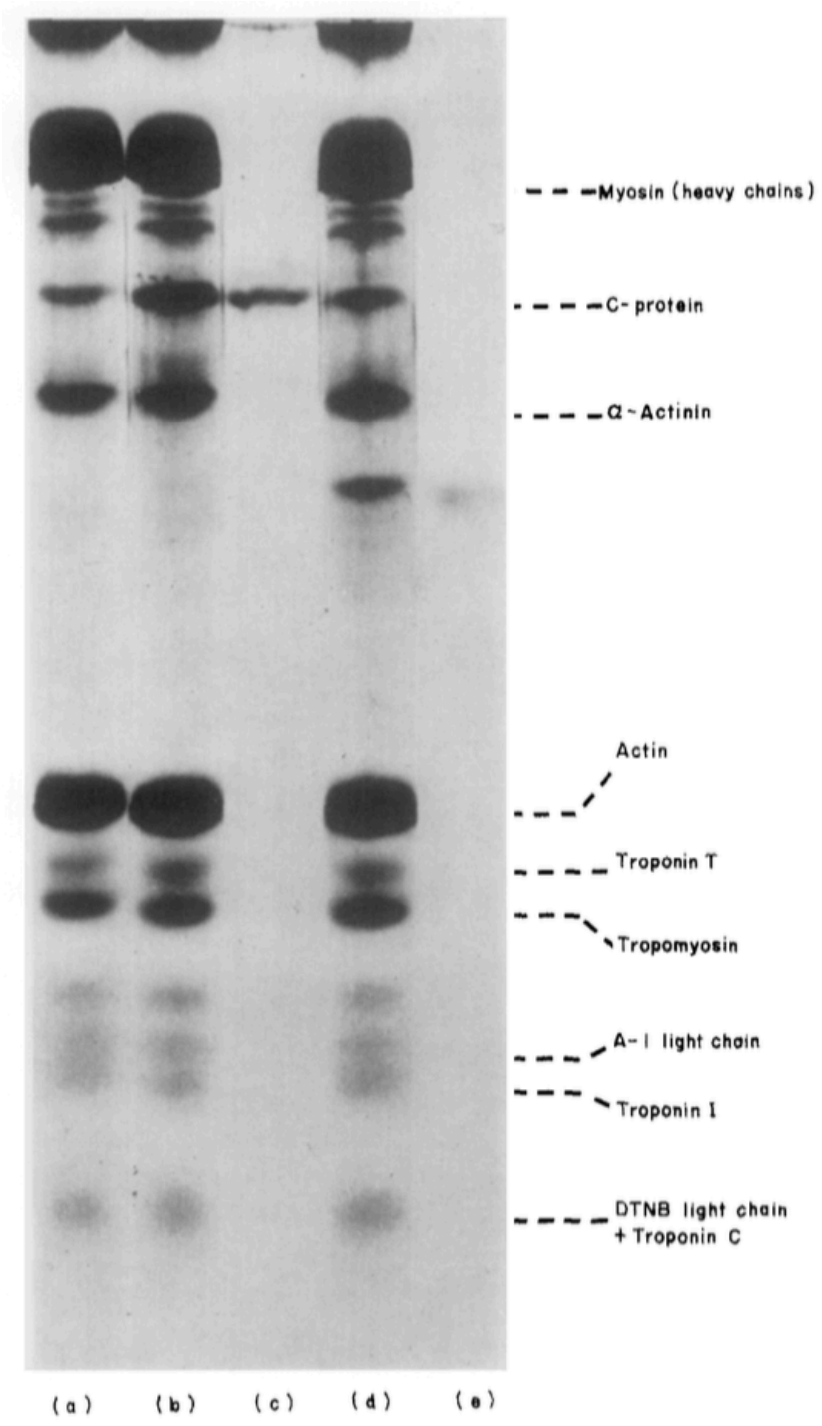


Figure 1 – SDS gel electrophoresis of rabbit myofibrils; a) myofibrils; b) myofibrils + C-protein; c) C-protein; d) myofibrils + F-protein; e) F-protein; adapted from Offer et al.[52]

1.5. Structure of cMyC

Three isoforms of MyBP-C exist in adult human muscle – fast and slow skeletal (encoded by MYBPC1 and MYBPC2 genes on chromosomes 12q23.3 and 19q33.3, respectively), and a cardiac isoform (cMyBP-C, gene MYBPC3 on chromosome 11p11.2).[53,54] Uniquely, the cardiac isoform contains an additional immunoglobulin-like domain at the N-terminus (C0), phosphorylation sites in between domains C1 and C2 (M motif) and a 28-amino acid insertion in the C5 domain. The whole protein consists of 12 domains, of which there are 8 immunoglobulin (IgC2)-like, 3 fibronectin (FN3) domains, plus the M domain mentioned above (Figure 2 + Figure 3).

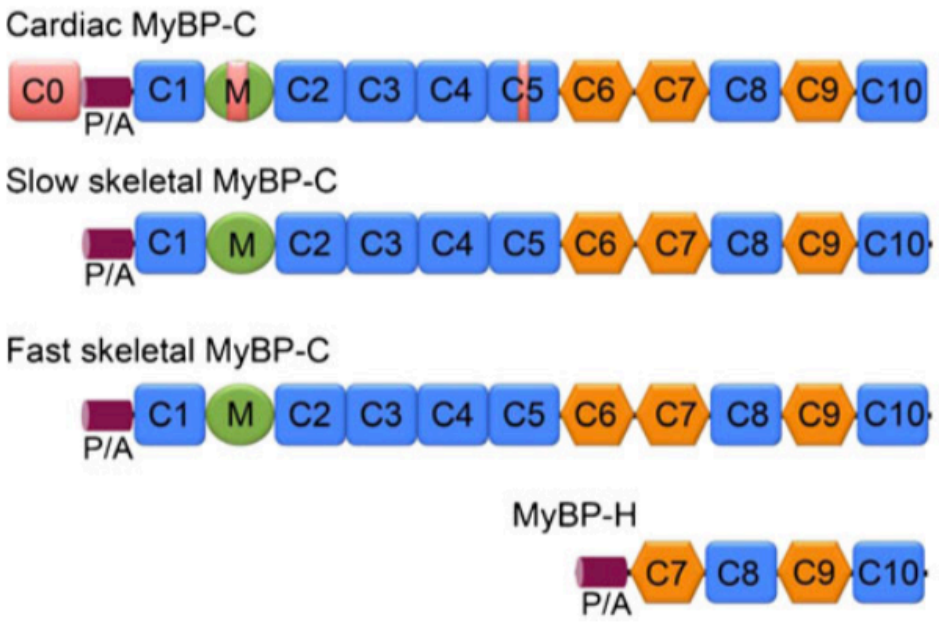


Figure 2 – Cardiac and skeletal isoforms of MyBP-C, adapted from Sadayappan et al.[55]

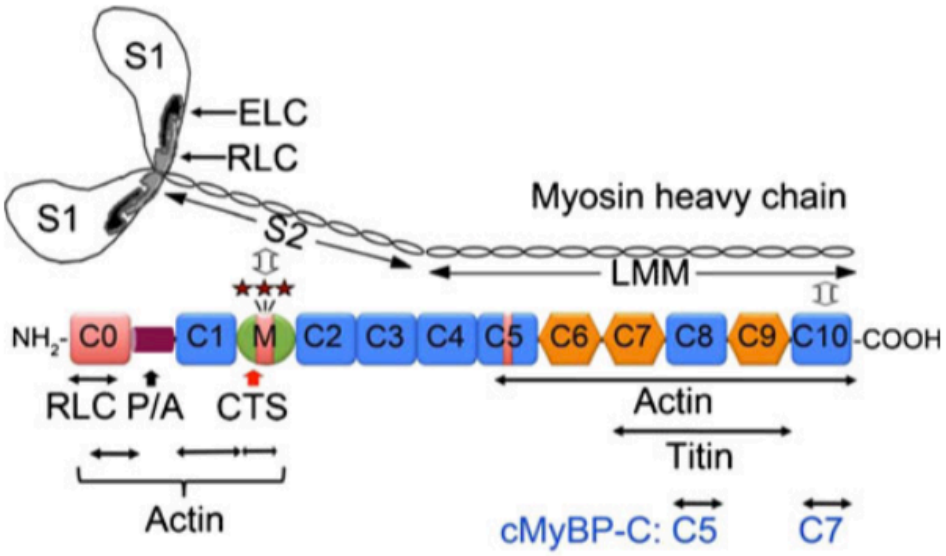


Figure 3 – cMyBP-C structure and regions interacting with myosin, adapted from Sadayappan et al.[55]

The four phosphorylation sites, designated A-D by Gautel et al.[54], are, amongst others, phosphorylated by protein kinase A (PKA; for sites A, B and C), protein kinase C (PKC) and calmodulin kinase (CAMK; for site B).[56–58] It appears that folding of the protein prohibits access to site D.[59]

In 2008 Luther et al., using electron microscopy, imaged 9 bands of cMyBP-C crossing the thick and thin filaments in perpendicular orientation in the C-zones of the A-band (Figure 4).[60]

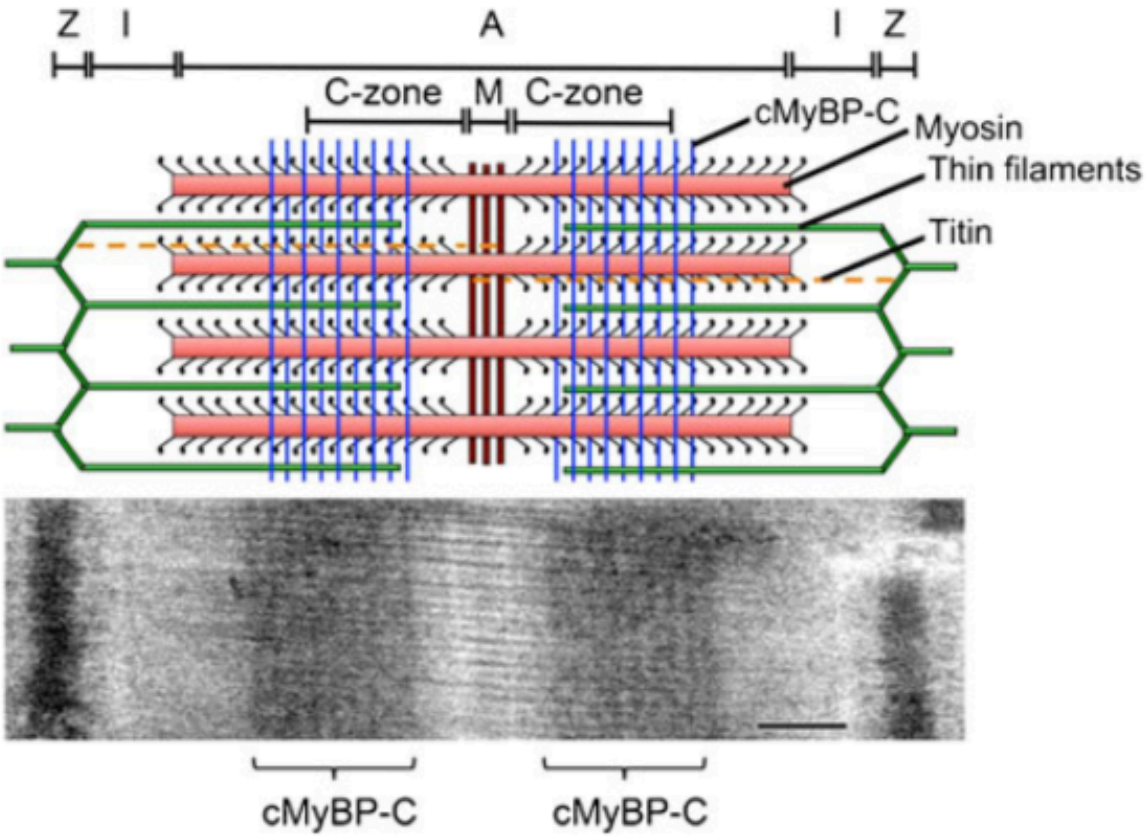


Figure 4 – Cardiac sarcomere (top) and 9 cMyBP bands crossing the A-band in the C-zone, adapted from Sadayappan et al.[55]; electron microscopic image obtained from rat myocardium, immunolabelled with anti-cMyBP-C antibodies (bottom); adapted from Luther et al.[60]

To date, the exact arrangement in the sarcomere remains unclear, and two models are being tested: 1) a trimeric collar model, where three cMyBP-C molecules form a collar around the thick filament core[61], 2) a rod model where cMyBP-C interacts with its C-terminal domains along the thick filament axis, with the N-terminal domains extending towards the thin filament.[62]

1.6. Function of cMyC

The uncertainty regarding the exact structural arrangement is further reflected in an incomplete understanding of the interaction between cMyBP-C and thick and thin filaments. Better understood are the effects of cMyBP-C phosphorylation, which is necessary for normal myocardial function and appears to protect from ischaemic injury.[63,64] These effects are predominantly mediated by phosphorylation at Ser-273, Ser-282 and Ser-302 sites; which diminish after ischaemia/reperfusion injury, or in the context of heart failure and hypertrophy[64], atrial fibrillation[65] or in cardiomyopathies[66]. More specifically, mouse models have shown that loss of phosphorylation (through phospho-ablation by residue substitution) is sufficient to cause hypertrophy and cardiac dysfunction.[64,67]

In the context of normal function, phosphorylation itself drives actin-myosin interaction and subsequently increases cross-bridge cycling rate – which in turn enhances cardiac contractility.[68–71]

1.7. Hypertrophic Cardiomyopathy

Gene defects affecting cMyBP-C have been extensively studied since the first description of two mutations causing hypertrophic cardiomyopathy (HCM) in separate kindreds 1995.[72,73] Better understood are the pathological consequences of gene defects affecting cMyBP-C. HCM affects about 0.25-1% of the population worldwide[74–76], and mutations in cMyBP-C are responsible for about 1/3 of symptomatic cases.[77] There are more than 350 unique mutations affecting cMyBP-C described to date[78] (for an up-to-date list, see uniprot.org[79]), >60% of mutations are C'-truncations – and are, intriguingly, rarely detected by western blot of myocardium from affected HCM patients[77] (in the mouse model, a homozygous C'-truncation results in cMyBP-C null mouse hearts – equivalent to a homozygous knockout). This

observation is attributed to cell surveillance mechanisms that protect affected cells from the adverse effects of the truncated proteins.[80] Thus, the phenotype of HCM is felt to be due to haploinsufficiency (a subtle reduction of the amount of cMyBP-C protein expressed since the healthy allele cannot fully compensate for the lack of protein expressed from the diseased allele).[55] This reduces the overall amount of cMyBP-C expressed, but means the protein that is expressed is normal and unaffected.[81] The other pathogenic variants of cMyBP-C, are missense mutations, resulting in single amino acid substitutions; with a range of associated phenotypes (from benign to severe). While they occur throughout the cMyBP-C protein (Figure 5), the domain linking C0 and C1 (enriched with proline and alanine residues; PA) seems to be exempt. More importantly, in particular with a view to the (later described) developed immunoassay, most missense mutations affect the C-terminal domains beyond C3. It also remains unclear whether, and how, individual missense mutations cause disease. Proposed effects are alteration of domain folding, direct impairment of the cMyBP-C function or, again, haploinsufficiency.[77]

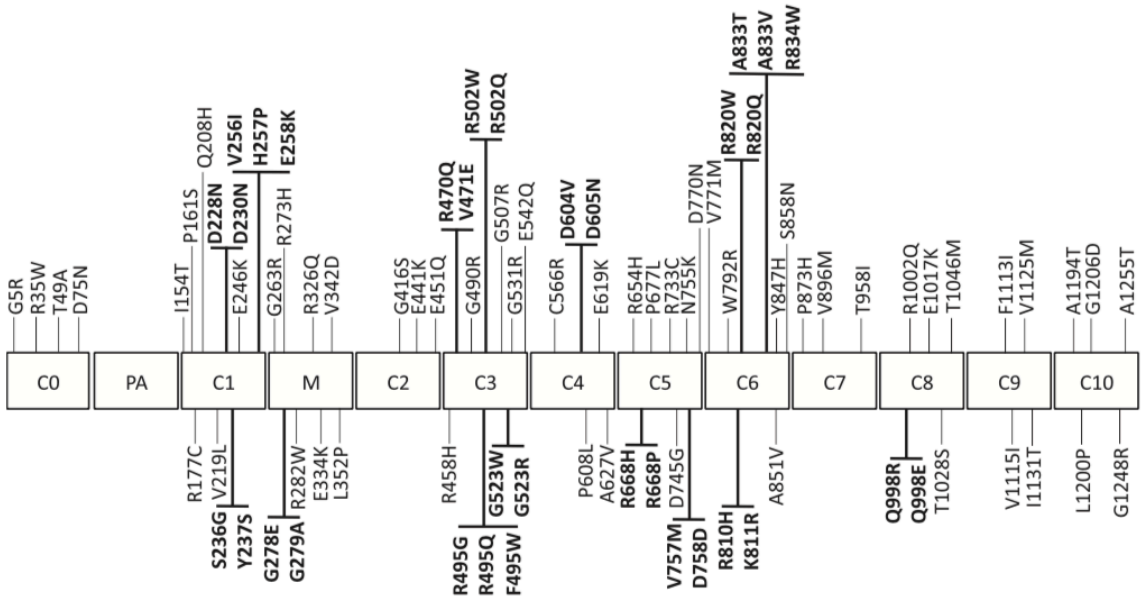


Figure 5 – Missense mutations of cMyBP-C by domain, in bold where there are multiple variants at the same codon. Adapted from Harris et al.[77]

1.8. Development of the in-house cMyC immunoassay

Over the past years the group has been working to improve the analytic performance of the assay for cMyC and initially created an in-house assay with a lower limit of quantification (LLoQ) of 80ng/L – in detail described by Baker et al.[50] In short, 3-month old Balb/c mice were immunized with either 1) 20 μg of recombinant C0C2 domains of cMyC or 2) four overlapping peptides spanning the sequence of the C0 region or the recombinant C5 domain of cMyC. Mouse spleen cells and cells of a myeloma cell line (P3X63Ag8.653) were fused and cultured. Candidate monoclonal antibodies were ranked using surface plasmon resonance (SPR) according to kinetic parameters. Competition studies were carried out comparing individual binding signals of each antibody in the presence of a competitor antibody, with a reduction of the binding signal >50% taken as an indication that the antibodies recognise overlapping epitopes. The best-performing antibodies (Clone 3H8 – 30 μL of 1 μg/mL; clone

1A4 – 30 µl of 1 µg/mL) were selected for the creation of a 'sandwich' electrochemiluminescence assay (MesoScale Discovery (MSD), Sector imager 2400). The standard curve was used to quantify and express cMyC concentrations as ng/L. This achieved an LLoQ of 80 ng/L (Figure 6 & Figure 7).

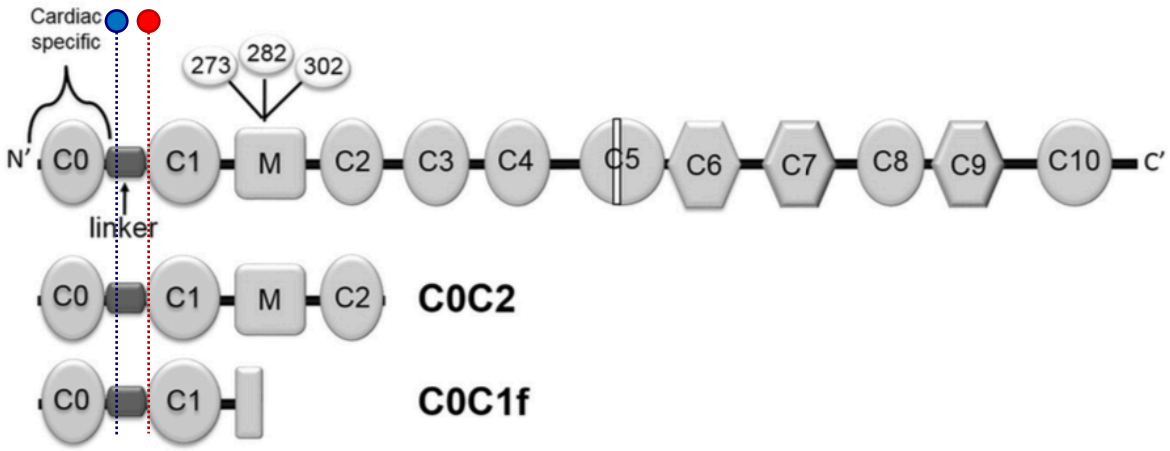
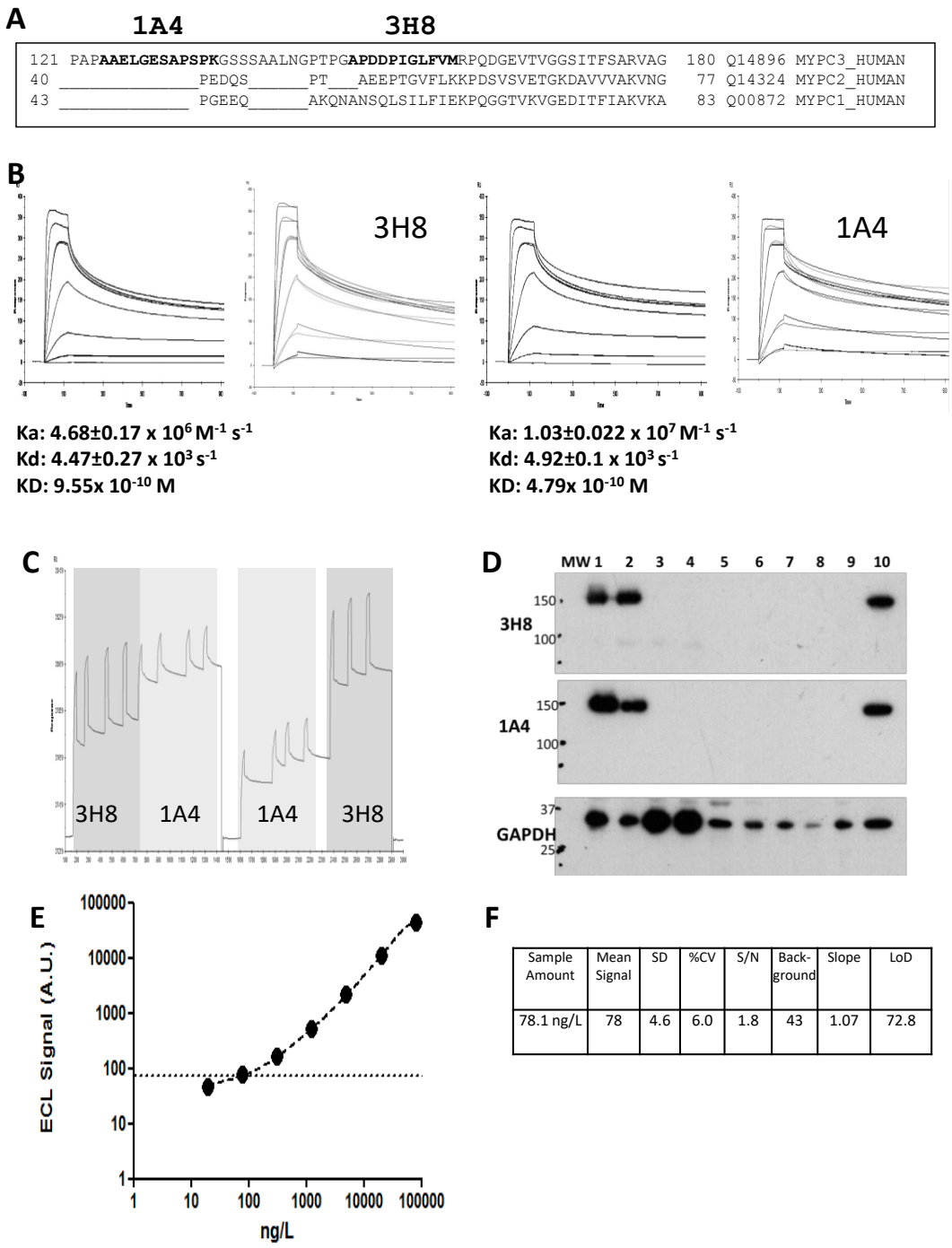


Figure 6 – Structure of full-length cMyBP-C; phosphorylation sites involved in the regulation of myocardial contractility – Ser-273, Ser-282 and Ser-302 – highlighted in the M-domain (where Calpain-dependent cleavage occurs)[82], and commonly detected N-terminal fragments, C0C2 and C0C1f. Binding sites for antibodies 1A4 (blue) and 3H8 (red) are highlighted. Adapted from Lipps et al.[83]



Sample Amount	Mean Signal	SD	%CV	S/N	Back-ground	Slope	LoD
78.1 ng/L	78	4.6	6.0	1.8	43	1.07	72.8

Figure 7 – The development of a quantitative immunoassay for human cMyC in serum. Panel A: Sequence alignment of cMyC with skeletal myosin binding protein C isoforms. The sequence recognised by monoclonal anti-cMyC antibodies 1A4 and 3H8 are shown in bold. The antibodies bind to cardiac-restricted sequences with organ specificity further verified by immunoblots (see Panel D). Panel B: SPR kinetic sensorgrams demonstrating

the kinetic parameters of clone 3H8 (left) and 1A4 (right). These antibodies were selected from over 50 hybridomas, and both antibodies are of high affinity. Panel C: Epitope competition sensorgram of 1A4 and 3H8 binding to the C0C2 region of cMyC conjugated to a CM5 biosensor chip. Although antibodies recognise near adjacent epitopes, there is no appreciable interference between them. Near adjacency is needed since cMyC is fragmented in the circulation raising the possibility of separation of capture and detection epitopes if they were widely spaced. Panel D: Immunoblot of rat and human tissue demonstrating specificity of 3H8 and 1A4 monoclonal antibodies. GAPDH was used as a loading control. Samples 1-9 are various rat tissue (1=ventricle, 2=atria, 3=rectus abdominus, 4=soleus, 5=spleen, 6=kidney, 7=aorta, 8=liver, 9=brain) and 10 is human ventricle. Panel E: Representative C0C2 standard curve from cMyC ECL assay indicating the limit of detection (dashed line). This, in-house assay on a MesoScale Discovery enhanced chemiluminescent detection platform, was used to measure cMyC appearance and disappearance in Figures 2 and 3 below. Panel F demonstrates the performance characteristics of the assay, with a LoD of approximately 80 ng/L. Figures adapted from Baker et al.[50]

1.9. In vivo models of myocardial infarction

Using the quantitative immunoassay described above, cMyC release kinetics were investigated in patients with ST-elevation myocardial infarction (STEMI, n = 20), undergoing therapeutic ablation of septal hypertrophy (TASH, n = 20) for hypertrophic cardiomyopathy (HCM; Figure 8), or having coronary artery bypass surgery (CABG, n = 20; Figure 9). In both models of myocardial infarction (STEMI, TASH), we detected an earlier peak of cMyC when compared to a high-sensitivity cTnT assay (STEMI, 9.3 ± 3.1 vs 11.8 ± 3.4 h, $p < 0.007$; TASH, 9.7 ± 1.4 vs 21.6 ± 1.4 h, $p < 0.0001$), a quicker accumulation (during first 4 h after TASH, 25.8 ± 1.9 vs 4.0 ± 0.4 ng/L/min, $p < 0.0001$) and faster disappearance (post-CABG, decay half-time 5.5 ± 0.8 vs 22 ± 5 h, $p < 0.0001$).[50]

These data suggest cMyC may fulfil the above described criteria needed to improve chest pain triage, which is currently heavily reliant on troponin. Figure 8 shows cMyC rises more rapidly

after acute myocardial injury. Figure 9 shows cMyC falls more rapidly and this may translate into a lower background concentration in those with vascular risk factors and/or underlying chronic heart disease. Unfortunately, these data also show that the in-house assay does not have the analytic performance needed to measure cMyC in serum from healthy patients. This is required to measure the population-defined 99th centile. Hence, the host department commissioned a contract research company to develop an assay using the same capture/detection monoclonal antibodies, but on a high-sensitivity platform.

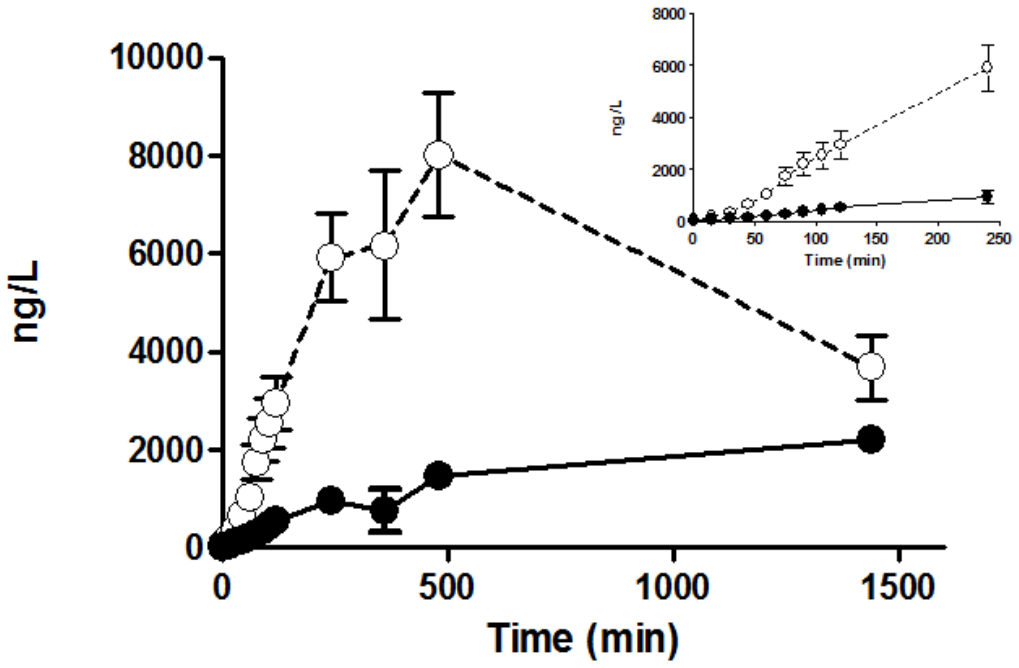


Figure 8 – The accumulation of cMyC v cTnT after myocardial injury caused by intracoronary ethanol. Venous blood was collected frequently over the first 2, and up to 24, hours after therapeutic alcohol septal ablation for hypertrophic cardiomyopathy (TASH) using ethanol infused selectively into a septal perforating branch coronary artery. Summary data of absolute quantification of cMyC (open symbols) v cTnT (closed symbols) over time following TASH (n =20). Inset figure is a zoom of the first 240 mins. Over this time interval cMyC accumulates

in the serum approximately 6-times faster than cTnT (slope 25.8±1.9 v 4.0±0.4ng/L/min, p<0.0001). Figures adapted from Baker et al.[50]

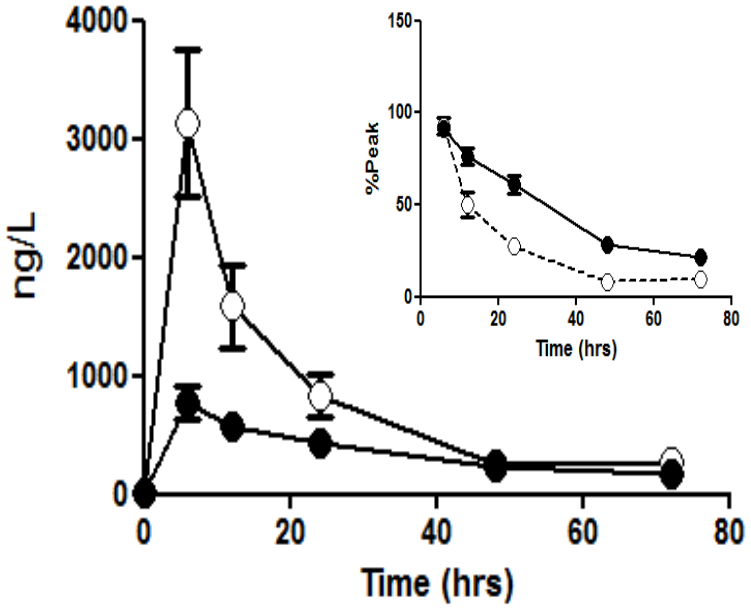


Figure 9 – The accumulation of cMyC (open symbols) v cTnT (closed symbols) after myocardial injury caused by surgical revascularisation. Venous blood was collected over 3 days following CABG. Summary data of absolute quantification of cMyC v cTnT over time following CABG (n =20). Inset figure is a zoom of the last 5 time points expressed as a % of peak concentration achieved in each patient. This normalization was used to remove the visual bias caused by the greater absolute concentration of cMyC. The decay half-time for cMyC is considerably shorter than for cTnT (5.5±0.8 hrs v 22±5 hrs, P<0.0001). Figures adapted from Baker et al.[50]

1.10. Development of a high-sensitivity cMyC immunoassay

We previously reported on the development of a high-sensitivity assay using the same pair of monoclonal antibodies as described above.[84] The new assay was developed on the Erenna platform (originally by Singulex Inc., California, USA), using the same antibody-pair (1A4, 3H8) used for the in-house assay. This achieved a lower limit of detection of 0.4 ng/L, LoQ of 1.2 ng/L (20% coefficient of variation (CV), and ≤10% CV at 99th centile). This was used to

measure cMyC in 360 stable patients without significant obstructive coronary artery disease and [hs-cTnT] <14 ng/L. cMyC was quantifiable in 359 patients (compared to 85 and 307 patients with quantifiable hs-cTnT and hs-cTnI levels, respectively) and correlated positively with both Troponin assays (R = 0.56 for cTnT, R= 0.77 for cTnI). Further, this facilitated the calculation of the 99th centile for cMyC at 87 ng/L. The study demonstrated in stepwise multiple logistic regression analysis that age, gender, creatinine, pulmonary hypertension, as well as the use of certain medication (statins, loop diuretics, beta-blockers) all statistically predicted cMyC concentrations ($R^2 = 0.198$, $p<0.05$).

1.11. Risk of false-negative results in HCM patients?

As summarised above, cMyBP-C mutations causing HCM are frequent but cause either truncation mutations resulting in haploinsufficiency (thus limited expression of the protein variant) or missense mutations with a phenotypically broad range. However, most missense mutations affect the C-terminal domains of cMyBP-C, and the (purposeful) antibody-alignment with the N-terminal domains C0-C1 makes it very unlikely that the newly developed assay is at risk of missing cMyBP-C elevations in a HCM patient. The only known variant affecting a domain bound by our antibodies is MET-158, substituting Valine with Methionine at position 158 (target of 3H8) – felt to be a non-pathogenic polymorphism.[85,86] The affected amino acid sequence is highlighted below – Figure 10.

1A4

100 EPMLAPAPAP AEATGAPGEA PAP**AAELGES APSPK**GSSSA

3H8

140 ALNGPTPG**AP DDPIGLF<u>V</u>MR** PQDGEVTVGG SITFSARVAG

Figure 10 – Amino acid sequence of cMyBP-C with variant MET-158 underlined; Antibodies 1A4 (blue) and 3H8 (red) at binding location

1.12. A Case for Testing a Novel Biomarker of Myocardial Injury?

Chest pain triage is fraught with difficulties as physicians are increasingly caught at the interplay of sensitivity and specificity. Arguably, tissue-specificity of cTn is close to 100%[24], but 'clinical specificity' for AMI is hampered by using a 99th centile as decision threshold. The technological advances in developing cTn assays to high-sensitivity tests come at the expense of losing this diagnostic specificity as analysers are increasingly able to provide quantifiable cTn levels in almost every individual. The emergency physician requires ultimate sensitivity and thus handles complex rule-in/rule-out algorithms to optimise care for the patient with suspected Acute Coronary Syndrome (ACS) at the front-door of the hospital. But ever-increasing sensitivity cannot be the sole answer in an attempt to overcome inherent biological disadvantages of cTn – even with high-sensitivity assays, the ESC advocates a delay of 3 hours after chest pain onset for the first blood draw to take place. This has to do with the biology of Troponin-release, and is not assay-specific, but results in many patients being caught up in an 'observe' zone of indeterminate risk. Without doubt, evermore-sensitive assays will bring a new reality of biomarker-interpretation to acute medical services around the world – the always-quantifiable level of a cardiac biomarker ought to be interpreted in the context of the clinical presentation, as opposed to an antiquated black & white approach.

cMyC is a promising novel biomarker of myocardial injury, which has favourable release-kinetics to act as a better adjudicator of acute versus chronic myocardial injury. The faster rise ought to yield a positive result (for rule-in of AMI) earlier; an overall more dynamic release profile aids the early discrimination of a delta-change which is both analytically and clinically meaningful, from a chronic elevation of a cardiac biomarker which is directly correlated with (increasing) age and the number of comorbidities (thus ruling-out AMI). The ultimate goal

being the reassurance of a larger proportion of patients with suspected, but now excluded, myocardial infarction. This would focus active care on the patients with confirmed acute myocardial injury – ideally within a timeframe that fulfils the expectations of healthcare policy and the worried patient.

This thesis aims to comprehensively compare the performance of our cMyC assay against the best commercially available signals – hs-cTnT (Roche Elecsys) and hs-cTnI (Abbott Architect): in (i) the quantification of myocardial tissue injury (Chapter 2), (ii) the immediate diagnosis of AMI, (iii) the risk-stratification and triage of early presenters and all-comers to the emergency department and (iv) the derivation and validation of a rapid rule-out/rule-in algorithm for effective triage of patients with suspected AMI. Finally, we will investigate the use of cardiac biomarkers in the Emergency Department of a central London hospital, allowing for the estimation of clinical impact cMyC could have on chest pain triage.

Chapter 2. Quantifying the release of biomarkers of myocardial necrosis from cardiac myocytes and intact myocardium

The findings were published previously and are reproduced with amendments for inclusion in the thesis.

Clin Chem 2017;63:990–996. Running Head: Biomarkers of necrosis and amount of MI

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Keywords:

Myocardial infarction, Biomarkers, High-sensitivity assay

2.1. Abstract

Background: Myocardial infarction is diagnosed when biomarkers of cardiac necrosis exceed the 99th centile, although guidelines advocate even lower concentrations for early rule-out. We examined how many myocytes and how much myocardium these concentrations represent. We also examined if dietary troponin can confound the rule-out algorithm.

Methods: Individual rat cardiac myocytes, rat myocardium, ovine myocardium or human myocardium were spiked into 400 µL aliquots of human serum. Blood was drawn from a volunteer after ingestion of ovine myocardium. High-sensitivity assays were used to measure cardiac troponin T (cTnT, Roche, Elecsys) troponin I (cTnI, Abbott, Architect) and myosin-binding protein C (cMyC, EMD Millipore, Erenna).

Results: The cMyC assay could only detect the human protein. For each rat cardiac myocyte added to 400 µL of human serum, cTnT and cTnI increased by 19.0 ng/L [95% CI 16.8–21.2] and 18.9 ng/L [95% CI 14.7–23.1], respectively. Under identical conditions cTnT, cTnI and cMyC increased by 3.9 ng/L [95% CI 3.6-4.3], 4.3 ng/L [95% CI 3.8-4.7] and 41.0 ng/L [95% CI 38.0-44.0]) per µg of human myocardium. There was no detectable change in cTnI or cTnT concentration after ingestion of sufficient ovine myocardium to increase cTnT and cTnI to $\approx 1 \times 10^8$ times their lower limits of quantification.

Conclusions: Based on pragmatic assumptions regarding cTn and cMyC release efficiency, circulating species and volume of distribution, 99th centile concentrations may be exceeded by necrosis of 40 mg of myocardium. This volume is much too small to detect by non-invasive imaging.

2.2. Introduction

Cardiac troponin (cTn), released in the setting of myocardial necrosis, is engrained in the universal definition of myocardial infarction.[11] This definition incorporates a diagnostic threshold concentration at the 99th centile for the general population. More recent guidelines suggest rule-in and rule-out cut-offs widely spaced around the 99th centile and utilize small changes in concentration between first and second blood draw (the delta values) to identify acute myocardial injury.[12] The guideline published by the European Society of Cardiology lists delta values derived from observational studies[87–89] and advocates changes in the concentration of hs-cTnT (Roche) <3ng/L and hs-cTnI (Abbott) <2 ng/L, to identify patients for early discharge (rule-out). The low magnitude of the absolute and delta concentrations used to rule-out MI have stimulated debate.[90–93] To our knowledge, no previous study has established how many myocytes, or how much myocardium, needs to undergo necrosis to exceed the 99th centile, rule-out cut-off or rule-out delta values. We set out to address this question by simulating myocardial injury using defined numbers of cardiac myocytes and quantities of myocardium spiked into human serum.

2.3. Methods

2.3.1 Rat cardiomyocytes

Adult rat ventricular myocytes were isolated from the hearts of male Wistar rats weighing ~200–250g (B&K Universal Ltd.) by a collagenase-based enzymatic method as described in De Nicola et al.[94] In brief, hearts were excised from terminally anesthetized and heparinized (60 mg/kg sodium pentobarbitone and 100U sodium heparin, intraperitoneally) rats. Excised hearts were immediately cannulated and initially perfused for 5 min with HEPES – Tyrode solution containing following (mmol/L): 130 NaCl, 4.5 $MgCl_2$, 0.4 NaH_2PO4, 0.75 $CaCl_2$, 4.2

HEPES, 20 Taurine, 10 Creatine and 10 Glucose. Hearts were then consecutively perfused with Ca^{2+}-free HEPES – Tyrode solution containing 100 μmol/L EGTA (10 min) and HEPES – Tyrode solution containing 100 μmol/L $CaCl_2$ and 1 mg/mL Type II collagenase (Worthington Biochemical Corp., 8 min). All solutions were gassed with 100% O_2 and maintained at 37 °C. Hearts were then removed from the perfusion apparatus, the ventricles were cut into small pieces, and agitated for a further 7 min at 37 °C. Isolated myocytes were separated from the undigested ventricular tissue by filtering through 200-micron nylon gauze, and the cells were allowed to settle by gravity (8 min). The supernatant was removed and replaced with HEPES – Tyrode solution containing 1% BSA and 500 μmol/L $CaCl_2$. Myocytes were again allowed to settle, the supernatant was removed, and the cells were finally pooled and re-suspended in 30 ml of HEPES – Tyrode solution containing 1 mmol/L $CaCl_2$. The pooled isolated myocytes were pelleted by brief centrifugation at 50 × g and washed at room temperature with modified M199 culture medium (Invitrogen) containing 2 mmol/L Creatine, 2 mmol/L carnitine and 5 mmol/L taurine supplemented with 100 IU/mL penicillin/streptomycin. Following further centrifugation at 50 × g, myocytes were finally re-suspended in modified M199 medium. Myocytes were then plated onto 6-well culture plates pre-coated with laminin and allowed to attach for 90 min in an incubator (37 °C, 5% CO2). Unattached cells were removed after pre-plating for 2hrs and the culture medium was replaced with fresh modified M199 medium, and the cells were maintained overnight. Cultured rat cardiomyocytes were subsequently re-suspended in Tyrode solution. Trypan blue staining revealed a viability of 45%. Cells were allowed to settle in Tyrode's solution. The solution was subsequently removed and replaced with 10ml Tris (20mmol, pH 7.5), then centrifuged at 1000 rpm for 3 minutes. The wash supernatant was discarded, and the cell pellet re-suspended in fresh Tris solution. Cell count was calculated using an automated cell counter (Bio-Rad

TC20™). The results of the automated cell counter were calibrated to manual cell counts attained by visual inspection with a haemocytometer. The 10mL of resuspended pellet was then ultrasonicated (6 x 10s bursts on ice, with 10s intervals on ice). Dilutions of this solution were then spiked into 400 μL of banked human serum.

Experiments with cultured rat myocytes were repeated using four different human serum samples to account for donor-dependent interaction between human serum and rat protein. Experiments were repeated once for two of the serum donors using a different stock solution of cultured myocytes, so as to account for variation between culture preparations. Cells were spiked into serum in increments of 10 cells, ranging from 1 cell to 90 or 100 cells (limited by the availability of cells and serum). These repeat experiments resulted in a total of 62 samples for assessment of linear correlation.

2.3.2 Human myocardium

Human myocardium was obtained from an explanted failing heart under Ethical Approval from the Royal Brompton and Harefield Trust BRU Biobank which complies with the Helsinki declaration of 1975. The tissue was transported in cardioplegia, and frozen at -80ºC. Frozen myocardium was weighed (the exact weight was recorded), and the tissue was crushed in a percussion mortar for 10 seconds. Buffer solution (50ml Tris pH7.5 with 1 tablet protease inhibitor [complete EDTA-free, Roche]) was added to the pulverized tissue (1 mL of buffer per 100 mg of tissue). The subsequent solution was ultrasonicated on ice (6 x 10s bursts on ice, with 10s intervals on ice). Following ultrasonication, the solution was centrifuged at 25000 rcf for 30 mins at 4 °C. The supernatant was frozen in liquid nitrogen and then stored at -80 °C. Dilutions of this solution were then spiked into 400 μL of banked human serum.

Experiments using human myocardium were limited in number by availability of myocardial tissue, and were repeated using serum obtained from three different donors. Myocardium was spiked into serum at a concentration of 1μg, 10 μg, and increments of 10 μg up to 100 μg. With addition of blank controls (serum + buffer), this resulted in a total of 36 samples for assessment of linear correlation.

2.3.3 Dietary troponin consumption:

Ovine left ventricular myocardium was boiled in water for 3 hours and then mechanically homogenized using a glass hand-held homogenizer. Buffer solution (50 mL Tris pH7.5 with 1 tablet protease inhibitor [complete EDTA-free, Roche]), was added to the homogenized tissue (1 mL of buffer per 100 mg of tissue). The subsequent solution was ultrasonicated on ice (6 x 10s bursts on ice, with 10s intervals on ice). Following ultrasonication, the solution was centrifuged at 25000 rcf for 30 mins at 4 °C. The supernatant was frozen in liquid nitrogen and then stored at -80°C. Dilutions of this solution were then spiked into 400 μL of banked human serum to generate a calibration curve. A healthy human volunteer with a baseline serum troponin (T and I) below the limit of detection had a 200 g dietary load of ovine left ventricular myocardium boiled for 3 hours. Serial venepuncture was performed from an antecubital fossa vein at 15, 60, 120, 180, 240, 1320, 1640 minutes after ingestion.

2.3.4 Biomarker measurement

The concentrations of cardiac troponin I (cTnI) and cardiac troponin T (cTnT) were measured using contemporary high-sensitivity assays (Abbott ARCHITECT [limit of detection (LoD) 1.9 ng/L] and Roche Elecsys [LoD 5 ng/L], respectively).[13,95,96] cMyC was measured by EMD

Millipore on the Erenna® platform using proprietary reagents as recently described [LoD of 0.4 ng/L].[84] A triplicate standard curve was run and used to interpolate the data.

For all samples, the biomarker concentration in the blank control was subtracted from the total biomarker concentration of each sample, to control for variation in troponin concentrations within the human serum or the background signal generated by buffer alone. Samples of Tris buffer added to banked human serum (serving as controls for each experiment) returned cTnT values between LoD and 6.98 ng/L, cTnI values between LoD and 5.00 ng/L, and cMyC values between 11.37-26.78 ng/L.

Likely compatibility of the high sensitivity troponin assays with rat cardiac troponin was assessed by basic local alignment search tool (BLAST) comparison of the amino acid sequence of rat troponin and the detection/capture epitopes for the high-sensitivity assay antibodies (supplemental Figure 14). The amino acid sequence for these epitopes was largely conserved between the human and rat proteins. This molecular suggestion of compatibility was borne out in the strong signal detected by the assays in rat and ovine cardiac tissues.

2.3.5 Statistical analysis

Linear regression analysis was used to assess correlation, and standardized residuals greater than ±3 standard deviations were excluded as outliers (n=1). Statistical analysis was conducted using SPSS version 22 (IBM Corp) and R version 3.3.0 (GUI 1.68, The R Foundation for Statistical Computing).

2.4. Results

The cMyC assay did not detect rat cMyC since the capture and detection antibodies are directed at human-specific sequences (Figure 7[50]). There is a strong linear correlation between

rat cardiomyocyte number and cTn concentration (cTnI; R^2=0.58, *P*<0.001, n=61) (cTnT; R^2=0.83, P<0.001, n=62; Figure 11). We were able to detect, in 400 μL of serum, a cTn increase resulting from a single cardiomyocyte. The slope coefficients for both cTnI and cTnT were similar (cTnI; slope = 18.9 $ng.L^{-1}$/cell [95% Confidence Interval (CI) 14.7–23.1]) (cTnT; slope = 19 $ng.L^{-1}$/cell [95% CI 16.8–21.2]) and the lines of regression did not deviate significantly from the origin (cTnI y-intercept = -44.6 $ng.L^{-1}$ [95% CI -128.8-39.5] and cTnT y-intercept = 24.4 $ng.L^{-1}$ [95% CI -18.9-67.7]) . One outlier was identified during linear regression with a standardized residual greater than 3 standard deviations. No other outliers were identified, and in the context of strong linear correlations either side of this point, this outlier was assumed to represent a user-operated pipetting error.

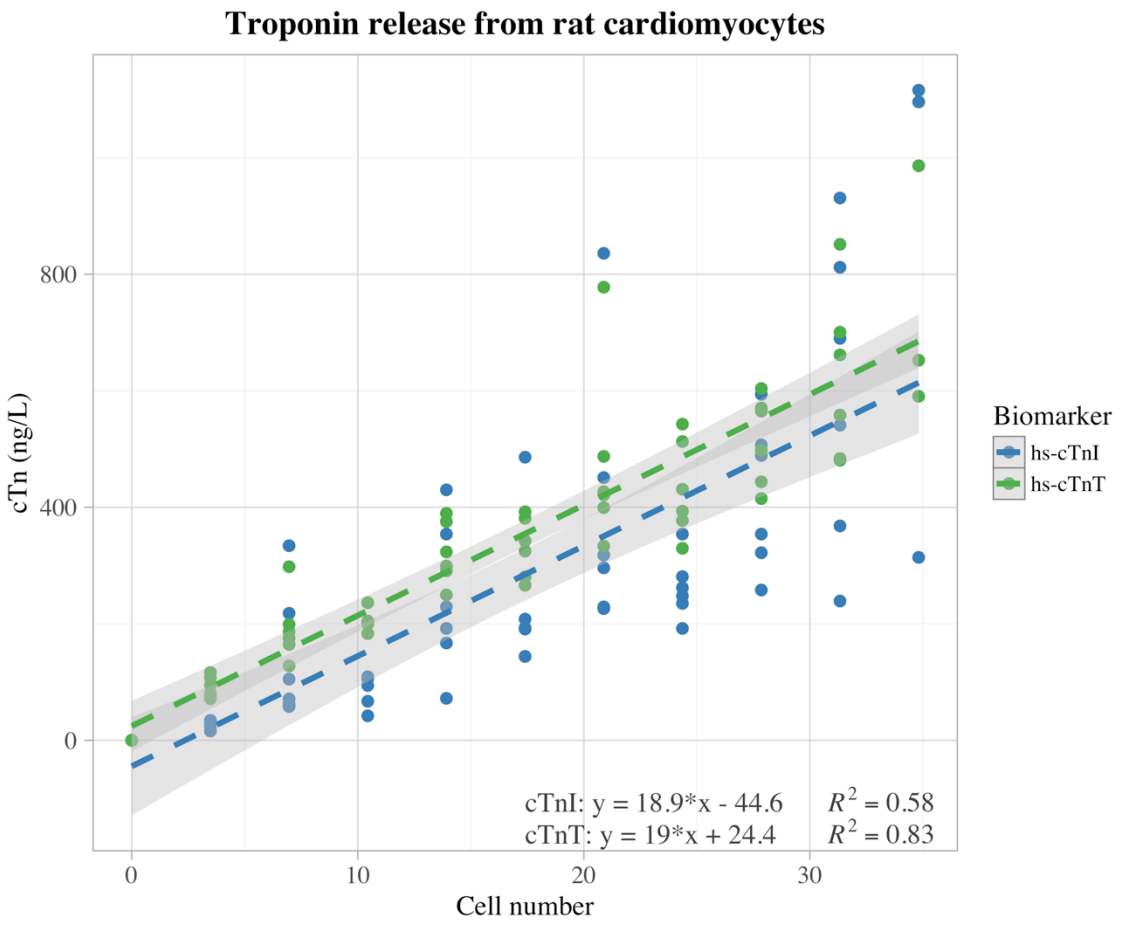

Figure 11 – Graph showing linear regression between number of rat cardiomyocytes and resultant cardiac biomarker concentration as measured by high-sensitivity assays (hs-cTnI and hs-cTnT) in 400 µL of human serum; cTnI (n=61, excluding one outlier; y=18.9 [95% CI 14.7–23.1] *x – 44.6 [95% CI -128.8-39.5]) cTnT (n=62; y=19 [95% CI 16.8–21.2] *x – 24.4 [95% CI -18.9-67.7]), both with spikes into serum from 4 different individuals. Light grey shading depicts the boundaries of the 95% confidence intervals, with dark grey illustrating their overlap

In experiments using human myocardium, both the cardiac troponins and cMyC were strongly linearly correlated with mass of myocardium (cTnI; R^2=0.92, P<0.001, n=36, slope = 4.3 ng.L^{-1}/µg [95% CI 3.8-4.7], y-intercept = 4.4 ng.L^{-1} [95% CI -20.1-28.8]), (cTnT; R^2=0.93, P<0.001, n=36, slope = 3.9 ng.L^{-1}/µg [95% CI 3.6-4.3], y-intercept = 19.0 ng.L^{-1} [95% CI -1.6-39.5]),

(cMyC; R^2=0.96, P<0.001, n=36, slope = 41.0 ng.L^{-1}/µg [95% CI 38.0-44.0], y-intercept = 91.1 ng.L^{-1} [95% CI -79.3-261.4], Figure 12).

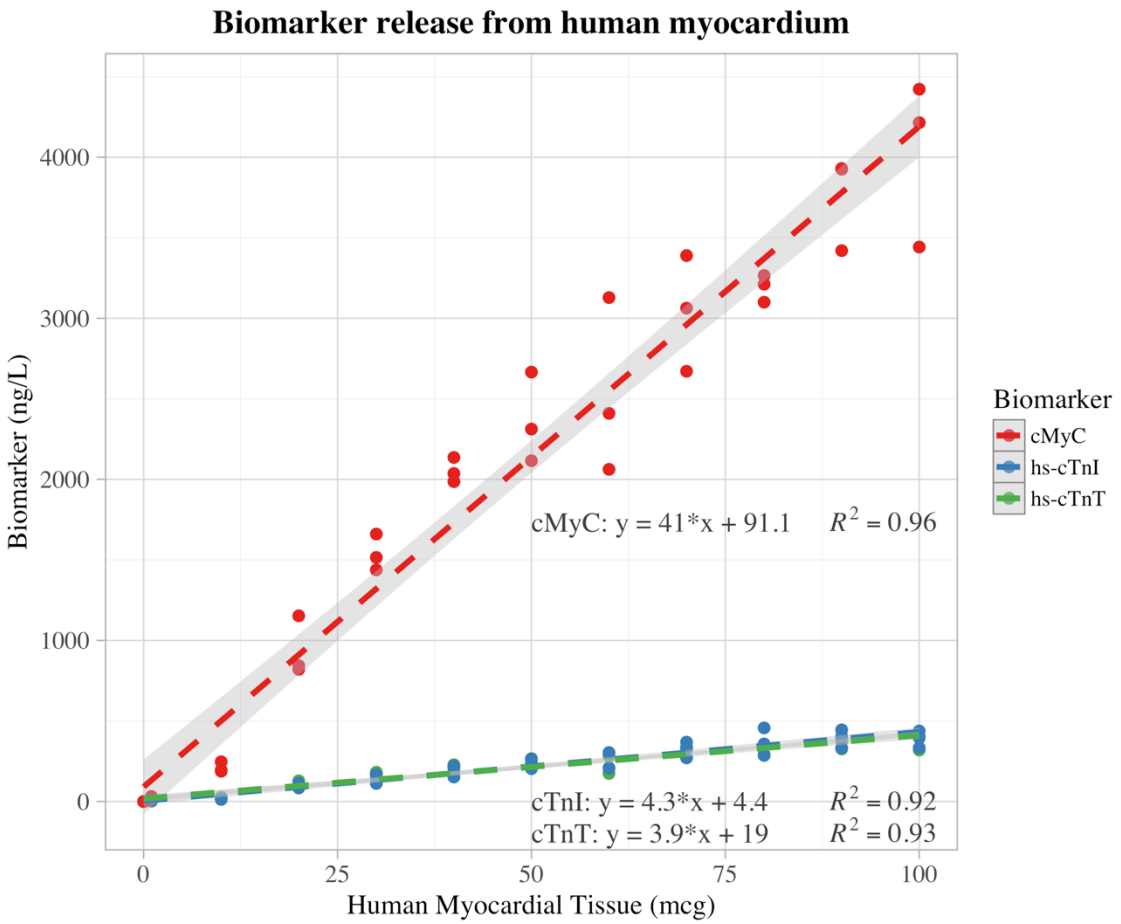

Figure 12 – Graph showing linear regression between mass of human myocardium and resultant cardiac biomarker concentration as measured by high-sensitivity assays (hs-cTnI and hs-cTnT) in 400 microliters of human serum; n=36 for each biomarker, each with spikes into serum from 3 different individuals. Light grey shading depicts the boundaries of the 95% confidence intervals. Regression equations: cTnI: y = 4.3 [95% CI 3.8-4.7] *x + 4.4 [95% CI -20.1-28.8], cTnT: y = 3.9 [95% CI 3.6-4.3] *x + 19 [95% CI -1.6-39.5], cMyC: y = 41 [95% CI 38.0-44.0] *x + 91.1 [95% CI -79.3-261.4]

Cooked ovine myocardium had a much greater troponin content than human myocardium, and a robust linear correlation was established between mass of myocardium and troponin

release (hs-cTnI; R^2=0.992, P<0.0001, n=12, slope = 4928 ng.L^{-1}/µg [95% CI 4616-5241]), (hs-cTnT; R^2=0.998, P<0.0001, n=12, slope = 11512 ng.L^{-1}/µg [95% CI 11225-11798]). Despite this extreme sensitivity, at all measured time points following an oral load of similarly processed ovine myocardium, the concentration of both cTnI and cTnT remained below the LoD for their respective assays when measured in human peripheral venous circulation (Figure 13).

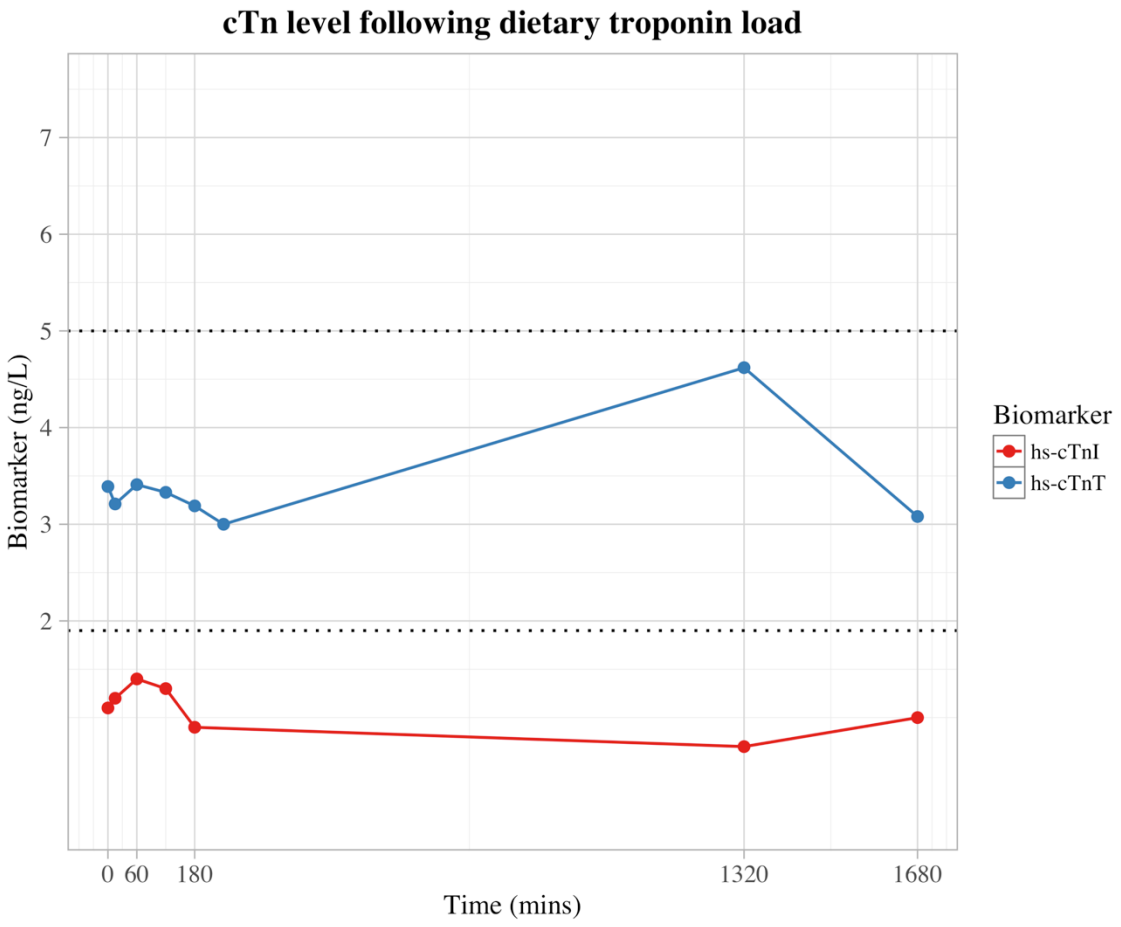

Figure 13 – Graph demonstrating available serum biomarker concentration after an oral load of cooked ovine myocardium at the following time-points: 0 min, 15 min, 1 hr, 2 hrs, 3 hrs, 4 hrs (missing for cTnI), 22 hrs, 28 hrs; all measured values remained below the Limit of Detection (LoD) for high-sensitivity assays (hs-cTnT and hs-cTnI) for the respective biomarkers (cTnT=5 ng/L, cTnI=1.9 ng/L; indicated on plot with dotted lines)

2.5. Discussion

This study documents the extreme sensitivity of the high-sensitivity cTn assays, which are capable of detecting release from a single cardiomyocyte in a 400 μL blood sample. All investigated biomarkers correlate strongly with the mass of human myocardium. The observation that each microgram of human myocardium releases less cTn than a single cardiomyocyte most likely results from the heterogeneous cellular makeup of the human heart samples and the difficulty in efficiently liberating sarcomeric protein.

Despite the extreme sensitivity of cTn assays, we were unable to detect exogenous cTn in the peripheral blood stream after an oral load.

Based on our results, if we assume a circulating plasma volume and biomarker distribution of 2.75 litres without clearance, the 99th centile concentrations for high-sensitivity assays for cTnT (Roche, 14 ng/L), cTnI (Abbott, 26 ng/L), and cMyC (87 ng/L) can be exceeded by necrosis of 0.025 g, 0.042 g, and 0.015 g of myocardium; respectively (for calculation see appendix). The recent guideline by the European Society of Cardiology defines 'rule-out' and delta values for cTn, below which cardiac injury is unlikely.[12] These values are close to the LoD concentrations of the high-sensitivity assays for cTnT and cTnI (5 ng/L and 1.9 ng/L, respectively). Our experiments suggest that 9 mg and 3 mg of human myocardial necrosis are required to increase cTnT and cTnI above their LoDs as measured by high-sensitivity assays, respectively. The corresponding value for cMyC (LoD 0.4 ng/L) is 0.07 mg. Our experiments simulate a scenario of complete myocardial necrosis, with subsequent rapid reperfusion and distribution of the coronary effluent into the systemic circulation. We have also ignored the circulating species of cTnI, cTnT and cMyC. Collectively these, and other unknown factors,

make it likely that diagnostic thresholds would in reality require myocardial necrosis more substantial than we have predicted.

The models of myocardial necrosis we have adopted are reductionist and convenient but differ markedly from necrosis of blood-perfused myocardium *in vivo*. Firstly, the process of cardiomyocyte necrosis *in vivo* is more complex than tissue homogenization. The vast majority of cTnI, cTnT and cMyC resides in the crystalline sarcomere and release from this compartment is slow. The cause for this slow and incomplete release is uncertain but is likely related to the quality of reperfusion since the temporal profile of cTnT differs markedly between alcohol septal ablation (low microvascular reflow) and cardioplegia (high microvasculature reflow).[50] In addition, myocardial cTnT can be readily released from myocardium by serum alone, without the need of specialist extraction buffers.[97] Although, we didn't measure the cTnI, cTnT or cMyC remaining in the insoluble fraction (pellet after centrifugation) following homogenization, it is likely extraction was inefficient since the concentration of cTnT we observed in human myocardium is lower than those published previously.[18,97] Secondly, the protracted release *in vivo* provides ample opportunity for post-translational modifications, that will be absent in our models of rapid myocardial homogenisation in calcium-free lysis buffers containing protease inhibitors. For example, cTnI, cMyC and cTnT appear in the circulation as peptides, as well as intact proteins.[50,51,98,99] In the case of cMyC, calpain-mediated cleavage is regulated by phosphorylation events within the M domain that impede the formation of an N-terminal peptide[63] that is both immunogenic[83] and negatively inotropic.[100]

Immunoassays may not have the same sensitivity for these modified forms of cTnI, cTnT and cMyC as they do for the parental unmodified protein.[98,101] If the cleavage event occurs between

the epitopes recognized by the capture and detection antibodies, the immunoassay will become insensitive. Conversely, other post-translational modifications are known to enhance the sensitivity of immunoassays. For example, the sensitivity of assays for cTnI can be enhanced by oxidation leading to intramolecular disulphide formation and also when cTnI is in a binary or ternary complex with cTnC and cTnT.[98] Furthermore, the abundance of circulating cTnI, cTnT and cMyC peptides changes over time in an individual patient as well as between patients.[50,98,99] There can also be marked differences between individual patients in the proportions of cTnT and cTnI that appear in apo versus binary and ternary forms.[98] These complexities were absent in our experiments where heart tissue was rapidly homogenized, protease inhibitors prevented protein cleavage, opportunities for oxidation were limited and there was no added calcium to maintain ternary complexes of cTnI/cTnC/cTnT.[98] These details illustrate the simplistic assumptions made in our *in vivo* extrapolation of volumes of myocardial necrosis needed to cross diagnostic thresholds. Nonetheless, they do not invalidate the extreme analytic sensitivity of the cTnT, cTnT and cMyC assays and the microscopic nature of the myocardial necrosis events that drive clinical decision-making.

Several publications have previously challenged the thresholds for AMI rule-out as well as the application of very small delta values in clinical practice.[90–92,102] In their analysis Chenevier-Gobeaux et al questioned the cut-off for rule-out at the limit of detection (5 ng/L for cTnT); prompting Peter Kavsak to comment that there might 'be other analytical factors at play that affected the performance of hs-cTnT'.[90,91] Clearly, novel ultra-sensitive cTnI assays (as available on Singulex Erenna and Clarity platforms[25,26]) would circumvent some of the issues above, but successful migration to a random-access laboratory analyser is still awaited.

Hickman et al.[92] further highlighted the challenges associated with such narrow decision-limits and the use of deltas when the cTn release might have plateaued – as is the case with late presentations to the emergency department and cardiac troponin release caused by events other than atherosclerotic plaque instability. Turer et al. have added to the diagnostic conundrum by describing low-level cTnT release in the context of ischemia without frank infarction.[102]

Although new myocardial scarring on cardiac magnetic resonance imaging (cMRI) has been correlated to cTn measured with a second generation assay[103], this is limited by the inability of cMRI to detect infarct size smaller than a gram of myocardium. To our knowledge, no previous study has directly correlated cardiac damage at a tissue or cellular level to biomarker concentrations measured using contemporary high-sensitivity assays.

While there is a significant body of evidence to suggest that di- and tri-peptides, derived from dietary protein can cross the gastrointestinal tract into the portal circulation, the absorption of larger intact polypeptides is controversial.[104] Based on the closely spaced capture/detection epitopes utilized by the cTnI and cTnT assays, 20-mer polypeptides should generate a signal (see appendix). Given the exceptional sensitivity of the high-sensitivity troponin assays, we hypothesized that we would detect longer cTnI and cTnT polypeptides in the systemic circulation. This hypothesis was supported by the fact that both of the high-sensitivity assays for cTnT and cTnI were substantially more sensitive to cTn in cooked ovine than in human myocardium. Following a meal of 200 g of ovine myocardium, venous blood was sampled from a healthy human volunteer at hourly time intervals. Assuming that, a) the human digestive tract is able to liberate all the cTnT in ovine myocardium in a manner similar to ultrasonication; and b) the entire quantity of liberated cTnT is able to cross into the systemic

circulation, extrapolation of the calibration curve indicates that serum cTnT should be increased by 335 milligrams/L. The high sensitivity cTnT assay has sensitivity to detect 5 ng/L increments in serum troponin. As such, only $1.5x10^{-6}$ % of the dietary cTnT needed to be released from the myocardium and absorbed into systemic circulation to produce a detectable increase in serum cTnT. Nonetheless, measured cTnT remained consistently below the LoD at all time points after the dietary load. The same observation was made with cTnI. Even with the unrealistic assumption of complete liberation of troponin from the full mass of ingested myocardium, the orders of magnitude involved suggest the gastrointestinal tract is virtually impervious to absorption of intact polypeptides of troponin.

In conclusion, we have, for the first time, correlated the 99th centile thresholds of cardiac troponin to the approximate mass of myocardium undergoing complete necrosis. Our experiments have revealed the exquisite sensitivity of the contemporary biomarker assays, with necrosis of just 40 mg of myocardium, equivalent to 0.015% of the heart, sufficient to increase serum concentrations above the 99th centile.

Acknowledgements

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Conflicts of Interest

Millipore Sigma (Hayward, California) was contracted to undertake the analyses of cMyC on a fee-for-service basis and hold no commercial interest. Marber is named as an inventor on a

patent held by King's College London for the detection of cMyC as a biomarker of myocardial injury.

2.6. Appendix

2.6.1 Calculation of mass of tissue having to be destroyed to increase cTnI above 99th centile:

a) cTnI is increased by 4.28 ng/L per 1 microgram (μg) of destroyed tissue in 400 microlitres of serum (as per experiment methodology).

b) We have assumed a circulating volume of 2,750,000 microliters (2.75 litres).

c) Thus 6,875 micrograms of tissue need to be destroyed to increase cTn by 4.28 ng/L at circulating volume.

d) The 99th centile of the high-sensitivity assay for cTnI is 26 ng/L

e) (26/4.28)*6875 micrograms = 41,764 micrograms = 41.7 milligrams

f) 41.7 milligrams is the mass of tissue that needs to be destroyed to increase cTnI above the 99th centile at the level of the circulating volume.

2.6.2 Supplemental figure – BLAST data:

Abbott ARCHITECT (hs-cTnI)

Capture (87-91)

87	***88***	***89***	***90***	***91***
G	**L**	**G**	**F**	**A**

Capture (24-40)

24	***25***	***26***	***27***	***28***	***29***	***30***	***31***	***32***

S	**N**	**Y**	**R**	**A**	**Y**	**A**	**T**	**E**
33	***34***	***35***	***36***	***37***	***38***	***39***	***40***	
P	**H**	**A**	**K**	**K**	**K**	**S**	**K**	

Detection 41-49

41	***42***	***43***	***44***	***45***	***46***	***47***	***48***	***49***
I	**S**	**A**	**S**	**R**	**K**	**L**	**Q**	**L**

Roche Elecsys (hs-cTnT)

Capture (136-147)

136	**137**	**138**	**139**	**140**	**141**	**142**	**143**	**144**	**145**	**146**	**147**
R	**I**	**E**	**R**	**R**	**R**	**A**	**E**	**R**	**A**	**E**	**Q**

Detection (125-131)

125	**126**	**127**	**128**	**129**	**130**	**131**
K	**E**	**E**	**E**	**E**	**L**	**V**

Figure 14 – Sensitivity of human troponin assay antibodies for the rat protein. Numbers represent the amino acid position in the human protein. Letters represent standard amino acid abbreviations, and reflect the amino acid in

the corresponding position of the human protein. Greyed-out amino acids are conserved between the human and rat troponin. Amino acids in white are not conserved and are different in the rat troponin.

Prelude to Chapter 3

Findings in chapter 2 highlight the extreme sensitivity of hs-cTn assays, but even more so of the novel cMyC assay. In parallel to the analysis of cMyC in large-scale, diagnostic chest pain trials, it was felt relevant to assess how transferable findings in European or American chest pain studies are to the UK environment. For this purpose, we undertook a single-centre prospective cohort study based on audit data obtained from hospital records collected routinely as part of clinical care at St Thomas' Hospital, London. This would allow estimation of the number of patients routinely undergoing cTn testing in a large teaching hospital and outline the clinical course and length-of-stay. Thus, the possible impact of a novel biomarker on chest pain triage – as performed in a healthcare environment relevant to the host institution – can be estimated in due course. The following paper represents a summary of the findings and discusses real-life challenges when using novel diagnostic algorithms.

The findings were published previously (DOI: 10.1177/2048872617746850) and are reproduced with amendments for inclusion in the thesis.

Chapter 3. A single centre prospective cohort study addressing the effect of a rule-in / rule-out troponin algorithm on routine clinical practice

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3.1. Abstract

Aims: In 2015 the European Society of Cardiology (ESC) introduced new guidelines for the diagnosis of acute coronary syndromes in patients presenting without persistent ST-segment elevation. These guidelines included the use of high-sensitivity troponin assays for 0 hour 'rule-in' and 'rule-out' of acute myocardial injury. Whilst these algorithms have been extensively validated in prospective diagnostic studies, the outcome of their implementation in routine clinical practice has not been described. The present study describes the change in the patient journey resulting from implementation of such an algorithm in a busy inner city Emergency Department.

Methods & Results: Data were prospectively collected from electronic records at a large Central London hospital over seven months spanning the periods before, during and after the introduction of a new high-sensitivity troponin rapid diagnostic algorithm modelled on the ESC guideline.

Over 213 days, 4644 patients had a high-sensitivity troponin T (hs-cTnT) measured in the Emergency Department. 40.4% of patients could be 'ruled-out' based on the hs-cTnT concentration at presentation, whilst 7.6% could be 'ruled-in'. Adoption of the algorithm into clinical practice was associated with a 37.5% increase of repeat hs-cTnT measurements within 1.5 hours for those patients classified as 'intermediate risk' on presentation.

Conclusions: Introduction of a 0hr 'rule-in' and 'rule-out' algorithm in routine clinical practice enables rapid triage of 48% of patients, and is associated with more rapid repeat testing in intermediate risk patients.

Keywords: High-sensitivity cardiac Troponin T; acute coronary syndrome; rule-in / rule-out algorithm.

3.2. Introduction

Chest pain and related complaints are estimated to account for 6% of all attendances to UK Emergency Departments (ED).[1] Determining which of these presentations represent an acute coronary syndrome, quickly and with high sensitivity and specificity, is an everyday challenge. The measurement of cardiac-specific biomarkers released into the circulation is invaluable, and the measurement of cardiac Troponin (cTn) I and T is engrained in the universal definition of myocardial infarction.[11] However, the slow release of cTn, in combination with the relative analytic insensitivity of conventional cTn assays, has necessitated serial measurements separated by at least 6 hours to increase both sensitivity and specificity. This period of diagnostic uncertainty prolongs the patient's hospital stay, delays their treatment and has an associated fiscal cost. The advent of high sensitivity troponin assays has encouraged investigators to examine shorter intervals between repeat troponin estimations. The high sensitivity assays have also allowed the testing of diagnostic cut off concentrations well below the population defined 99th centile to rapidly rule out acute myocardial injury. These innovations culminated in the European Society of Cardiology (ESC) releasing new guidelines in September 2015 for the management of patients without persistent ST elevation.[12,87,105,106] These guidelines adopt a 'rule-out' troponin value significantly below the 99th centile and a 'rule-in' value well above the 99th centile. Between these values of diagnostic clarity, the change in troponin level over the course of 1 hour can guide further rule-in or rule-out. In October 2015 we proposed introduction of the 0 hour rule-in / rule-out component of the ESC algorithm at St Thomas' Hospital (based in central London and home to a tertiary cardiac unit) and adopted the guideline, following an internal consultation process, during December 2015 – January 2016. This internal consultation process also involved extension of teaching to Emergency Department staff, both nursing and physician, as to the appropriate use of the

algorithm. All 'post-intervention' data were collected after implementation and associated staff training.

Whilst the ESC guidelines help streamline the diagnostic pathway, there has been little information regarding their impact on front-line medical services. The present study, based in the ED of a large Central London hospital, aims to a) prospectively assess the risk classification of patients based on 0 hour hs-cTnT measurement, and b) examine the effect of clinical implementation of the 0 hour component of the ESC guideline on the patient pathway. In particular, we document changes in the pattern of repeat troponin measurements and overnight admission.

3.3. Methods

Data was prospectively collected on all high-sensitivity cardiac troponin T (hs-cTnT) assays performed on serum from patients presenting to the ED of St Thomas' Hospital, between September 2015 and March 2016. This time-period of data collection spans the pre-intervention (September-November), transition (December), and post-intervention (January-March) phases of algorithm implementation. hs-cTnT assays were performed using the Roche Elecsys® platform (using a high-sensitivity reagent instead of a contemporary: 99th percentile of a healthy reference population reported at 14 ng/L, imprecision corresponding to 10% CV at 13 ng/L, limit of blank at 3 ng/L, limit of detection at 5 ng/L). The hs-cTnT value measured in the ED was matched to any subsequent hs-cTnT measurement on the same patient within 24 hours. Further information on admission, admitting specialty, and length of stay was collected from electronic discharge records. Data on presenting symptom was obtained from the system used for triage and clinical tracking in the Emergency Department (Ascribe Symphony); this captures the prime medical complaint however does not encompass

a physician's interpretation. Discharge diagnoses are locally recorded according to the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10) and were subsequently categorised into diagnostic groups by two adjudicators (JM & TEK).

The new algorithm for the diagnostic management of possible NSTE-ACS can be summarised as follows: hs-cTnT is measured on arrival to ED for patients with a history suggestive of ACS and an ECG without persistent ST elevation. ACS can be 'ruled-out' in low-risk patients with a hs-cTnT on presentation of <5 ng/L, and 'ruled-in' for those patients with an initial hs-cTnT of >50 ng/L (Figure 15). Although not adopted into our algorithm, the ESC advises that in patients with an initial hs-cTnT of 5-51 ng/L, a repeat hs-cTnT at 1 hour is performed, with rule-out if the initial hs-cTnT is <12ng/L and a change in hs-cTnT (ΔTnT) is <3 ng/L, and rule-in if ΔTnT is ≥5 ng/L.

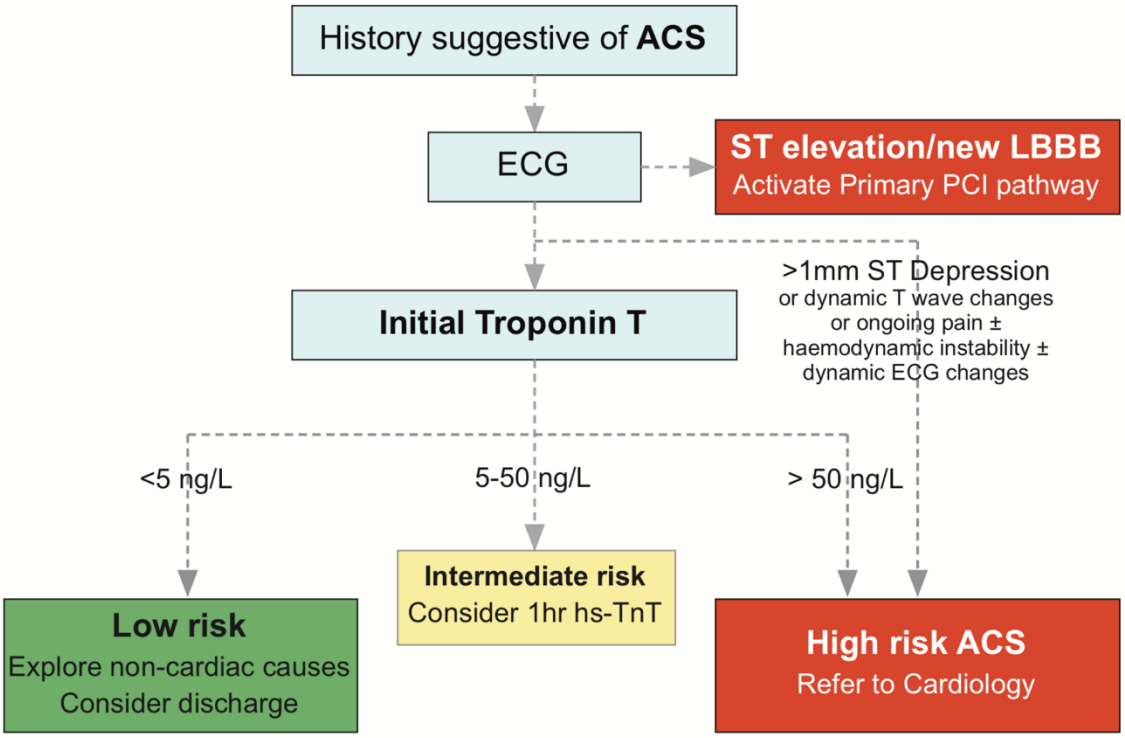

Figure 15 – The high-sensitivity troponin T (hs-cTnT) rapid diagnostic algorithm introduced at St Thomas' Hospital

For the purposes of our analysis, a patient was considered to have had a repeat hs-cTnT if a second sample was measured within 24 hours of the first. Patients were excluded from analysis if the first sample haemolysed. hs-cTnT measurements returned below the limit of blank (<3 ng/L) were all ascribed a value of 2.99 ng/L to allow for data analysis. Continuous variables were assessed for normality using Shapiro-Wilk Test. All data are expressed as medians [1st quartile, 3rd quartile] or means (standard deviation) for continuous variables (compared with the Mann-Whitney-U test or student's t-test), and for categorical variables as numbers and percentages (compared with Pearson chi-square). Hypothesis testing was two-tailed, and p values <0.05 were considered statistically significant. Statistical analysis was conducted using

SPSS version 22 (IBM Corp., Armonk, New York) and R, version 3.3.0 GUI 1.68 (The R Foundation for Statistical Computing), including ggplot2.

3.4. Results

Over a period of 213 days, spanning the introduction of the new diagnostic protocol, a total of 4644 patients had a hs-cTnT measurement in the ED. A summary of the presenting complaint of all patients with hs-cTnT measurements in the study period (September 2015 – March 2016) is presented in Table 1. In short, of the patients with a measured hs-cTnT (n=4644), chest pain was the primary presenting symptom in 45.7% (n=2120), and shortness of breath in 8.2% (n=382) – see Figure 16. Median age was 54 years [Interquartile Range (IQR), 41-70].

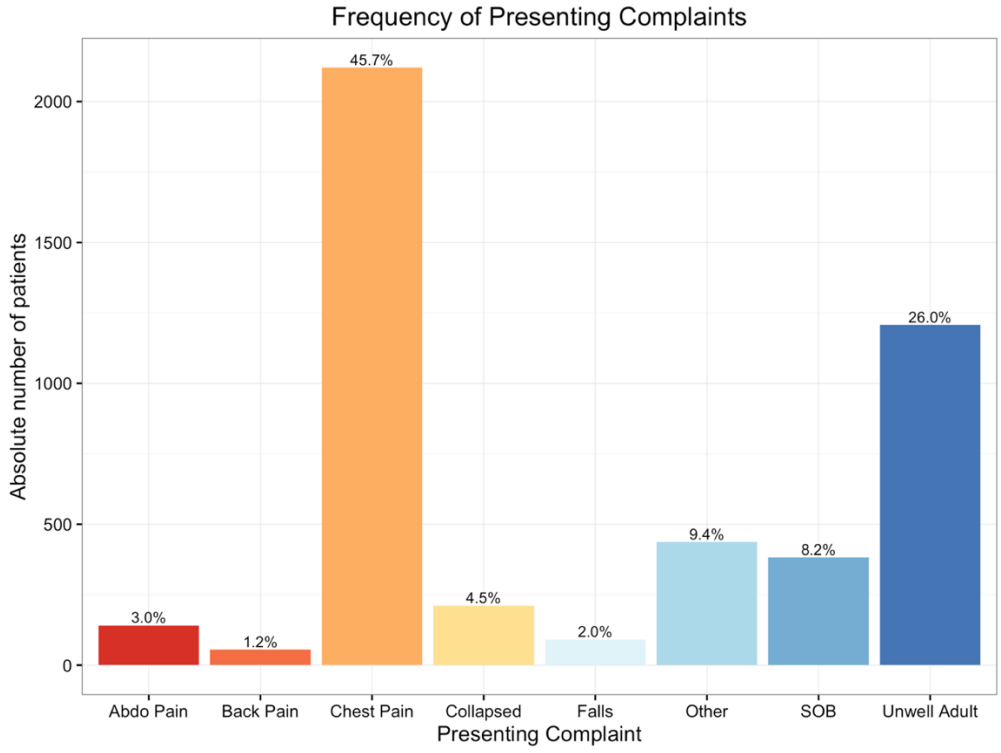

Figure 16 – Bar graph summarising the presenting complaint of all patients (n=4644) with a measured hs-cTnT in the entire study period; frequencies quoted as percentage of the cohort

Presenting complaint	All attendances	(%)
Abdominal pain	141	(3.0)
Back pain	55	(1.2)
Chest pain	2120	(45.7)
Collapsed adult	211	(4.5)
Falls	91	(2.0)
Shortness of breath	382	(8.2)
Other	437	(9.4)
Unwell adult	1207	(26.0)
Total	n = 4644	

Table 1 – Summary of all presenting complaints. Frequencies quoted as number (%); sample selection: all patients presenting to the Emergency Department with a hs-cTnT measured as part of their assessment between September 2015 and March 2016, age ≥18 years; 'Other' summarises non-cardiac presentations such as 'overdose' and 'limb problems'

3.4.1 0h risk stratification for whole sample period

Of the entire cohort, 40.4% had an initial hs-cTnT concentration below the 'rule-out' value of 5 ng/L at presentation, and 7.6% had a concentration above the 'rule-in' value of 50 ng/L (Figure 17). Of the patients presenting with chest pain (n=2120), 1026 (48.4%) had an initial hs-cTnT concentration below the 'rule-out' threshold, 107 (5%) had a concentration above the 'rule-in' threshold. Of the patients presenting with Shortness of Breath (n=382), 89 (23.3%) had an initial hs-cTnT concentration below the 'rule-out' threshold, 74 (19.4%) had a concentration above the 'rule-in' threshold.

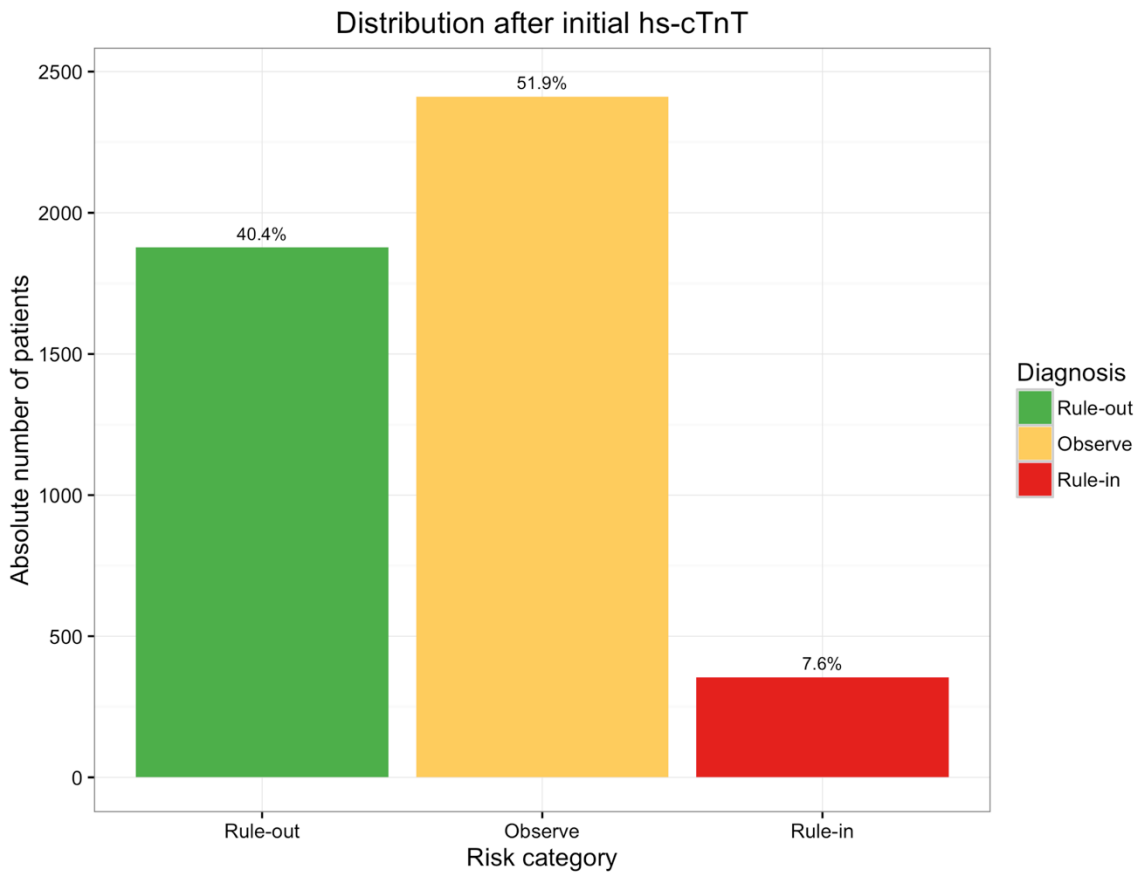

Figure 17 – Graph outlining the distribution of all hs-cTnT values measured on patients presenting to the Emergency Department during the monitoring period (September 2015 to March 2016; n=4644); the following thresholds applied: <5 ng/L 'Rule-out', 5-50 ng/L 'Observe', >50 ng/L 'Rule-In'

3.4.2 Retrospective analysis of deltas for all presentations

Although our algorithm incorporates only the rule-in/rule-out classification based on a 0 hour hs-cTnT measurement, retrospective analysis of the entire cohort demonstrates that 10.6% of those at intermediate risk (0 hour hs-cTnT 5-50ng/L) could have been ruled-in on repeat testing with a ΔTnT ≥5 ng/L, and 45.1% could have been ruled-out on the basis of an initial TnT <12ng/L and ΔTnT <3 ng/L.

3.4.3 Discharge diagnosis

1,876 patients were admitted from the ED during the entire study period. Amongst these, the prevalence of ischaemic heart disease in the discharge diagnosis was 21.2% (n=397); congestive cardiac failure was the discharge diagnosis in 5.8%; pulmonary embolism in 1.5%. Of those patients admitted with a Troponin value above the rule-in threshold (50 ng/L), 35.6% were diagnosed with ischaemic cardiac pathology (see Table 2; Figure 18 for details on all admitted patients; Figure 19 for subgroup analysis on all patients with a hs-cTnT at presentation >50 ng/L).

Coding diagnosis	All admitted patients	hs-cTnT >50 ng/L
Aortic dissection	8 (0.4)	0 (0)
IHD	397 (21.2)	88 (35.6)
Arrhythmia	159 (8.5)	17 (6.9)
CCF	108 (5.8)	26 (10.5)
Cardiac other	106 (5.7)	20 (8.1)
PE	28 (1.5)	5 (2.0)
OAD	100 (5.3)	7 (2.8)
Resp other	24 (1.3)	3 (1.2)
Infectious	189 (10.1)	17 (6.9)
Renal	52 (2.8)	15 (6.1)
GI	124 (6.6)	8 (3.2)
MSK	100 (5.3)	9 (3.6)
Other	481 (25.6)	32 (13.0)
Total	n = 1876	n = 247

Table 2 – Summary of discharge diagnoses. frequencies quoted as number (%); IHD = ischaemic heart disease; CCF = congestive cardiac failure; 'Cardiac other' includes myocarditis, valvular heart and pericardial disease; PE = pulmonary embolism; OAD = obstructive airways disease; 'Resp other' includes pleural effusion; Infectious includes lobar pneumonia, urinary tract infection and influenza; GI = gastrointestinal disorders including gastro-oesophageal reflux disease, gastroenteritis and symptomatic cholelithiasis; MSK = musculoskeletal disorder

including costochondritis, bony fractures and other injuries; 'Other' includes sickle-cell anaemia, malignancy and mental health disorder. Sample representative of the entire study period (September 2015 – March 2016) and comprises of all patients admitted from the Emergency Department.

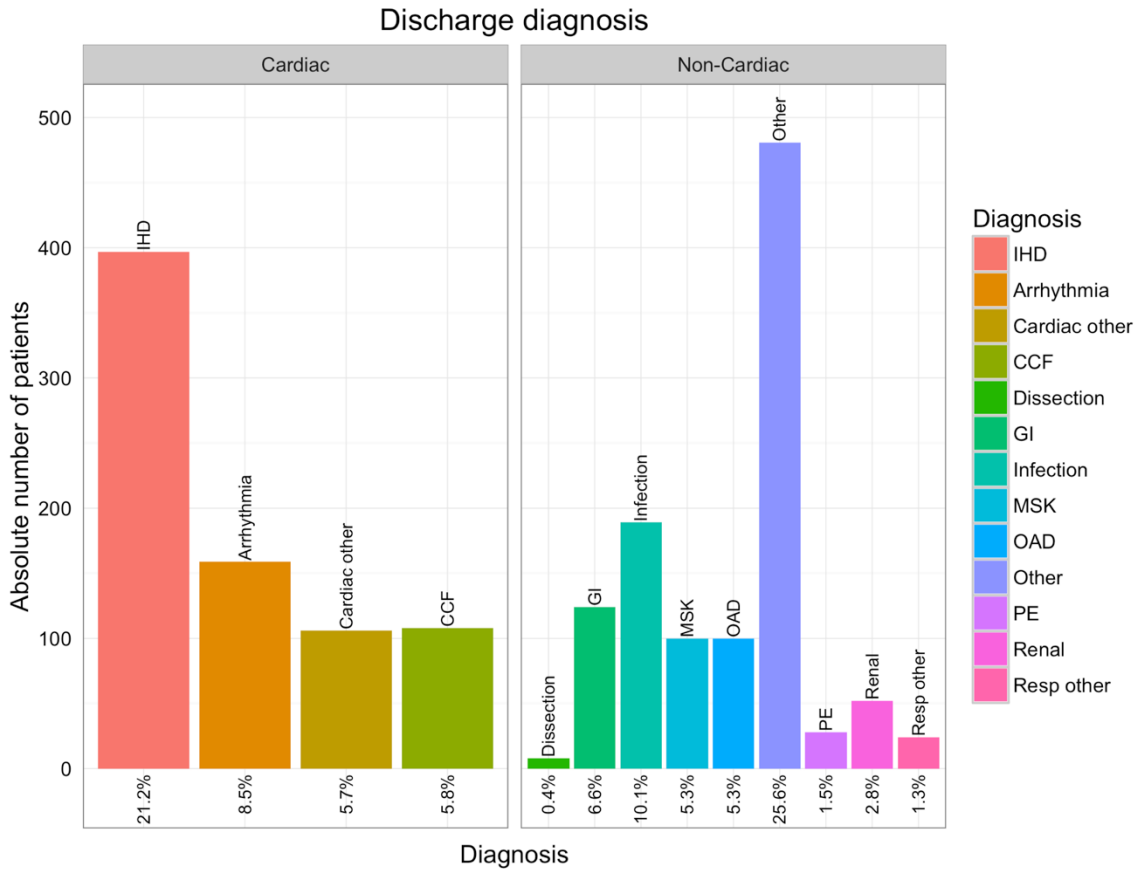

Figure 18 – Bar graph summarising the discharge diagnosis of all admitted patients in the monitoring period (September 2015 to March 2016; n=1876); frequencies quoted as percentage of the overall number of patients admitted following hs-cTnT testing; IHD = ischaemic heart disease; CCF = congestive cardiac failure; 'Cardiac other' includes myocarditis, valvular heart, conduction tissue and pericardial disease; PE = pulmonary embolism; OAD = obstructive airways disease; 'Resp other' includes pleural effusion; 'Infectious' includes lobar pneumonia, urinary tract infection and influenza; GI = gastrointestinal disorders including gastro-oesophageal reflux disease, gastroenteritis and symptomatic cholelithiasis; MSK = musculo-skeletal disorder including costochondritis, bony fractures and other injuries; 'Other' includes sickle-cell anaemia, malignancy and mental health disorder.

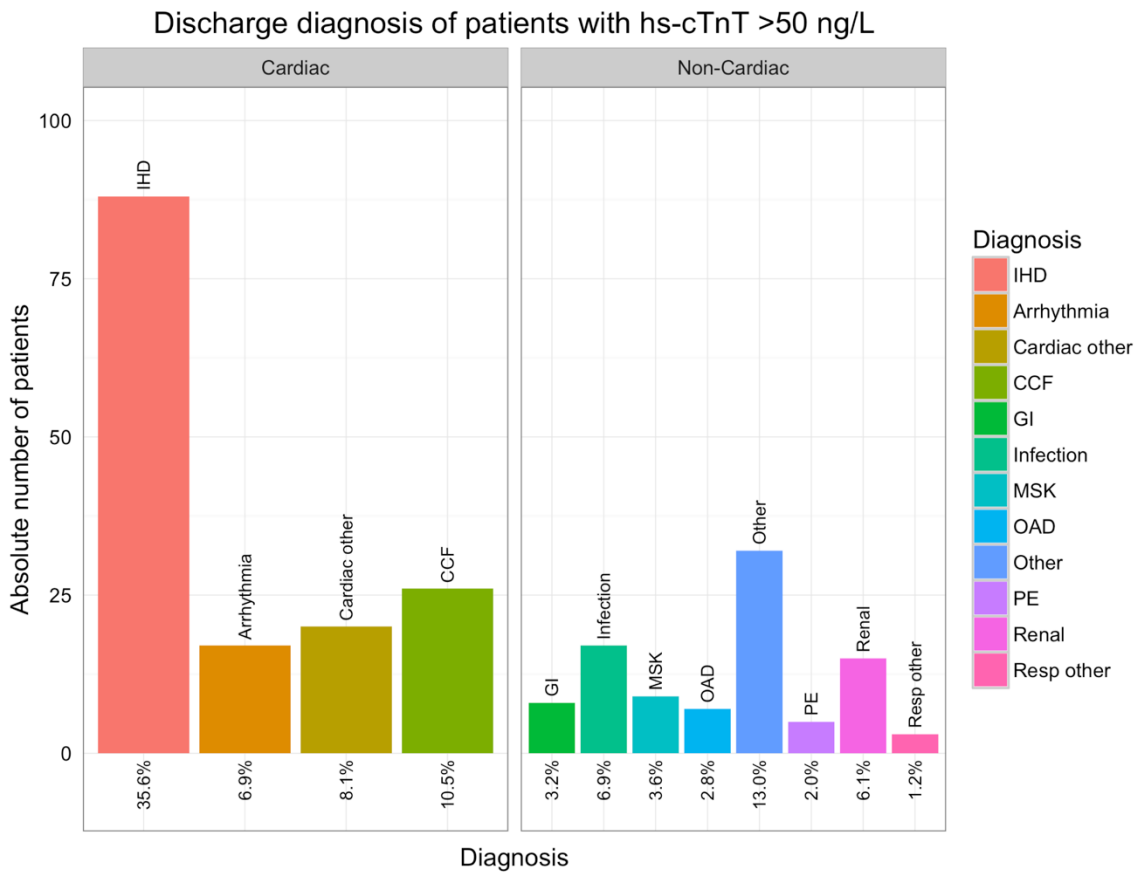

Figure 19 – Bar graphs summarising the discharge diagnosis of all admitted patients in the monitoring month with an initial hs-cTnT level >50 ng/L (n=247); frequencies quoted as percentage. Abbreviations and subgroups as in Figure 18

3.4.4 Repeat troponin samples in the post-intervention period

In the 3 months following introduction of the algorithm (i.e. the 'post-intervention period'), 946 patients (50.2%) had an initial hs-cTnT in the 5-50 ng/L zone of diagnostic uncertainty – of these, 443 (46.8%) had a repeat measurement within 24 hours. Of the patients undergoing further testing, 189 (42.7%) had a repeat measurement within 1.5 hours. Median time to repeat hs-cTnT measurement was 1.6hrs [1.3, 2.2] for the entire post-intervention period.

Eight hundred and ninety two patients presented with chest pain in the post-intervention period. Of these, 390 patients (43.7%) were in the observational group, of which 222 (56.9%)

had a repeat measurement within 24 hours. Of the patients undergoing further testing, 106 (47.7%) had a repeat measurement within 1.5 hours. The median time to repeat hs-cTnT measurement in the group presenting with chest pain was 1.5 hours [1.3, 2].

One hundred and fifty four patients presented with shortness of breath in the post-intervention period. Of these, 87 patients (56.5%) were in the observational group, of which 29 (33.3%) had a repeat hs-cTnT within 24 hours. Of the patients undergoing further testing, 10 (34.5%) had a repeat measurement within 1.5 hours. Median time to repeat in the group presenting with shortness of breath was 1.8 hours [1.4, 2.1].

3.4.5 Comparison of pre- and post-intervention periods for all presentations

Over the timeframe of implementation of the new algorithm we have demonstrated a gradual rise in the proportion of patients in the intermediate risk group (all presenting complaints) who had a repeat hs-cTnT measured within 1.5 hours. At month 1 (pre-implementation), only 3.3% of repeat hs-cTnT measurements in the intermediate-risk patients were within 1.5 hours, rising to 40.8% by month 7 (post-implementation) ($p<0.001$). In tandem, the median time to repeat troponin has fallen from 7.8 hours [4.7, 11.1] to 1.7 hours [1.3, 2.4] ($p<0.001$). This has been accompanied by a non-significant trend towards reduced overnight admissions in the low-risk group. In a month prior to implementation, of all patients with a hs-cTnT measurement <5 ng/L on presentation to ED, 12.7% were admitted for at least one night. This figure fell to 9.5% by month 7 ($p=0.26$, $n=525$). Early outcome data demonstrates that 30-day mortality in all patients with suspected ACS was not different before and after implementation of the new algorithm (1.8% versus 1.4% respectively, $p=0.38$).

3.5. Discussion

This study documents the rate of adoption of a rapid rule-in / rule-out algorithm for the routine clinical care of patients presenting with suspected NSTE-ACS, based on a single blood test at presentation. In this large cohort of over 4600 patients, 48% of all patients and 53% of patients with chest pain could be dichotomised into high- or low risk groups on the basis of a single hs-cTnT measured on presentation.

Multiple studies have prospectively validated the sensitivity and specificity of diagnostic algorithms based on high-sensitivity Troponin assays.[87,105–108] The unifying aim is to rapidly identify patients with ACS, facilitating prompt therapeutic intervention for those who need it, and prompt discharge for those who don't. However, since the ESC guidelines have been established, there is a dearth of studies that have addressed the fundamental question – can such an algorithm be implemented into routine clinical practice? As we have incorporated our algorithm into clinical practice, we have seen an increased rate of repeat testing, and a trend to faster repeats, in patient's classified into the intermediate risk group on presentation. Interestingly, despite the ESC endorsement of a 0/1h chest pain triage algorithm in their 2015 NSTEMI guidelines[109], we did not observe a significant reduction in overnight admissions in the low risk group when employing such a pathway in clinical practice. It appears that physicians are often more conservative than what guidelines advocate, despite an apparent sensitivity of (often) >99% for rule-out.

Whilst there is a clear trend in uptake of the protocol following its implementation, it is evident that it is still not being used universally across the services. This may reflect hesitancy amongst clinicians to discharge patients soon after presentation, without a significant period of monitoring. It is of paramount importance to involve all staff in understanding the rationale

for change, optimising operation procedures to ensure rapid turn-around times for sequential blood draws and to streamline a rapid assessment process; in order to reap the benefits of an earlier rule-out.

This study looks predominantly at the rule-in / rule-out power of the ESC algorithm at 0 hours, based on a hs-cTnT measurement at presentation. Whilst we have been able to retrospectively quantify the risk classification of patients based on ΔTnT, the translation of ΔTnT values into prospective clinical practice needs further evaluation. Although the ESC recommends a 0hr hs-cTnT ≥ 52ng/L as a rule-in threshold, our algorithm defines rule in as >50 ng/L for ease of clinical implementation.

Chest pain is clearly the typical presentation of NSTEMI. However, the ESC guideline appreciates that ACS can present atypically as 'epigastric pain, indigestion-like symptoms and isolated dyspnoea'.[12] The 0-1hr ESC algorithm suggests progression to biomarker risk stratification in the patient with 'suspected NSTEMI' and does not delineate that this suspicion must arise from the presence of typical chest pain. As such, presenting complaints like isolated shortness of breath and abdominal pain, that feature in Figure 16, can reasonably enter the troponin algorithm if the clinician has a high index of suspicion for ACS. Nonetheless, a limitation of this study is the underlying assumption that all patients who had a hs-cTnT measured in the Emergency Department correctly entered the diagnostic algorithm, i.e. had a clinical presentation compatible with a NSTE-ACS. Further, the 'presenting complaint' entered on the ED triage system is more a clerical than medically driven assessment and captures only the main complaint, and not a complex presentation. This may explain why a significant number of patients with an initial troponin in the 5-50 ng/L group did not go on to have a repeat (as it became evident that they should not have entered the algorithm in the first place).

Nonetheless, this is likely to represent the reality of a patient's clinical pathway in ED. The ESC guideline acknowledges that deviation from the protocol is appropriate in circumstances of clinical concern, and rapid rule-out is inappropriate for patients presenting very early after the onset of chest pain. Our study does not account for these possible extenuating circumstances. Importantly, despite our clinical practice moving toward faster repeat troponin measurements, the current study of ΔTnT is based on the repeat troponin at any time within 24 hours, whereas the ESC guideline is predicated on a repeat at 1 hour. In keeping with previously published observations[110], approximately 12% of initial troponin samples taken in the ED were haemolysed. These samples were excluded from analysis as they inevitably lead to deviation from the algorithm, and this study aimed to look at the routine functioning of the algorithm in clinical practice. However, it is important to acknowledge that in the real-world setting haemolysis is likely to affect the timings of samples. Finally, the troponin values available electronically to clinicians are rounded to the nearest integer, which may lead to some discrepancy between the true risk bracket that the patient belonged to and the risk bracket that they were ascribed to clinically in ED.

3.6. Conclusions

A 0 hour rule-in / rule-out algorithm, modelled on the 2015 ESC guideline, can be implemented with good uptake within the first few months of implementation. Although this has failed to demonstrate reduced overnight admission in the low-risk group, the algorithm clarifies the appropriate clinical pathway for up to 53% of chest pain patients at presentation. Further studies are needed to address the implications of 1 hour repeat testing in routine clinical practice.

Conflict of Interest:

Marber is named as an inventor on a patent held by King's College London for the detection of cardiac myosin binding protein-C as a biomarker of myocardial injury.

Acknowledgements:

Supported by the UK Department of Health through the National Institute for Health Research Biomedical Research Centre award to Guy's & St Thomas' National Health Service Foundation Trust, and the British Heart Foundation (FS/15/13/31320). Thanks to David Steed and Geoff Martin for their contribution to data collection.

Prelude to Chapter 4

Findings in chapter 3 demonstrate challenges when employing novel rule-out/rule-in pathways for chest pain triage, but importantly clarifies the burden chest pain triage places on acute care environments in a large UK hospital: about 650 patients undergo cTn testing every month at St Thomas' Hospital, and about 50% of these are assigned to the observe-zone when using a pathway inspired by the 2015 ESC guideline – thus requiring ongoing observation, repeat blood testing, and a longer hospital stay without diagnostic clarity. We have previously commented on a) the greater abundance of cMyC in the myocardium, thus potentially contributing to b) a more rapid rise to higher peak levels in the setting of iatrogenic myocardial infarction (in the context of alcohol septal ablation). To date, there is no evidence to suggest that cMyC is less tissue-specific. An analysis of a select group of patients with a diagnosis of Acute Myocardial Infarction and blood samples available very shortly after chest pain onset is presented in Chapter 4. This tested the hypothesis whether the previously observed, favourable release kinetics would translate into real-life: when measuring both, cMyC and hs-cTnI in samples obtained from patients at presentation to the emergency department, and at fixed time-points during the clinical course.

The findings were published previously (DOI: 10.1373/clinchem.2016.257188) and are reproduced with amendments for inclusion in the thesis.

Chapter 4. Temporal relationship between cardiac myosin-binding protein C and cardiac troponin I in type 1 myocardial infarction

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Running head: Myosin-binding protein C in myocardial infarction

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Keywords:

Cardiac myosin-binding protein C, cMyC, Troponin I, myocardial infarction

4.1. Letter to the Editor

Although acute chest pain is a common presenting symptom, the proportion of patients with acute myocardial infarction (AMI) that have diagnostic electrocardiographic changes on presentation is dwindling. Consequently, early triage of chest pain patients is becoming ever more reliant on assessment of the release of cardiac troponin I (cTnI) or cardiac troponin T (cTnT) into the systemic circulation. However these biomarkers are released relatively slowly reaching their peak many hours after symptom onset.[17] To achieve rapid triage the latest guidelines recommend the use of assays for cTnI and cTnT that have very high analytic sensitivity to rule-in and rule-out AMI based on concentrations markedly above and markedly below the population defined 99th centiles, respectively. The use of these widely spaced decision limits improves the specificity of rule-in and the sensitivity of rule-out. However, the majority of patients presenting with chest pain have cTn concentrations that place them between these decision limits; in an indeterminate zone. These patients require repeat testing and subsequent second or third rounds of triage based on rates of change of cTn concentration over time.[111] This introduces systemic delays in allocation of evidence-based treatments and prolongs stay in the pressured and precious environment of the emergency department.

We have described a new biomarker of cardiac injury – cardiac myosin-binding protein C (cMyC) – which rises more rapidly in the systemic circulation than cTnT after iatrogenic myocardial infarction in the cardiac catheterization laboratory.[50] The purpose of the study we describe here was to determine if the temporal differences between cMyC and cTn observed in the catheter laboratory could extend to patients presenting with symptoms of short duration due to spontaneous coronary atherosclerotic plaque rupture (type 1 AMI).

We identified 174 patients from the HighSTEACS cohort[107] with symptoms of less than 3 hours duration prior to the first blood draw (0h) and with serum/plasma available at 3 hours (3h) and at 6-12 hours (late) after presentation. Study participants presented to the Royal Infirmary of Edinburgh with suspected NSTEMI, were enrolled in the HighSTEACS trial and successfully completed all study-related blood draws, which were subsequently stored in the responsible biobank at early stages of the trial. To qualify for analysis, study samples had to be available from all 3 timepoints. Of these 174 patients, 26 were adjudicated as having type 1 myocardial infarction (see Shah et al.[107] for further details). To determine if the concentration trends for cMyC over time differ from those of cTnI we calculated the ratio of cMyC to cTnI ([MyC]/[cTnI] both expressed as ng/L) at each of the 3 blood sampling time points. cTnI was measured using the Abbott ARCHITECT$_{STAT}$ high-sensitive troponin I assay (Abbott Laboratories; limit of detection of 1.2 ng/L, upper reference limit (99th centile) of 34 ng/L in men and 16 ng/L in women). cMyC was measured using a high-sensitivity assay performed by Singulex on the Erenna platform using our proprietary reagents as recently described (limit of detection of 0.4 ng/L, upper reference limit (99th centile) approximately 80 ng/L[84]).

The demographics of our study population presenting early with Type 1 AMI are similar to those of the parent cohort (age 68.8 years, women 23.1%, primary symptom chest pain 84.6%, previous percutaneous coronary intervention 15.4%).

At each of the 3 timepoints we found a strong linear correlation between cMyC and cTnI, as we previously observed in an ambulatory population[84]: Spearman rho 0.795 ($P < 0.01$) at presentation, 0.902 ($P < 0.01$) at three hours and 0.888 ($P < 0.01$) at the late timepoint. Nonetheless, the ratio of cMyC to cTnI was found highest at presentation, thereafter decreasing significantly with time from presentation, mean ratio at presentation, 7.98 (median

2.72 – IQR 3.48); at 3 hours, 2.67 (median 1.83 – IQR 1.40) and at the late time point, 1.71 (median 0.63 – IQR 1.09) – all $P < 0.01$ by Friedman two-way analysis of variance by ranks. Furthermore, the ratio was also significantly greater than that we observed previously in a stable cohort without obstructive coronary artery disease and a cTnT < 14ng/L (mean 1.97 in Marjot et al.[84]).

Although the mean ratio of cMyC:cTnI was highest at presentation, there was substantial individual heterogeneity amongst the 26 subjects with type 1 AMI (Figure 20). The relative concentration of cMyC to cTnI at presentation soon after symptom onset in those with type 1 AMI was higher than in the same patients at later timepoints and in ambulatory patients at low risk. The more rapid rise of cMyC versus cTnI that we have observed in patients with type 1 AMI should enable their more rapid/accurate triage. However, the diagnostic performance of cMyC, with and without cTnI, needs further evaluation.

Acknowledgements:

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The study was approved by the national research ethics committee, and in accordance with the Declaration of Helsinki.

Conflicts of Interest:

Singulex was contracted to undertake the analyses of cMyC on a fee-for-service basis and holds no commercial interest. Marber is named as an inventor on a patent held by King's College London for the detection of cardiac myosin–binding protein C as a biomarker of myocardial injury.

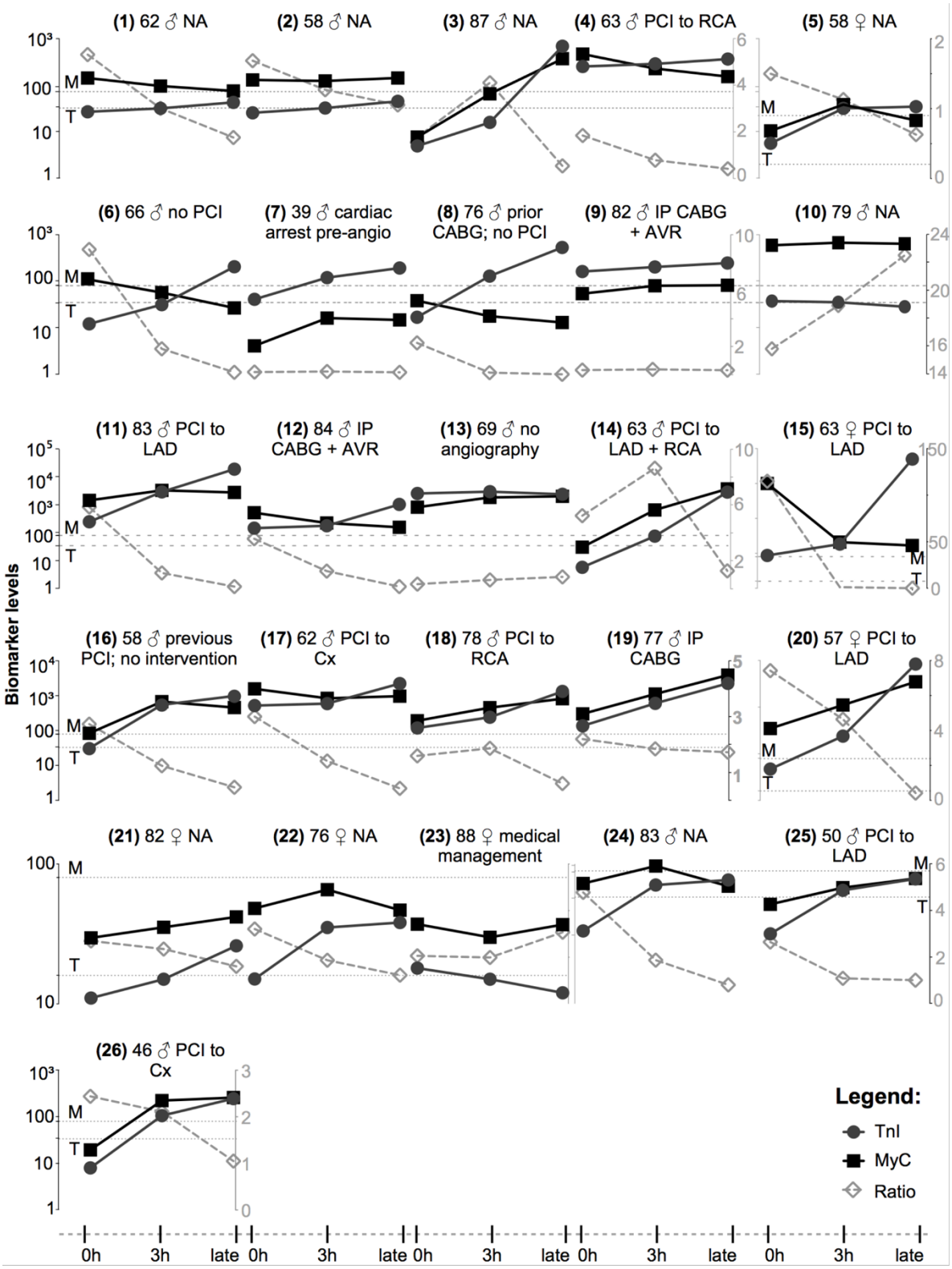
(1) 62 ♂ NA
(2) 58 ♂ NA
(3) 87 ♂ NA
(4) 63 ♂ PCI to RCA
(5) 58 ♀ NA
(6) 66 ♂ no PCI
(7) 39 ♂ cardiac arrest pre-angio
(8) 76 ♂ prior CABG; no PCI
(9) 82 ♂ IP CABG + AVR
(10) 79 ♂ NA
(11) 83 ♂ PCI to LAD
(12) 84 ♂ IP CABG + AVR
(13) 69 ♂ no angiography
(14) 63 ♂ PCI to LAD + RCA
(15) 63 ♀ PCI to LAD
(16) 58 ♂ previous PCI; no intervention
(17) 62 ♂ PCI to Cx
(18) 78 ♂ PCI to RCA
(19) 77 ♂ IP CABG
(20) 57 ♀ PCI to LAD
(21) 82 ♀ NA
(22) 76 ♀ NA
(23) 88 ♀ medical management
(24) 83 ♂ NA
(25) 50 ♂ PCI to LAD
(26) 46 ♂ PCI to Cx
Biomarker levels
M
T
Legend:
TnI
MyC
Ratio
0h
3h
late

Figure 20 – cTnI and cMyC concentration in early type 1 AMI. ID7, No previous cardiovascular disease history. 1 hour atypical chest pain without electrocardiographic changes. Died suddenly before cardiac investigation. ID8, Known extensive cardiovascular disease. Diffuse ischemic heart disease on angiography without target. Managed conservatively. ID10 Renal replacement therapy with extensive cardiovascular disease history. 1 hour severe typical chest pain. Managed conservatively.

Abbreviations: NA – no coronary angiography performed; M – cMyC 99th centile (80 ng/L); T – TnI 99th centile (gender-specific: 34 ng/L in men, 16 ng/L in women)

Prelude to Chapter 5

Findings in chapter 4 point towards an earlier rise of cMyC vs hs-cTnI after an acute plaque rupture event. In chapter 5 we describe the first large-scale analysis of cMyC performance in diagnosis and (potential) triage of chest pain patients using a presentation blood sample for classification of patients into rule-out, rule-in or observe categories. This was facilitated by close collaboration with colleagues in Basel (Christian Mueller et al.), who provided access to the APACE study – as part of the collaboration, we quantified cMyC concentrations in close to 7,500 samples from patients presenting with suspected AMI. The paper presented in Chapter 5 represents a secondary analysis – the candidate forged the collaborations, identified suitable patients, interpreted all cMyC concentrations, wrote the analysis plan, performed the statistical analysis and wrote the manuscript.

The findings were published previously (10.1161/CIRCULATIONAHA.117.028084) and are reproduced with amendments for inclusion in the thesis.

Chapter 5. Direct comparison of cardiac myosin-binding protein C with cardiac troponins for the early diagnosis of acute myocardial infarction

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Running title: Cardiac Myosin-binding Protein C diagnosing AMI

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5.1. Clinical Perspective

5.1.1 What is new?

- Cardiac myosin-binding protein C (cMyC) is a recently described novel biomarker of cardiac injury and in small "proof-of concept" studies its serum concentration rises and falls more rapidly than that of troponin T and I.
- This is the first study to assess the diagnostic and prognostic value of cMyC in patients presenting with possible acute myocardial infarction (AMI).
- A rule-in/rule-out pathway using the novel biomarker was designed to compare discriminative power in a clinical setting.

5.1.2 What are the clinical implications?

- Diagnostic accuracy of cMyC for AMI was similar to that of hs-cTnT and hs-cTnI in the entire cohort but superior for those with chest pain of less than 3 hours duration (early presenters) when compared to hs-cTnT.
- cMyC has correctly triaged more patients to "rule-out" or "rule-in" groups than either hs-cTnI or hs-cTnT leaving a much smaller proportion in the observation groups. This advantage may facilitate early discharge of low-risk patients.

5.2. Abstract

Background: Cardiac myosin-binding protein C (cMyC) is a cardiac-restricted protein that is more abundant than cardiac troponins (cTn) and is released more rapidly following acute myocardial infarction (AMI). We evaluated cMyC as an adjunct or alternative to cTn in the early diagnosis of AMI.

Methods: In 1954 unselected patients presenting to the emergency department with symptoms suggestive of AMI, concentrations of cMyC and high (hs) and standard (s) sensitivity cTn were measured at presentation. The final diagnosis of AMI was independently adjudicated using all available clinical and biochemical information without knowledge of cMyC. The prognostic endpoint was long-term mortality.

Results: Final diagnosis was AMI in 340 patients (17%). Concentrations of cMyC at presentation were significantly higher in those with vs. without AMI (median 237 ng/L vs. 13 ng/L, $p<0.001$). Discriminatory power for AMI, as quantified by the area under the receiver-operating characteristic curve was comparable for cMyC (AUC; 0.924), hs-cTnT (0.927) and hs-cTnI (0.922) and superior to cTnI measured by a contemporary sensitivity assay (0.909). Combination of cMyC with hs-cTnT or s-cTnI (but not hs-cTnI) led to an increase in AUC to 0.931 ($p<0.0001$) and 0.926 ($p=0.003$), respectively. Use of cMyC more accurately classified patients with a single blood test into rule-out or rule in categories: Net Reclassification Improvement (NRI) +0.149 vs hs-cTnT, +0.235 vs hs-cTnI ($p<0.001$). In early presenters (chest pain <3h), the improvement in rule-in/rule-out classification with cMyC was larger compared with hs-cTnT (NRI +0.256) and hs-cTnI (NRI +0.308; both $p<0.001$). Comparing the C statistics, cMyC was superior to hs-cTnI and s-cTnI ($p<0.05$ both) and similar to hs-cTnT at predicting death at 3 years.

Conclusions: cMyC at presentation provides discriminatory power comparable to hs-cTnT and hs-cTnI in the diagnosis of acute myocardial infarction, and may perform favorably in patients presenting early after symptom onset.

Trial-Registration: www.clinicaltrials.gov. Identifier, NCT00470587

Keywords: Cardiac myosin-binding protein C, cMyC, Troponin I, Troponin T, myocardial infarction, APACE

5.3. Introduction

Of the 130 million attendances to Emergency Departments (ED) in the United States each year, approximately 7 million (6%) are due to acute chest pain.[112] The assessment and triage of such patients has become increasingly complex as now only a small proportion of those with acute myocardial infarction (AMI) have the diagnostic ECG change of ST-segment elevation.[10] Consequently, the identification of patients with AMI has become almost totally dependent on the measurement in the systemic circulation of cardiac troponin I (cTnI) or cardiac troponin T (cTnT). These biomarkers are released slowly[17] – to overcome this hurdle, the analytic performance of the cTn assays has been enhanced markedly to measure the lower concentrations achieved before the late peak.[113] Hence, the best assays can reliably measure cTn concentrations below the 99th centile of the healthy population. These high-sensitivity (hs) assays are increasingly available and are the subject of national and international guidelines describing their use to achieve more rapid triage.[12,114] In particular, the European guidelines recommend the use of assays for hs-cTnI and hs-cTnT to rapidly rule-in and rule-out AMI. Algorithms using widely based decision limits based on concentrations well below the population defined 99th centile (for rule out) and above the 99th centile (for rule in) markedly improves the sensitivity of rule-out and specificity of rule-in . However, many patients presenting with chest pain have cTn concentrations that place them between these decision limits; in an indeterminate observation zone. These patients require repeat testing and subsequent second or third rounds of triage based on rates of change of cTn concentration over time.[12,111,115] European guidelines also do not support the use of rapid rule-out/rule-in pathways using hs-cTn in patients presenting 'too early' after chest pain onset – only after 3 hours is the rule-out threshold at the limit of detection guideline-compliant.[12] This introduces

systemic delays in allocation of evidence-based treatments and prolongs stay in the pressured and precious environment of the ED.

Originally discovered by Offer et al in 1973[52], the myosin-binding protein C family consists of three isoforms, specific for slow skeletal, fast skeletal and cardiac muscle – the latter being exclusively expressed in the heart from neonatal throughout human development.[116,117] Amongst others[118–121], we have identified cardiac myosin-binding protein C (cMyC, see Figure 21) as a new candidate biomarker of cardiac injury.[50]

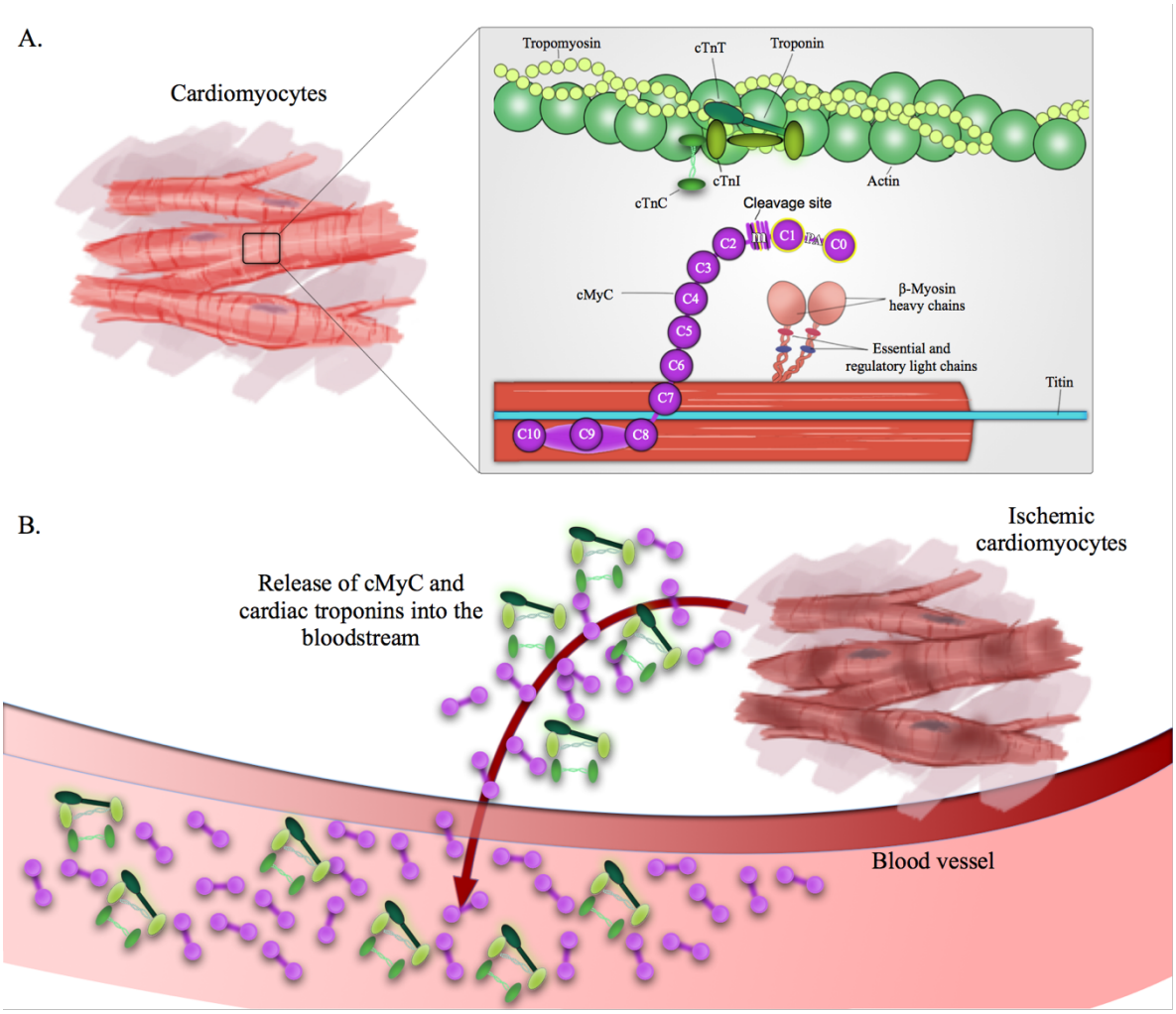


Figure 21 – Structure of cardiac Myosin-binding protein C and cardiac troponins in (A) healthy cardiomyocytes and (B) Ischaemia-induced cardiomyocyte damage. The highlighted N-terminal domain C0C1 is the binding site for the previously developed monoclonal antibodies used for detection of the cardiac-specific isoform of cMyC – see Baker et al.[50]

In common with cTnT and cTnI, cMyC expression is restricted to the heart but it is more abundant.[48] Moreover, cMyC rises more rapidly in the systemic circulation than hs-TnT after timed, iatrogenic AMI[50], perhaps as a result of its higher myocardial concentration.[122] Using a recently developed high-sensitivity assay for cMyC[84], a pilot study in 26 patients presenting early with AMI suggested that cMyC may rise more rapidly than hs-cTnI.[123]

The purpose of the current study is to compare the novel biomarker cMyC (measured on a research platform) against the most accurate currently available biochemical signals, hs-cTnI and hs-cTnT, for the early detection of AMI.

5.4. Methods

5.4.1 Study design and population

Advantageous Predictors of Acute Coronary Syndrome Evaluation (APACE) is an ongoing international multicentre diagnostic study (nine study centres in Switzerland, Spain, Poland, the Czech republic, and Italy) designed to advance the early diagnosis of AMI.[39,113,124,125] All patients older than 18 years presenting to the ED with acute chest discomfort possibly indicating AMI were eligible for recruitment if the onset of, or peak chest pain symptoms, were within the preceding 12 hours. Enrolment was independent of renal function, while patients with terminal kidney failure on chronic dialysis were excluded. For this analysis, the following patients were excluded (Figure 22): patients presenting with ST-segment elevation myocardial infarction; patients with missing levels of cMyC at presentation; patients in whom the final diagnosis remained unclear after adjudication and at least one hs-cTnT level was elevated. The latter group comprises of patients triaged and discharged following a negative gold-standard test at the time of enrolment (on a conventional cTn assay), who were later found to have an elevated hs-cTn result (comparison see table S1).

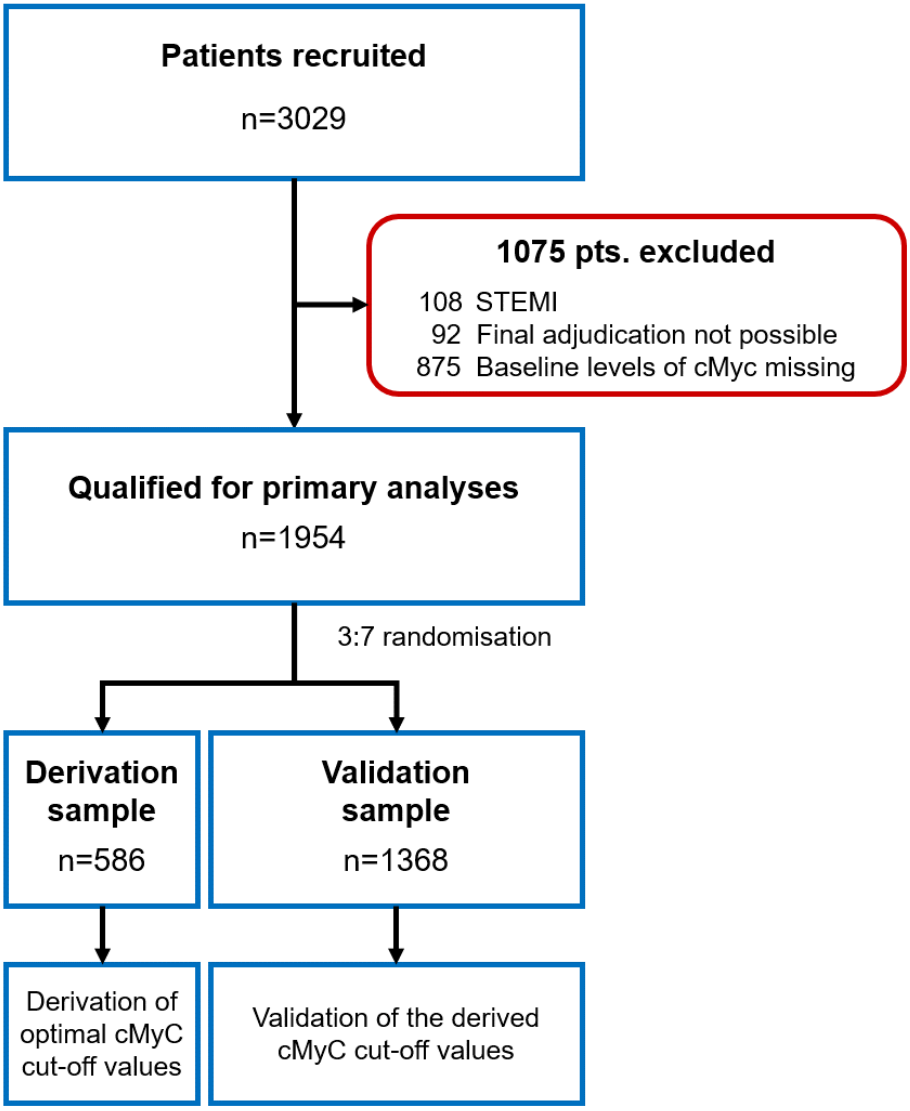


Figure 22 – Flowchart outlining recruitment numbers and exclusions from test cohort

A proportion of patients had no levels of cMyC measured at presentation due to insufficient sample volume. Demographics of the patients excluded due to missing cMyC values, compared to those of the test cohort, appear in the supplement (table S2). The protocol for routine clinical assessment is also described in the supplement. To obtain follow-up data, patients were contacted 3, 12, 24 and 36 months after discharge via telephone, email or letter.

Additionally, information regarding death during follow-up was obtained from the patient's hospital notes, the family physician's records and the national registry on mortality.

The study was carried out according to the principles of the Declaration of Helsinki and approved by the local ethics committees. Written informed consent was obtained from all patients. TK, RT and CM had full access to all the data in the study and take responsibility for its integrity and the data analysis. The authors designed the study, gathered, and analysed the data according to the STARD guidelines for studies of diagnostic accuracy (see supplement to original publication[126]), vouch for the data and analysis, wrote the paper, and decided to publish.

5.4.2 Adjudicated final diagnosis

Adjudication of the final diagnosis was performed centrally according to the 1st Universal Definition of MI, incorporating levels of hs-cTnT as the adjudicating biomarker.[127] It was based on extensive patient documentation derived from two sets of data: First, all clinical data derived from routine clinical investigations including all available medical records – patient history, physical examination, results of laboratory testing including serial local (h)s-cTn, radiologic testing, ECG, echocardiography, cardiac exercise stress test, lesion severity and morphology at coronary angiography – pertaining to the patient from the time of ED presentation to 90-day follow up. Second, study-specific assessment was collected, including 34 chest pain characteristics and serial hs-cTnT measurements in order to take advantage of the higher sensitivity and higher overall diagnostic accuracy offered by the more sensitive assays, as previously published.[113,124] In situations of disagreement about the diagnosis, cases were reviewed and adjudicated in conjunction with a third cardiologist. In brief, AMI was diagnosed when there was evidence of myocardial necrosis in association with a clinical setting consistent

with myocardial ischemia. Myocardial necrosis was diagnosed by at least one (h)s-cTn value above the 99th percentile together with a significant rise and/or fall.[11,128,129] All other patients were classified into the categories of unstable angina (UA), cardiac but non-coronary disease (e.g. tachyarrhythmias, perimyocarditis), non-cardiac chest pain and symptoms of unknown origin.

5.4.3 Measurement of cMyC, hs-cTnI, hs-cTnT, and s-cTnI

Blood samples for determination of cMyC, hs-cTnI, hs-cTnT, and s-cTnI were collected into heparin plasma and serum tubes at presentation to the ED and serially thereafter (at time points 1 h, 2 h, 3 h and 6 h). Serial sampling was discontinued when a diagnosis of AMI was certain and treatment required patient transfer to the coronary care unit or catheter laboratory. After centrifugation, samples were frozen at -80 ºC until they were assayed in a blinded fashion in a dedicated core laboratory. cMyC was measured using the previously established high-sensitivity assay on the Erenna platform that was performed by Millipore Sigma (Hayward, California).[84] The assay has a Limit of Detection (LoD) of 0.4 ng/L and a lower limit of quantification (LoQ) of 1.2 ng/L. The 99th percentile cut-off point determined previously (in patients without obstructive coronary artery disease on invasive angiography) is 87 ng/L.[84] Details of the assays used for hs-cTnI, hs-cTnT, and s-cTnI are described in the supplement.

5.4.4 Early guideline-based triage and Net Reclassification Improvement

The European Society of Cardiology (ESC) has published a rapid rule-in/rule-out pathway in the 2015 NSTEMI guidelines using hs-cTn at 0h and 1h to risk-stratify patients into 'rule-out', 'observe' and 'rule-in' categories.[12] Such categorization did not drive clinical decisions in this cohort, but it was used to compare the potential clinical utilities of cMyC and hs-cTn as triage tools. For this purpose, we have compared the categorical discrimination of hs-cTnT, hs-cTnI

and cMyC at presentation only (without subsequent delta measurements). In brief, the ESC pathway classifies patients – based on the presentation sample at 0h – into 'rule-out' with a hs-cTnT level <5 ng/L; hs-cTnI <2 ng/L; into 'rule-in' (for both assays) at ≥52 ng/L.[12] The ESC advocates the use of the pathway only in patients with ≥3 hours since chest pain onset; for completeness we have presented results for all patients, <3 and ≥3 hours since chest pain onset alone.

For cMyC we separated the cohort into derivation and validation cohorts (a randomized 3:7 split, for comparison see table S4); the 'rule-out' threshold was derived from a pre-defined sensitivity of ≥99.5%, 'rule-in' from a pre-defined specificity >95% for the gold-standard diagnosis of AMI. This resulted in a 'rule-out' threshold of ≤10 ng/L, and 'rule-in' threshold of >120 ng/L for cMyC (Figure 23). These thresholds were then used in the validation cohorts to compare cMyC against both hs-cTnT and hs-cTnI. Net Reclassification Improvement (NRI) operates as follows: each patient is first assigned a classification ('rule-out', 'observe' or 'rule-in') based on cut-off values of hs-cTnI/T in the presentation blood sample (the initial model). The same cohort is then reclassified to the same three groups based on the cMyC cut-off values (the new model). This reclassification may correctly or incorrectly reallocate a patient, e.g. a patient who went on to be diagnosed with an AMI may be correctly reclassified from 'observe' to 'rule-in', or incorrectly reclassified from 'observe' to 'rule-out'. The 'NRI' analysis defines separate categorical NRI values for those patients who were ultimately diagnosed with AMI (quoted as NRI_{AMI}) and those who were not (NRI_{noAMI}) – range -1 to +1; 'Dimensionless NRI' reflects the unweighted, net-movement of all patients regardless of final diagnosis (range -2 to +2). NRI_{AMI} is positive if there is a net movement of patients with adjudicated AMI into higher-risk classifications using cMyC (the new model). NRI_{noAMI} is

positive there is a net movement of patients without an adjudicated diagnosis of AMI into lower-risk classifications using cMyC (the new model).[130] NRI calculations were performed for the validation cohort, early presenters (<3 hours since onset of chest pain; ESC guideline not applicable) and late presenters (≥3 hours since onset; ESC guideline applicable); tables are presented in full where appropriate.

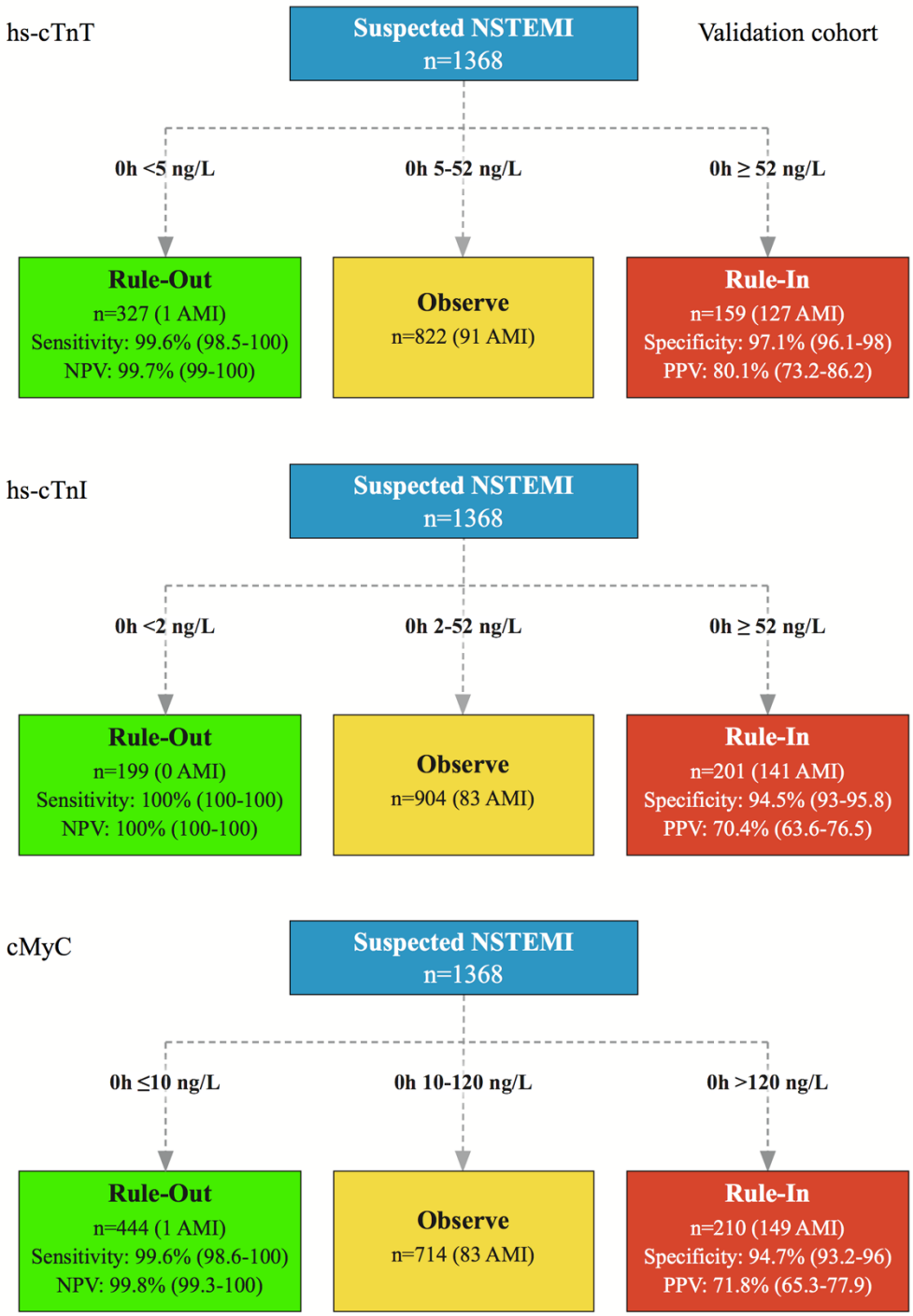


Figure 23 – Distribution of participants, depending on each biomarker used, according to ESC guideline[12] for hs-cTnT and hs-cTnI, and theoretical model for the novel biomarker cMyC; AMI = Acute Myocardial Infarction, based on the adjudicated gold-standard diagnosis

5.4.5 Statistical analysis

All data are expressed as medians [1st quartile, 3rd quartile] or means (standard deviation) for continuous variables (compared with the Mann-Whitney-U test or student's t-test), and for categorical variables as numbers and percentages (compared with Pearson chi-square). Hypothesis testing was two-tailed, and p values <0.05 were considered statistically significant. No adjustment for multiple comparisons was performed.

Discrimination power was quantified by the area under the receiver-operating characteristics curve (AUC) for each biomarker with all cases available, using 1,000 stratified bootstrap replicates to calculate Confidence intervals (CI). Logistic regression was used to combine cMyC levels with hs-cTnT, hs-cTnI or s-cTnI values for the assessment of an incremental value using two biomarkers at presentation. Sub-group analysis was performed for patients presenting early, defined as chest pain onset within 3 hours of presentation to the Emergency Department. This is a particular limitation of the published ESC guidance on the use of hs-cTn for risk-stratification, as the rapid rule-out/rule-in algorithms are only applicable to patients with chest pain onset >3 hours.

Predictive value of the biomarkers during follow-up was assessed two-fold: We calculated 1) Harrell's C statistic for each biomarker at presentation for endpoints AMI, death or the composite of AMI and all-cause mortality during follow-up (excluding the index event) – a higher C index indicates a higher probability of an event occurring during follow-up with higher biomarker values[131]; and 2) Kaplan-Meier survival curves. Cox regression analysis was performed as follows: All available biomarker levels were divided into 1) quintiles and 2) groups according to 'rule-out', 'observe' and 'rule-in' classification. Unadjusted Cox proportional hazard regression models were fitted for 30-day and 3-year follow-up for each

group with the lowest quintile (or risk group, respectively) normalized to a hazard ratio of 1 and assessed using the likelihood-ratio test. Cox coefficients and thus hazard ratios were not calculated if the lowest risk group did not suffer any events, which would invalidate the regression model. NRI statistics were calculated as categorical values.[130,132] The Integrated Discrimination Improvement (IDI) values quoted reflect a category-free (positive or negative) change in model-performance. Confidence intervals for cut-off thresholds, NRI and IDI statistics were derived using 1,000 bootstrap replicates. All statistical analyses were performed using R, version 3.3.0 GUI 1.68 (The R Foundation for Statistical Computing), including packages ggplot2, R Markdown, RStudio, PredictAbel, survival, Hmisc, compareC and ROCR.

5.5. Results

5.5.1 Baseline characteristics

A total of 1954 unselected patients eligible for this analysis were enrolled (Figure S1). Median age was 62 years, 31% were women, and 36% had a prior history of coronary artery disease (Table 3). Overall, 1469 patients (75%) had no significant electrocardiographic abnormalities at presentation to the ED. Median time since onset of chest pain was 5 hours [IQR 3, 12], with a median of 3 hours [IQR 2, 7] since peak chest pain severity.

The adjudicated final diagnosis was AMI in 340 (17%) patients, unstable angina in 10%, symptoms of cardiac origin other than coronary artery disease in 14%, non-cardiac symptoms in 54% and symptoms of unknown origin in 5%.

Median follow-up for the entire cohort was 772 days [IQR 731, 907]; of those not sustaining any events in the monitoring period (AMI or death), the median follow-up was 792 days [IQR 738, 923]. A total of n=165 (8%) patients died during 3-year follow-up. 1903 patients (97%)

exceeded 90 days of follow-up; of those who did not (n=51, 3%), 27 (1%) sustained a cardiovascular death.

Demographics	All patients (n = 1954)	AMI (n = 340)	Other diagnoses (n = 1614)	p value*
Age, years	62 ± 16	69 ± 13	60 ± 16	<0.001
Male	1341 (69)	256 (75)	1085 (67)	0.004
Risk factors				
Hypertension	1247 (64)	269 (79)	978 (61)	<0.001
Hyperlipidaemia	992 (51)	227 (67)	765 (47)	<0.001
Diabetes mellitus	369 (19)	92 (27)	256 (16)	<0.001
Current smoking	500 (25)	90 (27)	386 (24)	0.345
History of smoking	718 (38)	141 (42)	577 (36)	0.051
History				
Coronary artery disease	710 (36)	174 (51)	536 (33)	<0.001
Previous myocardial infarction	474 (24)	118 (35)	356 (22)	<0.001
Previous revascularisation (CABG or PCI)	553 (28)	127 (37)	426 (26)	<0.001
Peripheral artery disease	119 (6)	43 (13)	76 (5)	<0.001
Previous stroke	100 (5)	23 (7)	77 (5)	0.167
Vital status				
Heart rate, beats/min	79 ± 20	81 ± 20	79 ± 20	0.092
Systolic blood pressure, mm Hg	144 ± 24	145 ± 27	143 ± 24	0.421
Diastolic blood pressure, mm Hg	82 ± 15	81 ± 17	82 ± 15	0.299
Electrocardiographic findings				
ST-segment depression	193 (10)	93 (28)	100 (6)	<0.001
T-wave inversion	260 (13)	82 (24)	178 (11)	<0.001
No significant electrocardiographic abnormalities	1469 (75)	161 (49)	1308 (83)	<0.001
Laboratory assessment				
Estimated glomerular filtration rate, ml/min/1.73m2†	84 ± 26	74 ± 26	86 ± 25	<0.001
Presentation time				
Time since chest pain onset (hrs)	5 [3, 12]	5 [3, 12]	5 [3, 12]	0.898
Time since chest pain peak (hrs)	3 [2, 7]	3 [2, 7]	4 [2, 7]	0.408

Table 3 – Baseline demographics; * p values for comparison AMI group versus all other diagnoses; data are expressed as medians [1st quartile, 3rd quartile] or means ± standard deviation, for categorical variables as numbers (percentages); AMI = Acute Myocardial Infarction; IQR = Interquartile Range; CABG = Coronary Artery Bypass Graft; PCI = Percutaneous Coronary Intervention; † glomerular filtration rate was estimated using the Modification of Diet in Renal Disease (MDRD) formula

5.5.2 Distribution of biomarker concentrations

As shown in Figure 24, cMyC levels were significantly higher in patients with AMI (n=340) compared to patients with other diagnoses (AMI, median 237 ng/L [IQR 71, 876 ng/L; unstable angina, median 21 ng/L [IQR 13, 43 ng/L]; cardiac symptoms of origin other than coronary artery disease, median 33 ng/L [IQR 12, 96 ng/L]; non-cardiac symptoms, median 10 ng/L [IQR 6, 19 ng/L]; symptoms of unknown origin, median 11 ng/L [IQR 7, 16 ng/L]; $p < 0.001$ for all comparisons with AMI patients). Similarly, blood concentrations of hs-cTnT, hs-cTnI, and s-cTnI were significantly higher in AMI as compared to other final diagnoses (median biomarker concentrations displayed in tables S5+S6). Overall, blood concentrations of cMyC in relation to LoD were higher than those of hs-cTn in all diagnostic categories (Table S5). Non-cardiac sources of cMyC variation were previously investigated in an ambulatory cohort[84]; results of comparison within the groups with AMI and non-cardiac symptoms have been displayed in tables S7+S8.

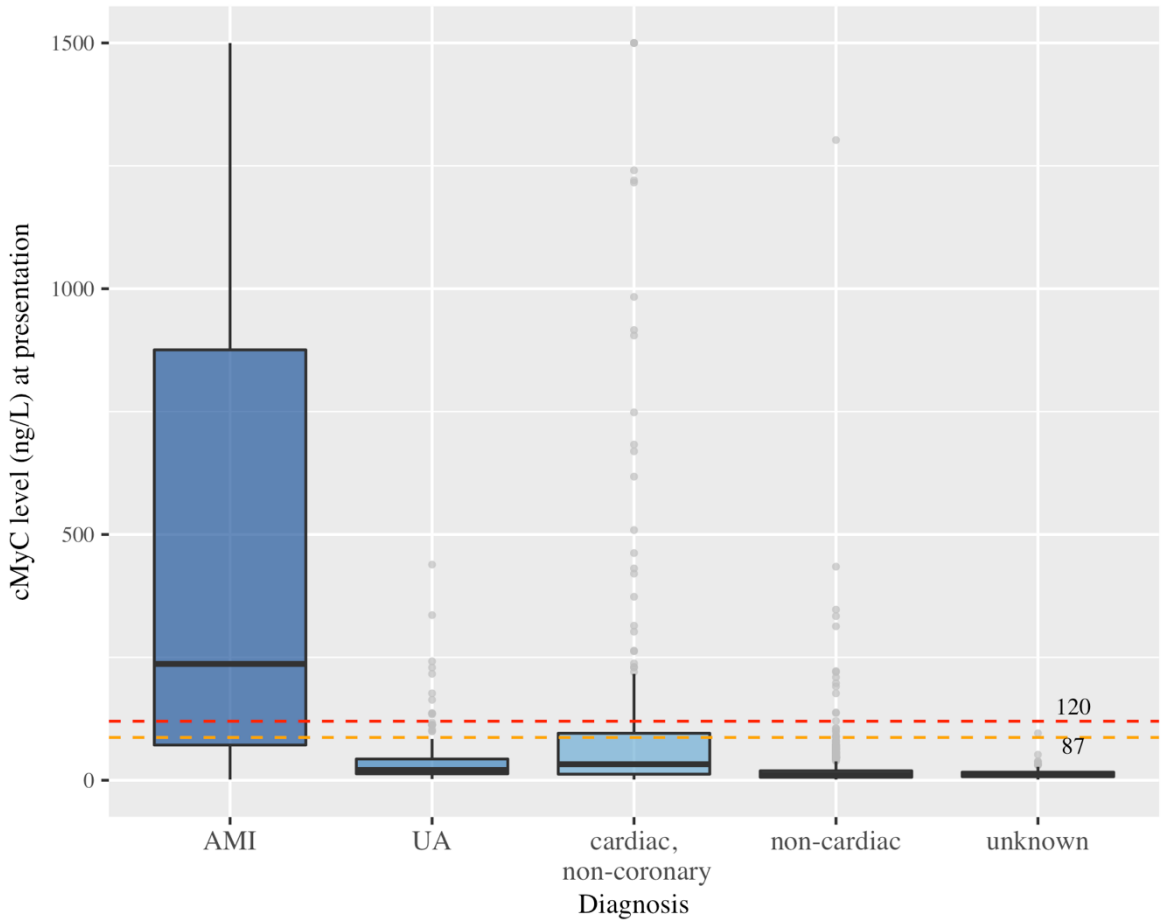


Figure 24 – Baseline distribution of cMyC levels at presentation to the emergency department in all patients based on adjudicated final diagnosis. Boxes represent interquartile ranges, whiskers extend to 1.5 * IQR from the hinges (y-axis capped at 1,500 ng/L, outliers represented by light grey bullets). 87 ng/L represents the 99th centile based on a previous study, 120 ng/L the cut-off threshold for diagnostic rule-in of AMI at presentation. AMI, median 237 ng/L [IQR 71, 876 ng/L]; unstable angina, median 21 ng/L [IQR 13, 43 ng/L]; cardiac symptoms of origin other than coronary artery disease, median 33 ng/L [IQR 12, 96 ng/L]; non-cardiac symptoms, median 10 ng/L [IQR 6, 19 ng/L]; symptoms of unknown origin, median 11 ng/L [IQR 7, 16 ng/L]; $p<0.001$ for all comparisons with AMI patients

5.5.3 Discrimination power

In blood drawn at presentation, the discrimination of cMyC for AMI, as quantified by the AUC, was 0.924 (95% confidence interval [CI], 0.910-0.939), compared to the AUCs for hs-

cTnT 0.927 (95% CI, 0.913-0.941; p=0.573 for direct comparison); hs-cTnI 0.922 (95% CI, 0.908-0.936; p=0.993 for direct comparison) and s-cTnI 0.909 (95% CI, 0.889-0.928; p=0.024 for direct comparison, Table 4, Figure 25).

All patients – comparison	**Area Under the Curve (95% Confidence Interval)**	**p value***	**n**
cMyC vs hs-cTnT	0.924 (0.910-0.939) vs 0.927 (0.913-0.941)	0.573	1554 controls, 322 AMI
cMyC vs hs-cTnI	0.923 (0.908-0.937) vs 0.922 (0.908-0.936)	0.993	1537 controls, 320 AMI
cMyC vs s-cTnI	0.924 (0.906-0.938) vs 0.909 (0.889-0.928)	0.024	1463 controls, 311 AMI
Early presenters (≤ 3 hours since chest pain onset) – comparison			
cMyC vs hs-cTnT	0.915 (0.887-0.941) vs 0.892 (0.857-0.922)	0.022	562 controls, 104 AMI
cMyC vs hs-cTnI	0.915 (0.889-0.939) vs 0.909 (0.879-0.935)	0.539	554 controls, 102 AMI
cMyC vs s-cTnI	0.914 (0.888-0.939) vs 0.892 (0.859-0.925)	0.060	529 controls, 103 AMI
All patients – Combination cMyC with…		p value†	n
hs-cTnT	0.935 (0.921-0.948)	0.002	1548 controls, 322 AMI
hs-cTnI	0.929 (0.913-0.943)	0.093	1537 controls, 320 AMI
s-cTnI	0.928 (0.909-0.943)	<0.001	1463 controls, 311 AMI

Table 4 – Area under the Receiver-Operating Characteristics Curve – Comparisons between biomarkers; *p value for direct comparison between biomarkers; †p value for direct comparison between AUC for combination (cMyC with cTn) and respective cTn on its own; AUC = Area under the Curve; AMI = Acute Myocardial Infarction

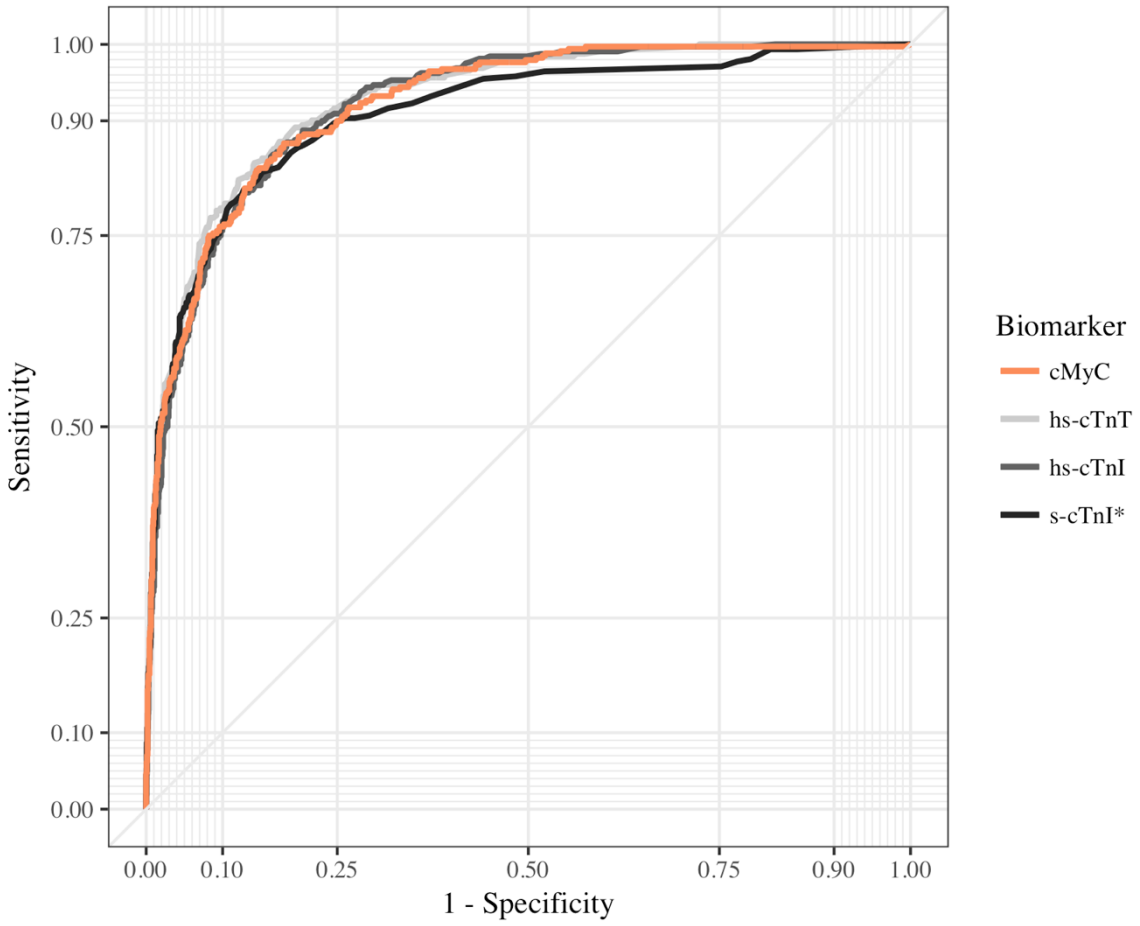


Figure 25 – ROC curves for individual biomarkers: Diagnostic performance of cMyC, hs-cTnT, hs-cTnI and s-cTnI in the early diagnosis of acute myocardial infarction (AMI), based on presentation blood sample and adjudicated AMI diagnosis. Receiver operating characteristic (ROC) curves describing the performance of cMyC (orange line; Area under the Curve (AUC) 0.924), hs-cTnT (light grey line; AUC 0.927), hs-cTnI (dark grey line; AUC 0.922) and s-cTnI (black line; AUC 0.909*); *p<0.05

5.5.4 Early presenters

In patients presenting within 3 hours of symptom onset (n=694, with AMI adjudicated in 16%) the AUC for cMyC was 0.915 (95% CI, 0.887-0.941), compared to the AUCs for hs-cTnT 0.892 (95% CI, 0.857-0.922; p=0.022); hs-cTnI 0.909 (95% CI, 0.879-0.935; p=0.539) and s-cTnI 0.892 (95% CI, 0.859-0.925; p=0.060) (Table 4).

5.5.5 Combination of cMyC with cTn

AUC for the combination of cMyC with hs-cTnT was 0.935 (95% CI, 0.921-0.948; p=0.002 for comparison with hs-cTnT alone), cMyC with hs-cTnI 0.929 (95% CI, 0.913-0.943; p=0.093 for comparison with hs-cTnI alone) and cMyC with s-cTnI 0.928 (95% CI, 0.909-0.943; p<0.001 for comparison with s-cTnI alone) (Table 4, Figure 26).

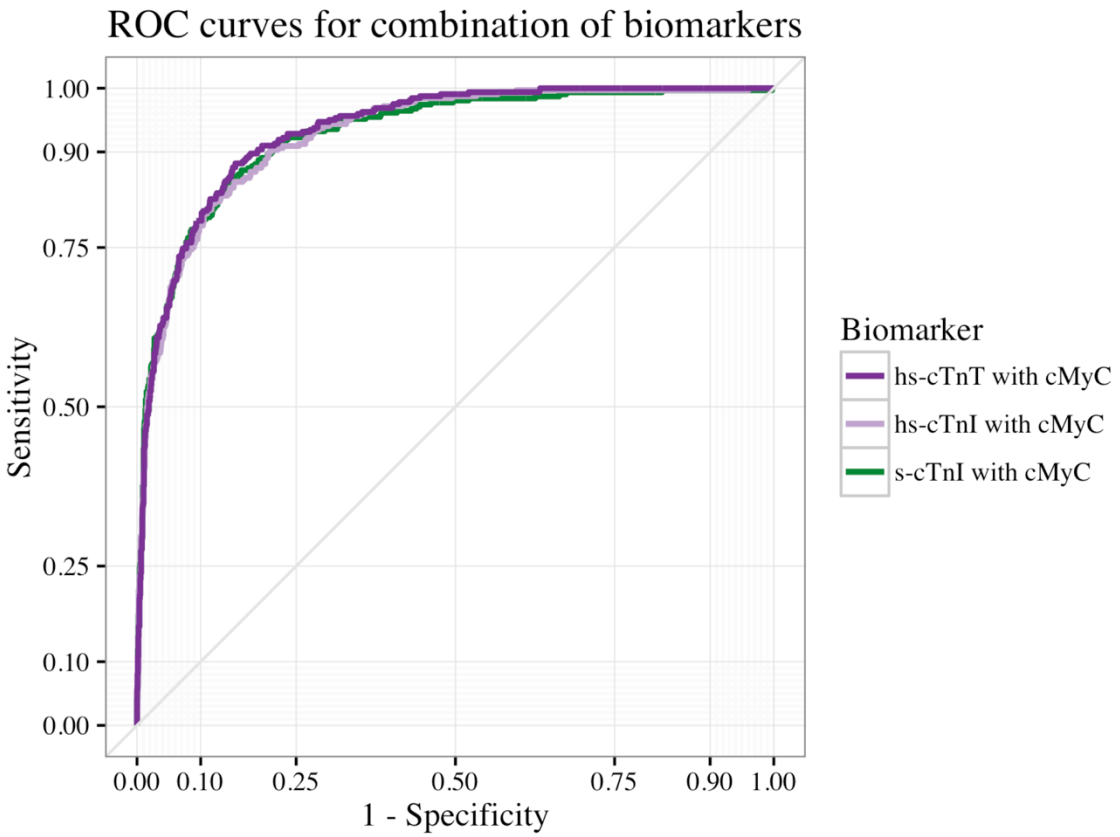


Figure 26 – ROC curves describing the diagnostic performance of the combination of cMyC with hs-cTnT (dark purple line; AUC 0.935*), hs-cTnI (light purple line; AUC 0.929) and s-cTnI (green line; AUC 0.928*); *p<0.05

5.5.6 Classification function of cut-off values for risk groups

Sensitivity, specificity, negative and positive predictive values were calculated for derivation (tables S9, S10) and validation cohorts based on cut-offs published in the 2015 ESC guideline[12]: in the validation cohort (n=1,368, 233 events), hs-cTnT has a sensitivity of 99.6% (95% CI, 98.5-100%) and NPV of 99.7% (95% CI, 99-100%) at the rule-out threshold of 5 ng/L, specificity of 97.1% (95% CI, 96.1-98%) and PPV 80.1% (95% CI, 73.2-86.2%) at the rule-in threshold (52 ng/L); hs-cTnI has a sensitivity of 100% (95% CI, 100-100%) and NPV of 100% (95% CI, 100-100%) at 2 ng/L, specificity of 94.5% (95% CI, 93-95.8%) and PPV 70.4% (95% CI, 63.6-76.5%) for rule-in – Table 5. After obtaining clinically meaningful cut-off thresholds in the internal derivation cohort (tables S9, S10; figure S2; based on sensitivity ≥99.5%, specificity >95%), these were tested in the validation cohort: at a threshold of 10 ng/L for rule-out, cMyC achieves a sensitivity of 99.6% (95% CI, 98.6-100%) and NPV of 99.8% (95% CI, 99.3-100%). At 120 ng/L for the rule-in threshold, cMyC achieves a specificity of 94.7% (95% CI, 93.3-95.9%) and PPV of 71% (95% CI, 64.9-77.2%); all data in Table 5 & Table 6.

Initial model	New model – cMyC (10/120) – Validation cohort					
hs-cTnT	No AMI (n=1089)			AMI (n=219)		
	Rule-out	Observe	Rule-in	Rule-out	Observe	Rule-in
Rule-out	249	77	0	0	1	0
Observe	190	509	32	1	66	24
Rule-in	0	7	25	0	9	118
NRI	0.081 (95% CI, 0.029-0.113)			0.068 (95% CI, 0.016-0.121)		
NRI (dimensionless) 0.149 (95% CI, 0.089-0.210); p value <0.001, IDI 0.050 (95% CI, 0.029-0.070)						

Thresholds	Sensitivity (95% CI)	NPV (95% CI)	Specificity (95% CI)	PPV (95% CI)
hs-cTnT 5 ng/L	99.6% (98.5-100%)	99.7% (99-100%)	29.9% (27.3-32.5%)	22.2% (19.6-24.8%)
hs-cTnT 52 ng/L	58.1% (51.6-64%)	92% (90.5-93.5%)	97.1% (96.1-98%)	80.1% (73.2-86.2%)
cMyC 10 ng/L	99.5% (98.6-100%)	99.8% (99.3-100%)	38.8% (35.8-41.7%)	24.6% (21.8-27.4%)
cMyC 120 ng/L	64.9% (58.5-71.2%)	93.1% (91.4-94.5%)	94.8% (93.5-96%)	71.5% (64.7-78%)

Table 5 – Net Reclassification Improvement (Validation cohort for hs-cTnT): NRI = Net Reclassification Improvement; IDI = Integrated Discrimination Improvement; CI = Confidence Interval; NPV = Negative Predictive Value; PPV = Positive Predictive Value; AMI = Acute Myocardial Infarction, based on the adjudicated gold-standard diagnosis

Initial model	New model – cMyC (10/120) – Validation cohort					
hs-cTnI	No AMI (n=1080)			AMI (n=224)		
	Rule-out	Observe	Rule-in	Rule-out	Observe	Rule-in
Rule-out	167	32	0	0	0	0
Observe	273	526	22	1	63	19
Rule-in	0	25	35	0	16	125
NRI	0.226 (95% CI, 0.174-0.258)			0.009 (95% CI, -0.044-0.062)		
NRI (dimensionless) 0.235 (95% CI, 0.174-0.296); p value <0.001; IDI 0.078 (95% CI, 0.057-0.098)						

Thresholds	Sensitivity (95% CI)	NPV (95% CI)	Specificity (95% CI)	PPV (95% CI)
hs-cTnI 2 ng/L	100% (100-100%)	100% (100-100%)	18.4% (16-20.8%)	20.3% (18-22.7%)
hs-cTnI 52 ng/L	62.9% (56.4-68.9%)	92.5% (90.9-93.9%)	94.5% (93-95.8%)	70.4% (63.6-76.5%)
cMyC 10 ng/L	99.6% (98.6-100%)	99.8% (99.3-100%)	39.4% (36.3-42.4%)	25.5% (22.9-28.5%)
cMyC 120 ng/L	64.3% (58.1-70.7%)	92.8% (91.2-94.3%)	94.7% (93.2-96%)	71.8% (65.3-77.9%)

Table 6 – Net Reclassification Improvement (Validation cohort for hs-cTnI): abbreviations as in table 5

All data for the groups of early (<3 hours of chest pain) and late presenters (≥3 hours of chest pain) is presented in tables S11 and S12. In short, in early presenters cMyC demonstrates comparable sensitivity to hs-cTnT (cMyC 100% vs 98.8%; p=0.317) for rule-out (at cMyC threshold 10 ng/L). Sensitivity is equivalent between cMyC and hs-cTnI (100% both; p=1). In the group of late presenters, there is no statistical difference between sensitivity and specificity achieved for cMyC when compared to hs-cTnT/I. Specificity for adjudicated diagnosis of AMI was individually assessed at the 99th centile in table S13.

5.5.7 Risk group reclassification

The distribution of patients in risk groups 'rule-out', 'observe' and 'rule-in' based on the initial blood test (either hs-cTnT, hs-cTnI or cMyC) is displayed in Figure 27 (validation cohort, n=1368, AMI in 17%). cMyC classified 443 patients (32.4%) safely as rule-out, compared to 348 (25.4%) with hs-cTnT and 206 (15.1%) with hs-cTnI – predominantly by reducing the size of the observation group.

In the validation cohort (Table 5, Table 6), cMyC at presentation was superior to hs-cTnT with NRI +0.149 (NRI_{noAMI} +0.081, NRI_{AMI} +0.068; p <0.001), and to hs-cTnI with NRI +23.5 (NRI_{noAMI} +0.226, NRI_{AMI} +0.009; p <0.001). In the cohort of early presenters (<3 hours of chest pain), cMyC was superior to hs-cTnT with NRI +0.256 (NRI_{noAMI} +0.256, NRI_{AMI} +0.128; p <0.001), and to hs-cTnI with NRI +0.308 (NRI_{noAMI} +0.257, NRI_{AMI} +0.051; p <0.001); table S11. In the cohort of late presenters (≥3 hours of chest pain), cMyC was superior to hs-cTnT with NRI +0.133 (NRI_{noAMI} +0.084, NRI_{AMI} +0.049; p <0.001), and to hs-cTnI with NRI +0.227 (NRI_{noAMI} +0.240, NRI_{AMI} -0.012; p <0.001); table S12.

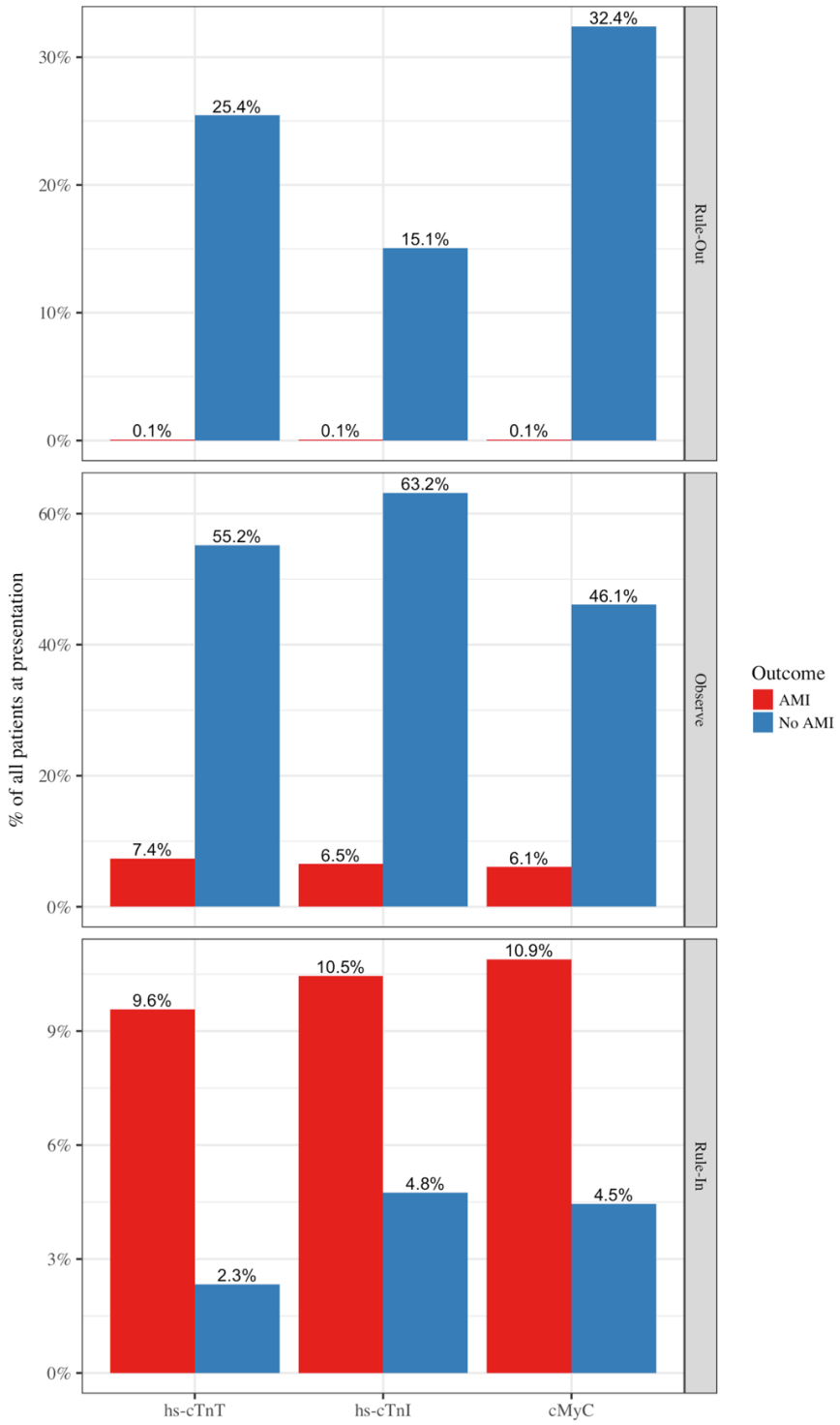
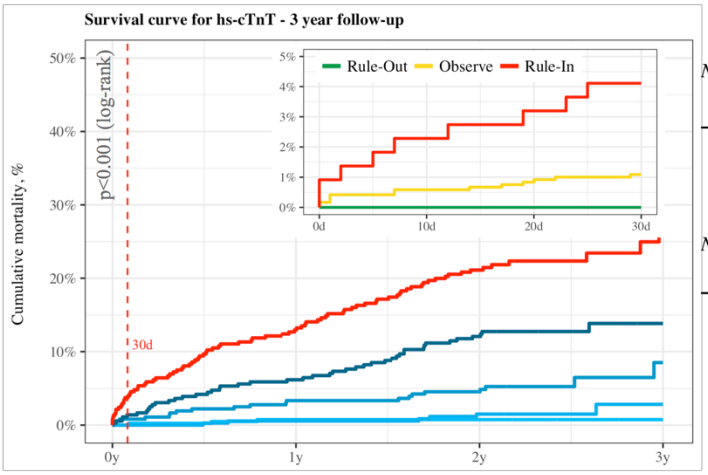


Figure 27 – Distribution of patients in different risk categories after presentation blood tests, based on ESC guideline 2015[12] for hs-cTnT and hs-cTnI, and newly developed cut-off thresholds for cMyC at ≤10 ng/L for rule-out and >120 ng/L for rule-in of myocardial infarction.

5.5.8 Prognostic performance of cMyC

As quantified by Harrell's C statistic calculated from the presentation sample (table S14), cMyC matched the performance of hs-cTnT in predicting AMI (excluding index event), death and the composite endpoint within a 3-year follow-up. Compared to hs-cTnI, there was a statistically different but numerically small improvement in predicting death and the composite endpoint at 3 years: cMyC C index 0.767 vs hs-cTnI 0.732 (p=0.001) and 0.746 vs 0.722 (p=0.008), respectively; AMI was comparable. cMyC was significantly better at predicting AMI, death or the composite endpoint when compared to cTnI.

For the calculation of cumulative hazard ratios (HR) for all-cause mortality using a Cox regression model, each biomarker was separated into quintiles. HR for hs-cTnT at three year follow-up was 2.3 (95% CI, 0.6-9.0) in the second quintile, 7.7 (95% CI, 2.3-25.8) in the third, 17.7 (95% CI, 5.5-57.1) in the fourth and 33.6 (95% CI, 10.6-106.3) in the fifth quintile – $p < 0.05$ for all except 2nd quintile. The HR for hs-cTnI was 6.6 (95% CI, 1.5-29.2), 11.3 (95% CI, 2.7-48.3), 25.1 (95% CI, 6.1-103.3) and 39.7 (95% CI, 9.7-161.8), respectively – $p < 0.05$ for all quintiles. The HR for cMyC was 2.6 (95% CI, 0.7-10.0), 7.8 (95% CI, 2.3-25.9), 17.2 (95% CI, 5.4-55.0) and 29.4 (95% CI, 9.3-93.2) – $p < 0.05$ for all except 2nd quintile. Survival curves for cMyC and hs-cTn assays are displayed in Figure 28 for three-year and 30-day follow-up.

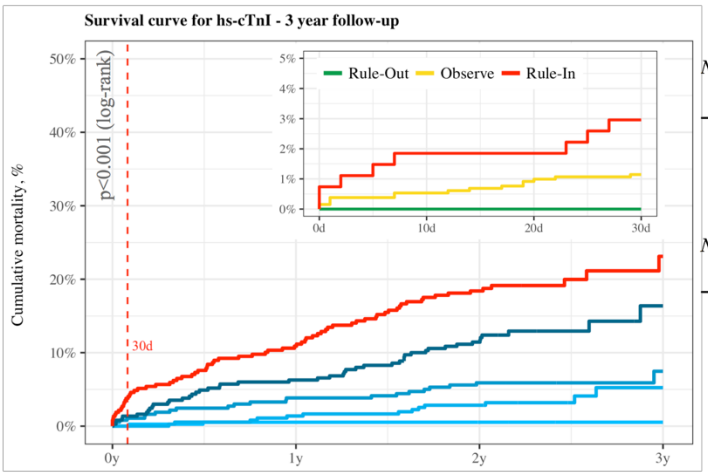


Number at risk at 30d

	10d	20d	30d
Rule-Out	457	457	456
Observe	1194	1187	1183
Rule-In	217	214	212

Number at risk at 3 years

	1y	2y	3y
1st quintile	407	391	351
2nd quintile	367	352	300
3rd quintile	364	343	296
4th quintile	359	329	275
5th quintile	371	318	259

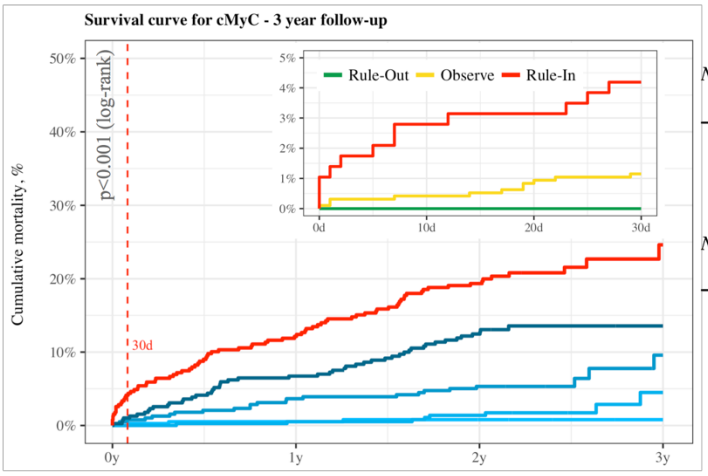


Number at risk at 30d

	10d	20d	30d
Rule-Out	271	271	271
Observe	1309	1303	1296
Rule-In	269	265	265

Number at risk at 3 years

	1y	2y	3y
1st quintile	379	367	315
2nd quintile	366	354	315
3rd quintile	368	344	299
4th quintile	368	340	288
5th quintile	368	319	261



Number at risk at 30d

	10d	20d	30d
Rule-Out	706	705	704
Observe	956	953	948
Rule-In	284	278	277

Number at risk at 3 years

	1y	2y	3y
1st quintile	390	377	327
2nd quintile	388	374	324
3rd quintile	390	365	320
4th quintile	391	356	297
5th quintile	387	335	283

1st quintile — 2nd quintile — 3rd quintile — 4th quintile — 5th quintile

Figure 28 – Kaplan-Meier Curves – Cumulative incidence of death in all patients based on biomarker value at presentation: all-comers underwent follow-up for up to 3 years. Survival curves are plotted for hs-cTnT, hs-cTnI and cMyC based on quintiles for a three year follow-up, and separated in risk groups 'Rule-Out', 'Observe' and 'Rule-In'[12] at 30 day follow-up. Amongst quintiles, the HR for hs-cTnT at three year follow-up was 2.3 (95% CI, 0.6-9.0) in the second quintile, 7.7 (95% CI, 2.3-25.8) in the third, 17.7 (95% CI, 5.5-57.1) in the fourth and 33.6 (95% CI, 10.6-106.3) in the fifth quintile. The HR for hs-cTnI was 6.6 (95% CI, 1.5-29.2), 11.3 (95% CI, 2.7-48.3), 25.1 (95% CI, 6.1-103.3) and 39.7 (95% CI, 9.7-161.8), respectively. The HR for cMyC was 2.6 (95% CI, 0.7-10.0), 7.8 (95% CI, 2.3-25.9), 17.2 (95% CI, 5.4-55.0) and 29.4 (95% CI, 9.3-93.2).

The quintiles comprise of the following tiers:

hs-cTnT: [0.0, 4.1) [4.1, 7.1) [7.1, 12.1) [12.1, 27.5) [27.5, 1750.0]

hs-cTnI: [0.2, 2.3) [2.3, 3.6) [3.6, 6.8) [6.8, 22.9) [22.9, 25351.6]

cMyC: [1.27, 6.92) [6.92, 12.24) [12.24, 24.19) [24.19, 71.71) [71.71, 4369.03]

5.6. Discussion

To our knowledge, cMyC is the first cardiac-restricted protein to be analysed as a diagnostic test for AMI since cTn. In this diagnostic multicentre study we compared its diagnostic performance to cTnI and cTnT, measured using the leading high-sensitivity assays recommended in current practice guidelines[12], in a well-characterized and large cohort of patients presenting with symptoms suggestive of AMI. Discrimination for MI with cMyC was similar to that of hs-cTnT and hs-cTnI and superior to s-cTnI. In patients presenting within 3 hours of chest pain onset, cMyC was superior to hs-cTnT, despite the latter's use as the adjudicating biomarker. Importantly, the exclusion of patients who were discharged on the basis of an undetectable contemporary cTn concentration, but were later found to have a quantifiable hs-cTn level above the 99th centile (n=92) leaves room for speculation whether an entirely different marker might have performed even better, if used for final adjudication. Using cut-offs for cMyC calibrated against those recommended in guidelines[12], cMyC correctly

and safely rules-out and rules-in AMI in a greater proportion of patients than either hs-cTnT or hs-cTnI. These findings indicate that cMyC may be better able to triage patients presenting to the ED with suspected AMI.

cTnT and cTnI have transformed the management of patients with suspected AMI and their importance is enshrined in the Universal Definition of Myocardial Infarction.[11] Consequently, AMI events are identified/adjudicated based on a significant rise (and/or fall) in cTnT/I blood concentration. This definition has harmonized clinical care and clinical research, but also introduced an inherent bias in favour of cTnT/I versus novel diagnostic biomarkers in studies such as ours. cMyC is not part of the troponin complex and has a distinct location within the cardiac sarcomere (Figure 21). For these reasons, our findings regarding the performance of cMyC against the hs-cTnT and hs-cTnI 'gold-standard', are notable. Since cMyC was not measured through the patients' journey, it is a matter of speculation if the outcome would have been different with cMyC as the adjudicating biomarker.

After iatrogenic or spontaneous AMI cMyC appears more rapidly in the systemic circulation than either hs-cTnT or hs-cTnI.[50,123] This is probably due to a combination of cMyC's greater myocardial abundance, distinct sarcomeric location and loose association with myosin and actin.[50] This biological distinctiveness of cMyC likely underpins the diagnostic advantage we observed over hs-cTnT/I in patients presenting within 3 hours of symptom onset. Moreover, the more rapid appearance of cMyC in the systemic circulation after cardiac injury is also likely to explain the net reclassification improvement over both hs-cTnT and hs-cTnI.

There are no large prospective clinical trials comparing the effect of different biomarkers of cardiac necrosis on clinical outcome. Nonetheless it is interesting to speculate what effect the improved classification of events by cMyC could have in clinical practice. The current

guidelines identify three risk groups, where only hs-cTn concentrations at the limit of detection or significantly above the 99th centile clearly triages patients towards rule-out or rule-in of AMI, respectively.[12] This leaves a significant proportion of patients within the 'observe' zone of clinical uncertainty requiring repeat cTn measurement and further investigation.[133] In the current study, of the patients who ultimately did not have AMI, the proportion in the observe-zone after the first measurement at presentation was 55.2% using hs-cTnT, 63.2% using hs-cTnI and 46.1% using cMyC. It is expected that the greater diagnostic certainty afforded on a single presentation blood draw by cMyC may reduce median time to discharge and costs of investigations.

As yet, near-patient, point-of-care devices have not been able to rule-out AMI since they have struggled to achieve the required analytic sensitivity to measure low concentrations of cTnT or cTnI. In addition, the development of reliable large-platform based hs-cTn assays has proved more challenging than expected. Until now, only two manufacturers have overcome the difficulties of developing and introducing hs-cTn assays into clinical practice[12], of which one had major quality issues initially.[134–136] These uncertainties and concerns have led to delays in the approval of these assays for clinical care in the United States.[137] The Food and Drug Administration has only recently ratified the use of the 5th generation hs-cTnT assay.[138] Since cMyC is more abundant and rises more rapidly, migration to a point-of-care format may be less challenging. Risk prediction appears grossly similar when comparing hs-cTn and cMyC and could therefore be performed on either. Notably, a cMyC level below 10 ng/L (the threshold resembling 25-times the Limit of Detection) offers both very high NPV and 30-day mortality rates approaching zero.

There are a number of limitations to our study. First, the diagnostic cut-offs for cMyC require external validation: Despite its size, a single cohort cannot entirely safeguard against calibration-issues and is inherently subject to potential, institutional bias. We have attempted to mitigate these risks by employing both randomization and bootstrapping, but in an ideal scenario the findings were validated in an independent cohort. Second, the analyses within this manuscript are confined to the concentration of necrosis biomarker on first blood draw. We have not analysed the effect on the grey zone of repeat blood draws after set intervals. This is an area of active research for which there is no consensus regarding resampling interval, magnitude of concentration change, use of absolute or relative change in concentration or effect of assay vendor.[113,114,124,139–141] Third, as a prospective diagnostic study, we cannot exactly quantify the clinical benefit associated with the use of cMyC as an alternative or addition to hs-cTn – further cluster-randomized studies will be required to address this issue. Fourth**,** we cannot comment on the accuracy of cMyC among patients with terminal kidney failure on renal replacement therapy or ST elevation myocardial infarction, since such patients were excluded from this study. Currently, biomarkers have no role in the assessment of patients with STEMI and hence this group was not analysed. Fifth, of 3029 patients recruited, 875 had no baseline cMyC measured; however, a comparison between the analysed cohort and the excluded patient sample has not demonstrated substantial differences in baseline characteristics (suppl. table 3S). Sixth, in patients with low levels of cMyC (e.g. the non-cardiac chest pain group), we have observed a significant difference in biomarker values dependent on certain underlying conditions (such as prior coronary artery disease; suppl. tables S7-8); however, this effect is muted in patients with AMI, and indeed did not negatively influence specificity. Finally, cMyC was measured using a research platform, whilst hs-cTnI and hs-cTnT were measured using widely available clinical laboratory analysers. The sandwich immunoassay is

comparable to the setup used to test for hs-cTn, but cMyC is not yet available on a random-access laboratory analyser for routine clinical use.

In summary, cMyC is a promising new biomarker of myocardial necrosis with overall discriminatory power comparable with the leading troponin assays in AMI diagnosis. A potential advantage of cMyC is its ability to more effectively rule-out AMI at presentation, particularly among those presenting early after symptom onset.

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Millipore Sigma was contracted to undertake the analyses of cMyC on a fee-for-service basis and holds no commercial interest. Prof Marber is named as an inventor on a patent held by King's College London for the detection of cardiac myosin–binding protein C as a biomarker of myocardial injury.

5.7. Supplement

5.7.1 Supplemental Methods

Routine clinical assessment

All patients underwent a clinical assessment that included medical history, physical examination, 12-lead ECG, pulse oximetry, standard blood test, and chest radiography according to local protocols and in accordance with the guidelines of the European Society of Cardiology (ESC).[12] Levels of cTn were measured at presentation and serially thereafter as long as clinically indicated. Treatment of patients was left to discretion of the attending physician.

Adjudication of the final diagnosis

AMI was defined and cTn levels interpreted as recommended in current guidelines.[11,127,129,142] In brief, AMI was diagnosed when there was evidence of myocardial necrosis with a significant rise and/or fall in a clinical setting consistent with myocardial ischemia. Patients with AMI were further subdivided into type 1 AMI (primary coronary events) and type 2 AMI (ischemia due to increased demand or decreased supply, for example tachyarrhythmia or hypertensive crisis).[11,143]

The adjudication of final diagnoses was performed centrally in the core lab (University Hospital Basel) for all patients incorporating levels of hs-cTnT (see test characteristics above). More specifically, two independent cardiologists not directly involved in patient care reviewed all available medical records (including patient history, physical examination, results of laboratory testing including hs-cTnT levels, radiologic testing, ECG, echocardiography, cardiac exercise test, lesion severity and morphology in coronary angiography, discharge summary) pertaining to the patient from the time of ED presentation to 90-day follow-up. Late samples were available for adjudication of final diagnosis in all patients. In general, serial sampling was

performed until at least 6h after presentation to the ED or onset of chest.[143] In situations of diagnostic disagreement, cases were reviewed and adjudicated in conjunction with a third cardiologist. While discharge diagnoses often were correct and in agreement with the final adjudicated diagnosis, there were also cases where those diagnoses needed to be revised, most often because more information became available from medical testing during early follow-up, and more rarely, because the discharge diagnosis was not in agreement with the Universal Definition of AMI.

The 99th percentile (14 ng/L) was used as cut-off for myocardial necrosis. Absolute cTn changes were used to determine significant changes based on the diagnostic superiority of absolute over relative changes.[144–149] Based on studies of the biological variation of cTn[30,150] as well as on data from previous chest pain cohort studies[144,151], a significant absolute change was defined as a rise or fall of at least 10 ng/L within six hours, or, in an assumption of linearity, as an absolute change of 6 ng/L within three hours. Predefined alternative diagnoses included 'unstable angina' (UA), 'Cardiac symptoms of origin other than coronary artery disease' and 'non-cardiac chest pain'.

Clinical Care: The (hs)-cTn assays and cut-off levels used for local clinical care

Routine clinical care comprised five different cTn assays at the different hospitals and at the different recruitment periods. The cTn assays used clinically in most of the participating institutions changed during the study from a conventional cTn assay to the hs-cTnT assay. In order to take advantage of the higher sensitivity and higher overall diagnostic accuracy offered by the hs-cTnT assay, patients were adjudicated using the hs-cTnT values in all patients. In patients in whom clinically a conventional cTn assay was used, the conventional cTn values

and the hs-cTnT values were available for the adjudication. In patients in whom clinically the hs-cTnT assay was used, only the hs-cTnT values were available for the adjudication.

The following conventional cTn assays were used: For the Roche cTnT 4th generation assay, the 10% CV level is 0.035 μg/l. The laboratories of the participating sites reported only two decimals; therefore 0.04 μg/l was used as a cut-off for myocardial necrosis. In order to fulfil the criteria of a significant change (30% of 99th percentile or 10% CV level), a patient would e.g. need to have a level of <0.01 μg/l at presentation and 0.04 μg/l at 6h. A patient would also qualify if the first level is 0.02 μg/l and the second 0.04 μg/l. A patient would not fulfil the criteria if the first level is 0.03 μg/l and the second is 0.04 μg/l. If the first level is 0.04 μg/l, the second level needs to be at least 0.06 μg/l.

For the Abbott Axsym cTnI ADV, the 10% CV level is 0.16 μg/l. A patient having 0.16 μg/l at presentation would meet the criteria for significant change if the second was ≥0.21 μg/l. A patient having <0.12 μg/l at presentation (limit of detection) would qualify if the second is >0.16 μg/l.

For the Beckmann Coulter Accu cTnI, the 10% CV level is 0.06 μg/l. A patient having 0.06ug/l at presentation would qualify if the second is ≥0.08 μg/l. A patient having 0.05 at presentation would qualify if the second is 0.07 μg/l, but not 0.06 μg/l. A patient having undetectable cTnI (cTnI <0.01 μg/l) at presentation would qualify if the second is ≥0.06 μg/l.

For the Siemens Dimension Vista s-cTnI, the 10% CV level is 40 ng/L. The limit of detection is 15 ng/L and the 99th percentile is 45 ng/L. An absolute change of 20 ng/L or more within 3-6h was considered significant.

For Elecsys hs-cTnT measured clinically, the same change criteria were applied as for hs-cTnT measured from the study blood samples.

Central adjudication: Definition of rise and/or fall in high-sensitivity cardiac troponin T (hs-cTnT)

Absolute changes in hs-cTnT were used to determine significant changes based on the diagnostic superiority of absolute over relative changes.[144–149] Based on studies of the biological variation of cTn[30,150] as well as on data from previous chest pain cohort studies[144,151], a significant absolute change was defined as a rise or fall of at least 10 ng/L within 6 hours or an absolute change of 6 ng/L within 3 hours. If later clinical samples (e.g., at 24, 48, or 72 hours) revealed a lower hs-cTnT level than that measured during the period of sampling in the ED, the later level was considered the true baseline level for the calculation of the change criteria.

Measurement of high-sensitivity cardiac troponin I, high-sensitivity cardiac troponin T and sensitive cardiac troponin I

After collection and subsequent centrifugation, samples were frozen at -80ºC until assayed in a blinded fashion in a dedicated core laboratory. The Roche hs-cTnT assay was measured on the Elecsys 2010 (Roche Diagnostics). The limit of blank and LoD were determined to be 3 and 5 ng/L, respectively. The 99th centile of a healthy reference population was reported at 14 ng/L with an imprecision corresponding to 10% CV at 13 ng/L.[95] This study does not include any measurements with hs-cTnT lots that required the revision of the calibration curve.[134–136,152,153] The Abbott hs-cTnI assay used was the final pre-commercial release version of the ARCHITECT High Sensitive STAT Troponin I assay (Abbott Laboratories, Abbott Park, IL, USA). Samples were thawed, mixed, and centrifuged (for 30 min at 3000 RCF and 4ºC for serum samples or for 10 min, twice, at 3000 RCF for plasma samples) prior to analysis and

according to manufacturer's instructions. The hs-cTnI assay has a 99th percentile concentration of 26.2 ng/L with a corresponding coefficient of variation (CV) of <5% and a limit of detection (LoD) of 1.9 ng/L.[154] The cTnI-ultra assay was performed with the use of the ADVIA Centaur immunoassay system (Siemens). Limit of detection is 6 ng/L; a 10% coefficient of variation was reported at 30 ng/L with the 99th percentile cut-off point of 40 ng/L.[155,156] Calculation of the glomerular filtration rate was performed using the abbreviated Modification of Diet in Renal disease formula.[157]

Measurement of cardiac myosin-binding protein C

We have previously described the creation, biophysical selection and organ specificity of mouse monoclonal antibodies recognising cardiac-restricted epitopes within the N-terminus of cMyC.[50] Two of these antibodies, 1A4 and 3H8, were used to create a sensitive sandwich immunoassay. In brief, Magnetic microparticles (MPs) for capture were prepared by binding 25 μg of mouse monoclonal (1A4) per mg of MPs. The coated MPs were diluted in assay buffer (proprietary mix with custom 450mM NaCl and 0.5% Triton X-100) to 100 μg/mL. Due to sample volume constraints, serum, plasma or analyte (recombinant C0C2 domain of cMyC)[50] were diluted 2.2 fold with standard diluent and 100μL added per well of a 96-well assay plate. Samples or standards were then exposed to 100μL of coated MPs and agitated for 2 hours at 25°C. MPs were retained via a magnetic bed with unbound material removed in a single wash step. Fluorescently-labelled mouse monoclonal (3H8) detection antibody was diluted in assay buffer to 100 ng/mL. To each well, 20 μL of detection antibody was added and the MPs agitated for 1 hour at 25°C, retained via a magnetic bed and then washed 4 times to remove any unbound detection reagent. The MPs were then transferred to a new plate and all buffer was aspirated. The MPs were then exposed to 20 μL/well of elution buffer B for 5 minutes at

25°C before transfer to a 384-well plate containing 10 μL/well of neutralization buffer D. Fluorescent label was then detected by single molecule counting using the Erenna system with a dwell time of 60s per well. Three signal outputs were obtained from the Erenna System: Detected Events (DEs; low end signal), Event Photons (EPs; low end and higher end signal), and Total Photons (TPs; high end signal).

5.7.2 Supplemental Tables

Biomarker	N	Median ng/L [IQR]
cMyC at 0h	60	36 [24-62]
hs-cTnI at 0h	56	11 [7-21]
hs-cTnT at 0h	60	21 [16-28]
All patients	92	
cMyC at 0h	60	36 [24-62]
hs-cTnI at 0h	78	11 [6-19]
hs-cTnT at 0h	92	22 [17-29]

Table S1 – Comparison of biomarkers in patients excluded because of uncertain final diagnosis (e.g. patients discharged based on negative result on conventional cTn assay, who then tested positive on high-sensitivity cTn assay); comparison is performed for all patients with a measured cMyC at baseline (N=60) and all patients including missing values (N=92)

Demographics	**All patients (n = 1954)**	**Excluded patients (n=875)**	**p value*** for comparison derivation vs validation
Age, years	62 ± 16	59 ± 16	<0.001
Male	1341 (69)	587 (67)	0.441
Risk factors			
Hypertension	1247 (64)	384 (44)	<0.001
Hyperlipidaemia	992 (51)	421 (48)	0.206
Diabetes mellitus	348 (18)	136 (16)	0.155
Current smoking	476 (24)	244 (28)	0.051
History of smoking	1194 (61)	553 (63)	0.297
History			
Coronary artery disease	710 (36)	272 (31)	0.008
Previous myocardial infarction	474 (24)	199 (23)	0.408
Previous revascularisation (CABG or PCI)	553 (28)	237 (27)	0.535
Peripheral artery disease	119 (6)	52 (6)	0.947
Previous stroke	100 (5)	53 (6)	0.352
Vital status			
Heart rate, beats/min	79 ± 20	81 ± 21	0.234
Systolic blood pressure, mm Hg	144 ± 24	143 ± 25	0.711
Diastolic blood pressure, mm Hg	82 ± 15	82 ± 15	0.569
Electrocardiographic findings			
ST-segment depression	193 (10)	75 (9)	0.313
T-wave inversion	260 (13)	89 (10)	0.026
No significant electrocardiographic abnormalities	1469 (75)	681 (79)	0.193
Laboratory assessment			
Estimated glomerular filtration rate, ml/min/1.73m2†	84 ± 26	87 ± 25	0.008
Presentation time			
Time since chest pain onset, hours	5 [3, 12]	4 [1, 9]	<0.001
Time since chest pain peak, hours	3 [2, 7]	2 [5, 5]	<0.001

Table S2 – Demographics – group qualifying for primary analysis (n=1954) vs patients excluded due to missing cMyC values at baseline: * p values for comparison included versus excluded patient groups; data are expressed as medians [1st quartile, 3rd quartile] or means ± standard deviation, for categorical variables as numbers (percentages); CABG = Coronary Artery Bypass Graft; PCI = Percutaneous Coronary Intervention; † glomerular filtration rate was estimated using the Modification of Diet in Renal Disease (MDRD) formula

Table S3 – STARD checklist for studies of diagnostic accuracy – please online supplement to original publication[126]

Demographics	All patients (n = 1954)	Derivation (n = 586)	Validation (n = 1368)	p value* for comparison derivation vs validation
Age, years	62 ± 16	62 ± 16	62 ± 16	0.777
Male	1341 (69)	393 (67)	948 (69)	0.357
Acute Myocardial Infarction	340 (17)	107 (18)	233 (17)	0.512
Risk factors				
Hypertension	1247 (64)	362 (62)	885 (65)	0.239
Hyperlipidaemia	992 (51)	290 (49)	702 (51)	0.489
Diabetes mellitus	348 (18)	99 (17)	249 (18)	0.505
Current smoking	476 (24)	148 (25)	328 (24)	0.602
History of smoking	1194 (61)	372 (63)	864 (63)	0.906
History				
Coronary artery disease	710 (36)	200 (34)	510 (37)	0.202
Previous myocardial infarction	474 (24)	136 (23)	338 (25)	0.515
Previous revascularisation (CABG or PCI)	553 (28)	153 (26)	400 (29)	0.176
Peripheral artery disease	119 (6)	33 (6)	86 (6)	0.652
Previous stroke	100 (5)	27 (5)	73 (5)	0.577
Vital status				
Heart rate, beats/min	79 ± 20	80 (20)	79 (21)	0.895
Systolic blood pressure, mm Hg	144 ± 24	145 ± 25	143 ± 24	0.058
Diastolic blood pressure, mm Hg	82 ± 15	82 ± 15	82 ± 15	0.765
Electrocardiographic findings				
ST-segment depression	193 (10)	53 (9)	140 (10)	0.475
T-wave inversion	260 (13)	64 (11)	196 (14)	0.05
No significant electrocardiographic abnormalities	1469 (75)	456 (80)	1013 (76)	0.075
Laboratory assessment				
Estimated glomerular filtration rate, ml/min/1.73m2†	84 ± 26	84 ± 25	84 ± 26	0.441
Presentation time				
Time since chest pain onset, hrs	5 [3, 12]	5 [2, 12]	5 [3, 12]	0.804
Time since chest pain peak, hrs	3 [2, 7]	4 [2, 7]	3 [2, 7]	0.528

Table S4 – Demographics for derivation and validation cohorts; * p values for comparison validation to derivation cohort; data are expressed as medians [1st quartile, 3rd quartile] or means ± standard deviation, for categorical variables as numbers (percentages); CABG = Coronary Artery Bypass Graft; PCI = Percutaneous Coronary Intervention; † glomerular filtration rate was estimated using the Modification of Diet in Renal Disease (MDRD) formula

Adjudicated diagnosis	cMyC (ng/L)	hs-cTnT (ng/L)	hs-cTnI (ng/L)	s-cTnI (mg/L)
AMI	237 [71-876]	62 [28-139]	97 [21-456]	0.175 [0.039-0.722]
Unstable angina	21 [13-43]	11 [7-17]	6 [4-12]	0.009 [0.005-0.020]
cardiac symptoms of origin other than coronary artery disease	33 [12-96]	15 [7-32]	10 [4-30]	0.017 [0.005-0.044]
non-cardiac symptoms	10 [6-19]	6 [4-10]	3 [2-5]	0.005 [0.004-0.011]
symptoms of unknown origin	11 [7-16]	6 [3-10]	3 [2-5]	0.005 [0.001-0.010]

Table S5 – Blood concentrations of cMyC, hs-cTnT, hs-cTnI and s-cTnI at presentation in the five diagnostic categories; AMI = acute myocardial infarction; data is quoted in median ng/L [Interquartile Range] for cMyC and hs-cTn assays, and mg/L [IQR] for s-cTnI

cMyC >87 ng/L	AMI	UA	non-coronary	non-cardiac	unknown	p	N
	N=237	N=18	N=72	N=22	N=1		
cMyC at 0h	559 [215-1228]	135 [113-207]	168 [120-329]	157 [101-222]	96 [96-96]	<0.001	350
hs-cTnI at 0h	230 [74-725]	42 [17-74]	81 [34-227]	29 [15-43]	3 [3-3]	<0.001	327
hs-cTnT at 0h	92 [51-182]	29 [24-43]	50 [35-88]	26 [19-47]	8 [8-8]	<0.001	337
adjusted R2: cMyC and hs-cTnI 0.230, cMyC and hs-cTnT 0.504, hs-cTnT and hs-cTnI 0.608							

hs-cTnI >26 ng/L	AMI	UA	non-coronary	non-cardiac	unknown	p	N
	N=226	N=21	N=67	N=30	N=0		
cMyC ng/L at 0h	524 [208-1230]	60 [30-134]	158 [99-344]	75 [36-167]	NA	<0.001	344
hs-cTnI ng/L at 0h	235 [84-739]	60 [53-98]	91 [49-229]	42 [30-65]	NA	<0.001	344
hs-cTnT ng/L at 0h	93 [54-183]	22 [14-32]	50 [35-88]	27 [15-47]	NA	<0.001	332
adjusted R2: cMyC and hs-cTnI 0.230, cMyC and hs-cTnT 0.528, hs-cTnT and hs-cTnI 0.602							

hs-cTnT >14 ng/L	AMI	UA	non-coronary	non-cardiac	unknown	p	N
	N=290	N=63	N=135	N=150	N=0		
cMyC ng/L at 0h	328 [97-998]	48 [24-83]	91 [46-165]	34 [19-57]	NA	<0.001	638
hs-cTnI ng/L at 0h	134 [33-557]	14 [7-38]	28 [14-87]	10 [6-19]	NA	<0.001	600
hs-cTnT ng/L at 0h	70 [36-147]	21 [17-26]	31 [20-52]	20 [16-27]	NA	<0.001	638
adjusted R^2: cMyC and hs-cTnI 0.274, cMyC and hs-cTnT 0.567, hs-cTnT and hs-cTnI 0.617							

Table S6 – Blood concentrations of biomarkers above 99th centiles at presentation; AMI = acute myocardial infarction; UA = unstable angina; data is quoted in median ng/L [Interquartile Range]

	AMI group (N=340)			p	N
Gender – male vs female	207 [62-814]	vs	361 [91-1006]	0.096	256
Age – <65 vs ≥65	237 [62-938]	vs	237 [74-826]	0.925	122
Body Mass Index (BMI) – <30 vs ≥30	264 [72-898]	vs	219 [76-616]	0.414	257
Hypertension – absent vs present	272 [64-885]	vs	230 [73-874]	0.935	71
Hyperlipidaemia – absent vs present	321 [92-840]	vs	211 [57-887]	0.140	113
Diabetes mellitus – absent vs present	282 [75-1004]	vs	182 [64-535]	0.059	245
Current smoking – absent vs present	257 [66-894]	vs	213 [78-822]	0.681	249
History of smoking – absent vs present	219 [70-837]	vs	274 [72-894]	0.701	198
Coronary artery disease – absent vs present	308 [79-973]	vs	206 [60-785]	0.135	166
Estimated glomerular filtration rate, ml/min/1.73m2* – <60 vs ≥60	345 [87-953]	vs	208 [60-828]	0.111	101
	Non-cardiac chest pain group (N=1052)				
Gender – male vs female	10 [6-19]	vs	10 [5-18]	0.108	716
Age – <65 vs ≥65	8 [5-13]	vs	18 [11-32]	<0.001	701
Body Mass Index (BMI) – <30 vs ≥30	10 [6-19]	vs	11 [6-21]	0.282	815
Hypertension – absent vs present	7 [5-12]	vs	14 [9-29]	<0.001	509
Hyperlipidaemia – absent vs present	8 [5-15]	vs	14 [8-28]	<0.001	634
Diabetes mellitus – absent vs present	10 [6-17]	vs	16 [10-35]	<0.001	916
Current smoking – absent vs present	11 [7-21]	vs	8 [5-14]	<0.001	769
History of smoking – absent vs present	10 [6-17]	vs	13 [7-23]	<0.001	707
Coronary artery disease – absent vs present	9 [6-15]	vs	18 [11-32]	<0.001	784
Estimated glomerular filtration rate, ml/min/1.73m2* – <60 vs ≥60	30 [16-53]	vs	9 [6-16]	<0.001	107

Table S7 – Non-Cardiac sources of cMyC variation; MI = myocardial infarction, based on the adjudicated gold-standard diagnosis; CABG = Coronary Artery Bypass Graft; PCI = Percutaneous Coronary Intervention; data is quoted in median [Interquartile Range]; N = number of patients with the condition on the left-hand side of the demographic factors (e.g. 'Hypertension – absent in 509 patients'); *glomerular filtration rate was estimated using the Modification of Diet in Renal Disease (MDRD) formula

Non-cardiac chest pain group	R^2	B	SE B	β_i	p
	0.077				
Constant		-32.439	11.893		0.006
Hypertension		2.040	3.860	0.020	0.597
Hyperlipidaemia		1.326	4.127	0.013	0.748
Diabetes mellitus		1.139	5.047	0.007	0.822
Current smoking		-1.914	3.978	-0.017	0.631
History of smoking		-3.531	3.680	-0.033	0.338
Coronary artery disease		9.658	4.552	0.083	0.034
Creatinine on admission		0.357	0.072	0.157	0.000
Age		0.399	0.113	0.127	0.000
Body Mass Index (BMI)		-0.007	0.328	-0.001	0.982
	0.075				
Constant		-34.087	7.050		0.000
Coronary artery disease		10.680	3.662	0.093	0.004
Creatinine on admission		0.351	0.070	0.155	0.000
Age		0.428	0.099	0.137	0.000

AMI group	R^2	B	SE B	β_i	p
	0.028				
Constant		516.589	496.148		0.299
Hypertension		-1.724	121.878	-0.001	0.989
Hyperlipidaemia		141.837	106.276	0.082	0.183
Diabetes mellitus		-236.237	108.644	-0.129	0.030
Current smoking		-30.010	136.079	-0.016	0.826
History of smoking		15.745	107.937	0.010	0.884
Coronary artery disease		-175.913	102.811	-0.108	0.088
Creatinine on admission		0.727	0.933	0.045	0.436
Age		-0.459	4.339	-0.007	0.916
Body Mass Index (BMI)		4.402	11.964	0.023	0.713
	0.014				
Constant		674.182	52.552		0.000
Diabetes mellitus		-220.143	100.580	-0.119	0.029

Table S8 – Multiple regression to determine influence of baseline variables on cMyC levels; AMI = Acute Myocardial Infarction; R^2 = fit of the regression model; B = beta estimate; SE B = standard errors of beta estimate; β_i = standardized beta estimate

Initial model	**New model – cMyC (10/120) – Derivation cohort**					
hs-cTnT	No AMI (n=465)			AMI (n=103)		
	Rule-out	Observe	Rule-in	Rule-out	Observe	Rule-in
Rule-out	105	28	0	0	0	0
Observe	95	221	8	0	41	10
Rule-in	0	0	8	0	3	49
NRI	0.127 (95% CI, 0.061-0.173)			0.068 (95% CI, 0.0-0.136)		
NRI (dimensionless)	0.195 (95% CI, 0.113-0.277); p value <0.001			IDI	0.065 (95% CI, 0.037-0.093)	
Thresholds	Sensitivity (95% CI)	NPV (95% CI)		Specificity (95% CI)	PPV (95% CI)	
hs-cTnT 5 ng/L	100% (100-100%)	100% (100-100%)		28.8% (24.8-33.1%)	23.8% (19.4-27.8%)	
hs-cTnT 52 ng/L	50.5% (41.3-60.4%)	89.9% (87.4-92.6%)		98.3% (97-99.3%)	86.9% (77.2-94.1%)	
cMyC 10 ng/L	100% (100-100%)	100% (100-100%)		41.3% (36.8-45.9%)	27.3% (22.9-31.9%)	
cMyC 120 ng/L	57.1% (47.5-67%)	91.1% (88.5-93.6%)		96.6% (94.8-98.1%)	78.9% (68.8-87.6%)	

Table S9 – Derivation cohort – hs-cTnT

Initial model	**New model – cMyC (10/120) – Derivation cohort**					
hs-cTnI	No AMI (n=457)			AMI (n=96)		
	Rule-out	Observe	Rule-in	Rule-out	Observe	Rule-in
Rule-out	61	10	2	0	0	0
Observe	141	224	3	0	37	5
Rule-in	1	4	11	0	6	48
NRI	0.287 (95% CI, 0.217-0.336)			-0.010 (95% CI, -0.081-0.060)		
NRI (dimensionless)	0.276 (95% CI, 0.191-0.361); p value <0.001			IDI	0.090 (95% CI, 0.062-0.119)	
Thresholds	Sensitivity (95% CI)	NPV (95% CI)		Specificity (95% CI)	PPV (95% CI)	
hs-cTnI 2 ng/L	100% (100-100%)	100% (100-100%)		15.9% (12.7-19.3%)	20% (16.3-23.8%)	
hs-cTnI 52 ng/L	55.9% (46.2-66%)	91.3% (88.8-93.7%)		96.5% (94.9-98%)	77.3% (66.7-86.4%)	
cMyC 10 ng/L	100% (100-100%)	100% (100-100%)		42.7% (38.3-47.4%)	26.9% (22.2-31.1%)	
cMyC 120 ng/L	55.3% (45.8-64.8%)	91.2% (88.5-93.6%)		96.5% (94.8-98%)	77.1% (66.7-86.1%)	

Table S10 – Derivation cohort – hs-cTnI; NRI = Net Reclassification Improvement; IDI = Integrated Discrimination Improvement; CI = Confidence Interval; NPV = Negative Predictive Value; PPV = Positive Predictive Value; AMI = Acute Myocardial Infarction, based on the adjudicated gold-standard diagnosis

Initial model	**New model – MyC (10/120) – chest pain for <3hrs**					
hs-cTnT	No AMI (n=382)			AMI (n=78)		
	Rule-out	Observe	Rule-in	Rule-out	Observe	Rule-in
Rule-out	99	28	0	0	1	0
Observe	83	161	6	0	38	10
Rule-in	0	0	5	0	1	28
NRI	0.128 (95% CI, 0.055-0.181)			0.128 (95% CI, 0.044-0.213)		
NRI (dimensionless)	0.256 (95% CI, 0.157-0.356); p value <0.001			IDI	0.086 (95% CI, 0.052-0.119)	
Thresholds	Sensitivity (95% CI)	NPV (95% CI)		Specificity (95% CI)	PPV (95% CI)	
hs-cTnT 5 ng/L	98.8% (95.8-100%)	99.2% (97.5-100%)		33.3% (28.6-38.1%)	23.3% (19-27.9%)	
hs-cTnT 52 ng/L	37.2% (25.9-48.2%)	88.5% (85.3-91.4%)		98.7% (97.5-99.7%)	86.1% (73.3-96.9%)	
cMyC 10 ng/L	100% (100-100%)	100% (100-100%)		46.4% (41.5-51.6%)	27.5% (22.3-32.5%)	
cMyC 120 ng/L	49% (36.8-60%)	90.4% (87.4-93.2%)		97.2% (95.3-98.7%)	77.8% (65.2-89.7%)	

Initial model	**New model – MyC (10/120) – chest pain for <3hrs**					
hs-cTnI	No AMI (n=381)			AMI (n=79)		
	Rule-out	Observe	Rule-in	Rule-out	Observe	Rule-in
Rule-out	76	11	1	0	0	0
Observe	109	169	4	0	39	8
Rule-in	0	5	6	0	4	28
NRI	0.257 (95% CI, 0.185-0.310)			0.051 (95% CI, -0.032-0.133)		
NRI (dimensionless)	0.308 (95% CI, 0.210-0.406); p value <0.001			IDI	0.101 (95% CI, 0.067-0.135)	
Thresholds	Sensitivity (95% CI)	NPV (95% CI)		Specificity (95% CI)	PPV (95% CI)	
hs-cTnI 2 ng/L	100% (100-100%)	100% (100-100%)		23.2% (19-27.1%)	21.3% (16.8-25.7%)	
hs-cTnI 52 ng/L	40.3% (29.5-51.2%)	88.7% (85.5-91.7%)		97.2% (95.5-98.7%)	74.8% (60.7-87.5%)	
cMyC 10 ng/L	100% (100-100%)	100% (100-100%)		47.1% (42.4-52.2%)	28.2% (22.9-33.3%)	
cMyC 120 ng/L	45.6% (34.7-57.3%)	89.6% (86.7-92.6%)		97.1% (95.3-98.7%)	76.6% (64.1-87.8%)	

Table S11 – Net Reclassification Improvement – Onset of chest pain <3 hours prior to presentation; NRI = Net Reclassification Improvement; IDI = Integrated Discrimination Improvement; CI = Confidence Interval; NPV = Negative Predictive Value; PPV = Positive Predictive Value; AMI = Acute Myocardial Infarction, based on the adjudicated gold-standard diagnosis

Initial model	**New model – MyC (10/120) – chest pain for ≥3hrs**					
hs-cTnT	No AMI (n=1172)			AMI (n=244)		
	Rule-out	Observe	Rule-in	Rule-out	Observe	Rule-in
Rule-out	255	77	0	0	0	0
Observe	202	569	34	1	69	24
Rule-in	0	7	28	0	11	139
NRI	0.084 (95% CI, 0.034-0.114)			0.049 (95% CI, 0.0-0.098)		
NRI (dimensionless)	0.133 (95% CI, 0.076-0.190); p value <0.001			IDI	0.044 (95% CI, 0.025-0.063)	
Thresholds	Sensitivity (95% CI)	NPV (95% CI)		Specificity (95% CI)	PPV (95% CI)	
hs-cTnT 5 ng/L	100% (100-100%)	100% (100-100%)		28.4% (25.8-31%)	22.6% (20.1-25.1%)	
hs-cTnT 52 ng/L	61.4% (55.6-67.3%)	92.3% (90.9-93.9%)		97% (96-97.9%)	81.1% (75.3-86.7%)	
cMyC 10 ng/L	99.6% (98.7-100%)	99.8% (99.3-100%)		37.3% (34.8-40.3%)	24.9% (22-27.8%)	
cMyC 120 ng/L	66.9% (61-72.6%)	93.2% (91.8-94.5%)		94.7% (93.4-96%)	72.5% (66.7-78.1%)	

Initial model	**New model – MyC (10/120) – chest pain for ≥3hrs**					
hs-cTnI	No AMI (n=1156)			AMI (n=241)		
	Rule-out	Observe	Rule-in	Rule-out	Observe	Rule-in
Rule-out	152	31	1	0	0	0
Observe	305	581	21	1	61	16
Rule-in	1	24	40	0	18	145
NRI	0.240 (95% CI, 0.190-0.270)			-0.012 (95% CI, -0.061-0.036)		
NRI (dimensionless)	0.227 (95% CI, 0.170-0.285); p value <0.001			IDI	0.075 (95% CI, 0.056-0.094)	
Thresholds	Sensitivity (95% CI)	NPV (95% CI)		Specificity (95% CI)	PPV (95% CI)	
hs-cTnI 2 ng/L	100% (100-100%)	100% (100-100%)		15.9% (14-18%)	19.9% (17.7-22%)	
hs-cTnI 52 ng/L	67.5% (61.3-73.7%)	93.3% (91.9-94.7%)		94.4% (93.1-95.6%)	71.5% (65.4-77%)	
cMyC 10 ng/L	99.6% (98.6-100%)	99.8% (99.3-100%)		38.1% (35.3-41%)	25.2% (22.4-28.1%)	
cMyC 120 ng/L	66.9% (60.7-72.4%)	93.2% (91.7-94.6%)		94.6% (93.3-95.9%)	72.1% (66.2-78.3%)	

Table S12 – Net Reclassification Improvement – Onset of chest pain ≥3 hours prior to presentation; NRI = Net Reclassification Improvement; IDI = Integrated Discrimination Improvement; CI = Confidence Interval; NPV = Negative Predictive Value; PPV = Positive Predictive Value; AMI = Acute Myocardial Infarction, based on the adjudicated gold-standard diagnosis

	cMyC at 87 ng/L	hs-cTnI at 26 ng/L	hs-cTnT at 14 ng/L
Sensitivity	69.6% (95% CI, 64.9-74.2%)	70.6% (95% CI, 65.6-75.5%)	91% (95% CI, 87.8-94.1%)
Specificity	93% (95% CI, 91.7-94.3%)	92.3% (95% CI, 90.8-93.5%)	76.4% (95% CI, 74.3-78.6%)
NPV	93.6% (95% CI, 92.3-94.7%)	93.8% (95% CI, 92.5-94.9%)	97.6% (95% CI, 96.7-98.4%)
PPV	67.7% (95% CI, 62.9-72.4%)	65.4% (95% CI, 60.2-70.6%)	44.4% (95% CI, 40.7-48.2%)

Table S13 – Specificity of biomarkers at presentation for adjudicated diagnosis of Acute Myocardial Infarction at the 99th centile

n=1876	cMyC	hs-cTnT	p value*	est.cov
FU AMI				
Harrell's C Statistic	0.725	0.706	0.251	0.000
Somers' D ± *SD*	0.450 ±0.045	0.411 ±0.048		
FU death				
Harrell's C Statistic	0.765	0.782	0.142	0.000
Somers' D ± SD	0.530 ±0.034	0.564 ±0.031		
FU composite EP				
Harrell's C Statistic	0.745	0.749	0.667	0.000
Somers' D ± SD	0.489 ±0.029	0.498 ±0.029		

n=1857	cMyC	hs-cTnI	p value	est.cov
FU AMI				
Harrell's C Statistic	0.724	0.714	0.577	0.000
Somers' D ± SD	0.447 ±0.047	0.429 ±0.047		
FU death				
Harrell's C Statistic	0.767	0.732	0.001	0.000
Somers' D ± SD	0.535 ±0.034	0.464 ±0.036		
FU composite EP				
Harrell's C Statistic	0.746	0.722	0.008	0.000
Somers' D ± SD	0.492 ±0.029	0.443 ±0.030		

n=1774	cMyC	s-cTnI	p value	est.cov
FU AMI				
Harrell's C Statistic	0.719	0.504	<0.001	0.000
Somers' D ± SD	0.438 ±0.047	0.007 ±0.002		
FU death				
Harrell's C Statistic	0.763	0.507	<0.001	0.000
Somers' D ± SD	0.527 ±0.035	0.014 ±0.011		
FU composite EP				
Harrell's C Statistic	0.741	0.503	<0.001	0.000
Somers' D ± SD	0.483 ±0.030	0.007 ±0.008		

Table S14 – Prognosis – Harrell's C and Somers' D statistics; FU = Follow-up event, AMI = Acute Myocardial Infarction (based on the adjudicated gold-standard diagnosis), composite EP = endpoint combining death and AMI during FU (excluding index event), Somers' D quoted ± SD = Standard error of Somers' D, est.cov = estimated covariance between two C indices; *p value for direct comparison between biomarkers

Prelude to Chapter 6

Chapter 5 represents the first study to assess the diagnostic and prognostic value of cMyC in patients presenting with possible AMI. Based on the AUC, cMyC was equivalent to hs-cTnT/I in its diagnostic accuracy, and a rule-in/rule-out pathway was designed to compare classification power in a clinical setting. Using this pathway, cMyC would have correctly triaged more patients to 'rule-out' or 'rule-in' groups than either hs-cTnI or hs-cTnT – with a smaller proportion of patients left in the observation group. However, the rule-in/rule-out pathway only addressed the use of cMyC as a triage-tool at presentation to the emergency department – upon first blood draw. From this analysis, it was unclear as to whether the release-profile of cMyC, e.g. quantified as a delta between first and second blood draw 1 hour apart, would add to the discrimination power or enhance ongoing triage. The goal of Chapter 6 was to design a complete rule-in/rule-out pathway for the use of cMyC as a complete triage tool, and investigate whether cMyC could be used as a 'triage-booster' – e.g. as an additional blood test performed with the first hs-cTn assay, to enhance triage and expedite risk-stratification into rule-in and rule-out groups. Similar to Chapter 5, Chapter 6 is a secondary analysis of a pre-existing study – APACE, a multi-centre international diagnostic trial enrolling all patients with suspected AMI. The work was made possible by close collaboration with colleagues in Basel (Christian Mueller et al.). The candidate forged the collaborations, identified suitable patients, interpreted all cMyC concentrations, wrote the analysis plan, performed the statistical analysis and wrote the manuscript. The findings are not published in manuscript-form yet, but were presented as part of a rapid-fire abstract presentation at the ESC Congress 2018 (Munich). Findings are reproduced with amendments for inclusion in the thesis.

Chapter 6. Derivation and Validation of a 0/1h-algorithm to diagnose Myocardial Infarction using Cardiac Myosin-binding Protein C

Summary

Introduction: Rapid triage and treatment are the cornerstones of improving management of patients presenting with suspected Acute Myocardial Infarction (AMI). Cardiac myosin binding protein C (cMyC) is a cardiac-restricted protein that is more abundant than Troponin (cTn) and is released rapidly following AMI. We have previously demonstrated more effective rule-out and rule-in of AMI using a single blood sample at presentation.

Purpose: In this study, we aimed to (i) investigate the diagnostic performance of cMyC, hs-cTnI and hs-cTnT measured in 0- and 1-hour blood samples as a single- and dual-marker strategy; (ii) derive and validate a 0/1h-triage-algorithm to diagnose AMI based on cMyC and (iii) compare it to the well-established ESC 0/1h-algorithms using hs-cTnT and hs-cTnI.

Methods: In a prospective international diagnostic study enrolling patients presenting with suspected AMI to the ED, cMyC (Erenna), hs-cTnT (Elecsys) and hs-cTnI (Architect) were determined at baseline and after one hour (1,390 complete datasets for hs-cTnI, 1,431 for hs-cTnT). Patients presenting with STEMI were excluded. The final diagnosis was centrally adjudicated by two independent cardiologists using all available data including coronary angiography, echocardiography, follow-up data, and serial measurements of hs-cTnT (but not hs-cTnI). Discriminatory power of each biomarker was evaluated by calculating the area under the receiver-operating characteristic curve; markers were combined using logistic regression. We evaluated the performance of >390,000 different cut-off combinations for cMyC in a derivation set (random 50:50 split) and applied the best performing algorithm to the validation set. Safety of rule-out was quantified by the negative predictive value (NPV) for AMI; the

accuracy of rule-in by the positive predictive value (PPV); efficacy by the proportion assigned to rule-out or rule-in based on the 0/1h-samples. Only cut-off combinations achieving an a-priori defined NPV ≥99% and PPV ≥70% were selected.

Results: Prevalence of AMI was 17%; median age was 61 years [49;74], 32% were female. The diagnostic accuracy of cMyC at presentation (0h) for AMI was high (AUC 0.919 [95% CI, 0.901-0.937]) and overall comparable to both hs-cTnI (0.916 [0.898-0.934], p=0.616) and hs-cTnT (0.921 [0.903-0.939], p=0.810). A dual-marker strategy, combining baseline levels of cMyC and hs-cTn, increased the diagnostic accuracy with hs-cTnI (0.925 [0.909-0.941], p= 0.008) and with hs-cTnT (0.930 [0.914-0.946], p=0.006). For the combination of baseline levels and absolute 1h-changes, the diagnostic performance of cMyC was comparable to hs-cTnI (AUC 0.924 [0.908-0.940] vs 0.917 [0.899-0.935], p=0.194), and inferior to hs-cTnT (AUC 0.925 [0.907-0.942] vs 0.945 [0.931-0.958], p=0.003). When baseline cMyC was added to baseline levels and 1h-changes of hs-cTn, the diagnostic accuracy further improved with hs-cTnI (AUC 0.926 [0.909-0.942], p=0.012), but not significantly with hs-cTnT (AUC 0.947 [0.935-0.960], p=0.186).

The best performing cMyC cut-off combination [0h <10 (for patients presenting >3h after chest pain onset) or 0h <26 AND Δ0-1h <8 for rule-out; ≥136 OR Δ0-1h ≥15 for rule-in (all values ng/L)] was selected in a derivation cohort and applied to the validation cohort.

Comparing cMyC with hs-cTnT in the validation cohort, accuracy for rule-out by the best performing cMyC 0/1h-algorithm was high in the hs-cTnT validation cohort: NPV 99.3% [95% CI, 98.5-99.9%]; sensitivity 97.6% [94.7-100%]; and comparable to the ESC hs-cTnT 0/1h-algorithm: NPV 99.7% [99.2-100%; p=0.332]; sensitivity 99.2% [97.3-100%, p=0.317). Accuracy for rule-in was high: PPV 70% [62.2-77.6%]; specificity 93.3% [91.1-95.1%]; but

inferior to the ESC hs-cTnT 0/1h-algorithm: PPV 78.9% [71.7-85.87%; p=0.007]; specificity 95.6% [93.9-97.2%; p=0.011]. Proportion of patients assigned to either rule-out or rule-in based on the 0/1h-samples significantly increased from 75.28% using hs-cTnT to 79.19% using cMyC (p<0.001). Direct rule-out (feasible in patients presenting >3 hours after chest pain onset) or rule-in based on a single blood draw at ED presentation increased from 35.75% using hs-cTnT to 47.21% using cMyC (p<0.001)

Comparing cMyC with hs-cTnI in the validation cohort, accuracy for rule-out by the novel cMyC 0/1h-algorithm was high: NPV 99.3% (95% CI, 98.5-99.9%); sensitivity 97.4% (94.1-100%); and comparable to the ESC hs-cTnI 0/1h-algorithm: NPV 99.1% (98.2-99.9%; p=0.78); sensitivity 97.4% (94.1-100%; p=1). Similarly, accuracy for rule-in was high: PPV 70.9% (63.1-78.5%); specificity 93.4% (91.2-95.3%); and comparable to the ESC hs-cTnI 0/1h-algorithm: PPV 71.2% (63.3-78.9%; p=0.91); specificity 93.5% (91.4-95.6%; p=0.85). Proportion of patients assigned to either rule-out or rule-in based on the 0/1h-samples significantly increased from 70.1% using hs-cTnI to 80.7 % using cMyC (p<0.05). Direct rule-out (feasible in patients presenting >3 hours after chest pain onset) or rule-in based on a single blood draw at ED presentation increased from 28.8% using hs-cTnI to 48.5% using cMyC (p<0.05).

Conclusion: A newly developed cMyC AMI rule-in/rule-out pathway identifies a greater proportion of patients suitable for safe rule-out as compared with the ESC 0/1h-algorithm using hs-cTnT or hs-cTnI and thus reduces the number of patients in a diagnostic grey zone.

6.1. Introduction

Rapid triage and treatment are the cornerstones of improving management of patients presenting with suspected Acute Myocardial Infarction (AMI). The European Society of Cardiology has first published a rapid rule-out/rule-in algorithm for diagnosis and treatment of patients with suspected Non-ST elevation Myocardial Infarction using a 0/1hr-approach in 2015.[12] This algorithm has pioneered a move from a 'diagnostic' to a risk-stratification approach: While prior strategies have focused on cTn values above the population-derived 99th centile to identify patients presenting with AMI[12,158,159], the ESC demoted this threshold in 2015 and, instead, focused on early rule-out of patients with undetectable levels of cTn and early rule-in of patients with a high cTn result and resulting high positive-predictive value for AMI. This approach was only made possible through the use of high-sensitivity cTn assays, with a focus on the two commercially available assays at the time (hs-cTnT, Elecsys; hs-cTnI, Architect). Naturally, such widely spaced decision limits leave a proportion of patients with quantifiable but only moderately elevated cTn levels in an indeterminate 'observe' zone. To further enhance triage, the ESC algorithm employs (absolute) delta-change values between 1st and 2nd blood draws to identify patients with – likely – biologically relevant cTn changes, thus indicating acute myocardial injury. These deltas are assay-specific, compounded by the slow release-profile of cTn[18] and remarkably close to the analytic abilities of the respective laboratory platform.[31,160,161] Despite these challenges, the use of deltas does indeed increase sensitivity and specificity – reducing the number of patients in the indeterminate 'observe'

zone. However, even the second blood draw leaves up to 40% of patients in this diagnostic grey zone.[133,162]

We have recently demonstrated more effective rule-out and rule-in of AMI using a novel biomarker in a single blood sample at presentation[126]: Cardiac myosin-binding protein C (cMyC) is a cardiac-restricted protein that is more abundant than Troponin (cTn)[50,122] and is released rapidly following AMI.[123] In a retrospective analysis, cMyC reduced the size of the ESC observe zone at presentation by 9-17% when compared to hs-cTnT/I. Several other publications have demonstrated the incremental benefit to chest pain triage when using delta-change values with hs-cTn.[158,163–165] In this study, we aimed to (i) derive and validate a 0/1h-triage-algorithm to rapidly rule-out or rule-in AMI based on cMyC and (ii) compare it to the well-established ESC 0/1h-algorithm using hs-cTnT or hs-cTnI.

6.2. Methods

In a prospective international diagnostic study enrolling patients presenting with suspected AMI to the ED, cMyC (Erenna), hs-cTnT (Elecsys) and hs-cTnI (Architect) were determined at baseline and after one hour.

6.2.1 Study design and population

Advantageous Predictors of Acute Coronary Syndrome Evaluation (APACE) is an ongoing international multicentre diagnostic study (nine study centres in Switzerland, Spain, Poland, the Czech Republic, and Italy) designed to advance the early diagnosis of AMI.[39,45,124,125] All patients older than 18 years presenting to the ED with acute chest discomfort possibly indicating AMI were eligible for recruitment if the onset of, or peak chest pain symptoms, were within the preceding 12 hours. Enrolment was independent of renal function, while patients with terminal

kidney failure on chronic dialysis were excluded. For this analysis, the following patients were excluded: patients presenting with ST-segment elevation myocardial infarction; patients with missing levels of cMyC at presentation; patients in whom the final diagnosis remained unclear after adjudication and at least one hs-cTnT level was elevated. The latter group comprises of patients triaged and discharged following a negative gold-standard test at the time of enrolment (on a conventional cTn assay), who were later found to have an elevated hs-cTn result – which might imply a missed MI on the basis of a (comparably insensitive) contemporary cTn assay. These patients (n=92) were followed-up, but did not undergo gold-standard adjudication as per the study protocol, and their clinical course would have been determined by a (negative) cTn result.

A proportion of patients had no levels of cMyC measured at presentation due to insufficient sample volume. The protocol for routine clinical assessment is has been described previously.[126] For follow-up, patients were contacted 3, 12, 24 and 36 months after discharge via telephone, email or letter. Additionally, information regarding death during follow-up was obtained from the patient's hospital notes, the family physician's records and the national registry on mortality.

The study was carried out according to the principles of the Declaration of Helsinki and approved by the local ethics committees. Written informed consent was obtained from all patients. TK, RT and CM had full access to all the data in the study and take responsibility for its integrity and the data analysis. The authors designed the study, gathered, and analysed the data according to the STARD guidelines for studies of diagnostic accuracy, vouch for the data and analysis, wrote the paper, and decided to publish.

6.2.2 Adjudicated final diagnosis

Adjudication of the final diagnosis was performed centrally according to the 1st Universal Definition of MI, incorporating levels of hs-cTnT as the adjudicating biomarker.[127] Two sets of data were used: First, all clinical data derived from routine clinical investigations including all available medical records – patient history, physical examination, results of laboratory testing including serial local (h)s-cTn, radiologic testing, ECG, echocardiography, cardiac exercise stress test, lesion severity and morphology at coronary angiography – pertaining to the patient from the time of ED presentation to 90-day follow up. Second, a study-specific assessment was collected, including 34 chest pain characteristics and serial hs-cTnT measurements to take advantage of the higher sensitivity and higher overall diagnostic accuracy offered by the more sensitive assays, as previously published.[113,124] In situations of disagreement about the diagnosis, cases were reviewed and adjudicated in conjunction with a third cardiologist. In brief, AMI was diagnosed when there was evidence of myocardial necrosis in association with a clinical setting consistent with myocardial ischemia. Myocardial necrosis was diagnosed by at least one (h)s-cTn value above the 99th percentile together with a significant rise and/or fall.[128,129,142] All other patients were classified into the categories of unstable angina (UA), cardiac non-coronary disease (e.g. tachyarrhythmias, perimyocarditis), non-cardiac chest pain and symptoms of unknown origin.

6.2.3 Measurement of cMyC, hs-cTnT and hs-cTnI

Blood samples for determination of cMyC, hs-cTnI and hs-cTnT were collected into heparin plasma and serum tubes at presentation to the ED and serially thereafter (at time points 1h, 2h, 3h and 6h). Serial sampling was discontinued when a diagnosis of AMI was certain and treatment required patient transfer to the coronary care unit or catheter laboratory. After

centrifugation, samples were frozen at -80 ºC until they were assayed in a blinded fashion in a dedicated core laboratory. cMyC was measured using the previously established high-sensitivity assay on the Erenna platform that was performed by EMD Merck Millipore (Hayward, California).[84] The assay has a Limit of Detection (LoD) of 0.4 ng/L and a lower limit of quantification (LoQ) of 1.2 ng/L. The 99th percentile cut-off point determined previously (in patients without obstructive coronary artery disease on invasive angiography) is 87 ng/L.[84]

The Roche hs-cTnT assay was measured on the Elecsys 2010 (Roche Diagnostics). The limit of blank and LoD were determined to be 3 and 5 ng/L, respectively. The 99th-percentile of a healthy reference population was reported at 14 ng/L with an imprecision corresponding to 10% CV at 13 ng/L.[95] This study does not include any measurements with hs-cTnT lots that required the revision of the calibration curve.[134–136,152,153] The Abbott hs-cTnI assay used was the final pre-commercial release version of the ARCHITECT High Sensitive STAT Troponin I assay (Abbott Laboratories, Abbott Park, IL, USA). Samples were thawed, mixed, and centrifuged (for 30 min at 3000 RCF and 4ºC for serum samples or for 10 min, twice, at 3000 RCF for plasma samples) prior to analysis and according to manufacturer's instructions. The hs-cTnI assay has a 99th percentile concentration of 26.2 ng/L with a corresponding coefficient of variation (CV) of <5% and a limit of detection (LoD) of 1.9 ng/L.[154] Calculation of the glomerular filtration rate was performed using the abbreviated Modification of Diet in Renal disease formula.[157]

6.2.4 Derivation and Validation of the cMyC rule-out/rule-in algorithm

We evaluated the performance of >390,000 different cut-offs combinations in a derivation set (random 50:50 split) of the patients having 0/1hr results available for both hs-cTnT and cMyC and applied the best performing algorithm to the validation sets (50% of patients with hs-cTnT

and cMyC measurements, and 50% of patients with hs-cTnI and cMyC measurements). Based on previously obtained results[126], we entered values in numeric proximity to the published cut-off values (10 ng/L for rule-out, 120 ng/L for rule-in) into a 5-dimensional matrix listing all possible cut-off combinations for direct rule-out (see also Figure 31; ESC 'A'), rule-out upper limit (ESC 'B') with delta for rule-out (ESC 'C'), direct rule-in (ESC 'D') and delta for rule-in (ESC 'E') – see Figure 29. A specifically developed program written in R then uses this matrix to iterate through all possible combinations, hereby calculating NPV (defined as Σ true negatives / Σ (true negatives + false negatives)), PPV (defined as Σ true positives / Σ (true positives + false positives)), and risk-group distributions for all cut-off combination. Safety of rule-out was quantified by the NPV for AMI; the accuracy of rule-in by the PPV; efficacy by the proportion assigned to rule-out or rule-in based on the 0/1h-samples. Only cut-off combinations achieving an a-priori defined NPV ≥99% and PPV ≥70% were selected and evaluated with respect to triage-efficacy. The most efficient combinations were selected from the derivation cohort and applied to the validation cohort.

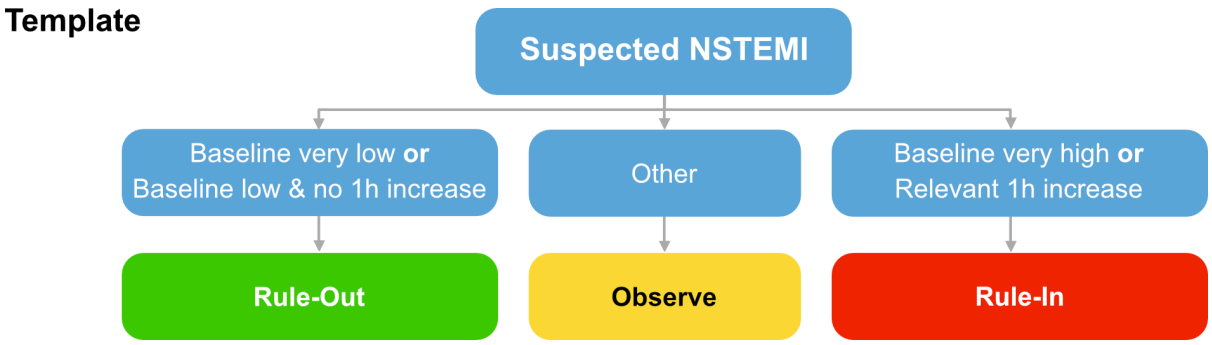


Figure 29 – Template for cMyC 0/1h-triage algorithm, adapted from the 2015 ESC pathway for hs-cTn[109]

For analysis of the effectiveness of a combination of hs-cTn rule-out/rule-in pathways with cMyC the following – step-wise – approaches were used (Table 7, Table 8, Figure 30):

Rule-Out	Observe	Rule-In
cMyC <A ng/L		
hs-cTnT <5 ng/L		
cMyC <B & cMyC delta <C		
hs-cTnT <12 ng/L & hs-cTnT delta <3 ng/L		
		hs-cTnT ≥52 ng/L
		hs-cTnT delta ≥5 ng/L
	Remainder	

Table 7 – Combined algorithm using hs-cTnT and cMyC 0/1h samples

Rule-Out	Observe	Rule-In
cMyC <A ng/L		
hs-cTnI <2 ng/L		
cMyC <B & cMyC delta <C		
hs-cTnI <5 ng/L & hs-cTnT delta <2 ng/L		
		hs-cTnI ≥52 ng/L
		hs-cTnI delta ≥6 ng/L
	Remainder	

Table 8 – Combined algorithm using hs-cTnI and cMyC 0/1h samples

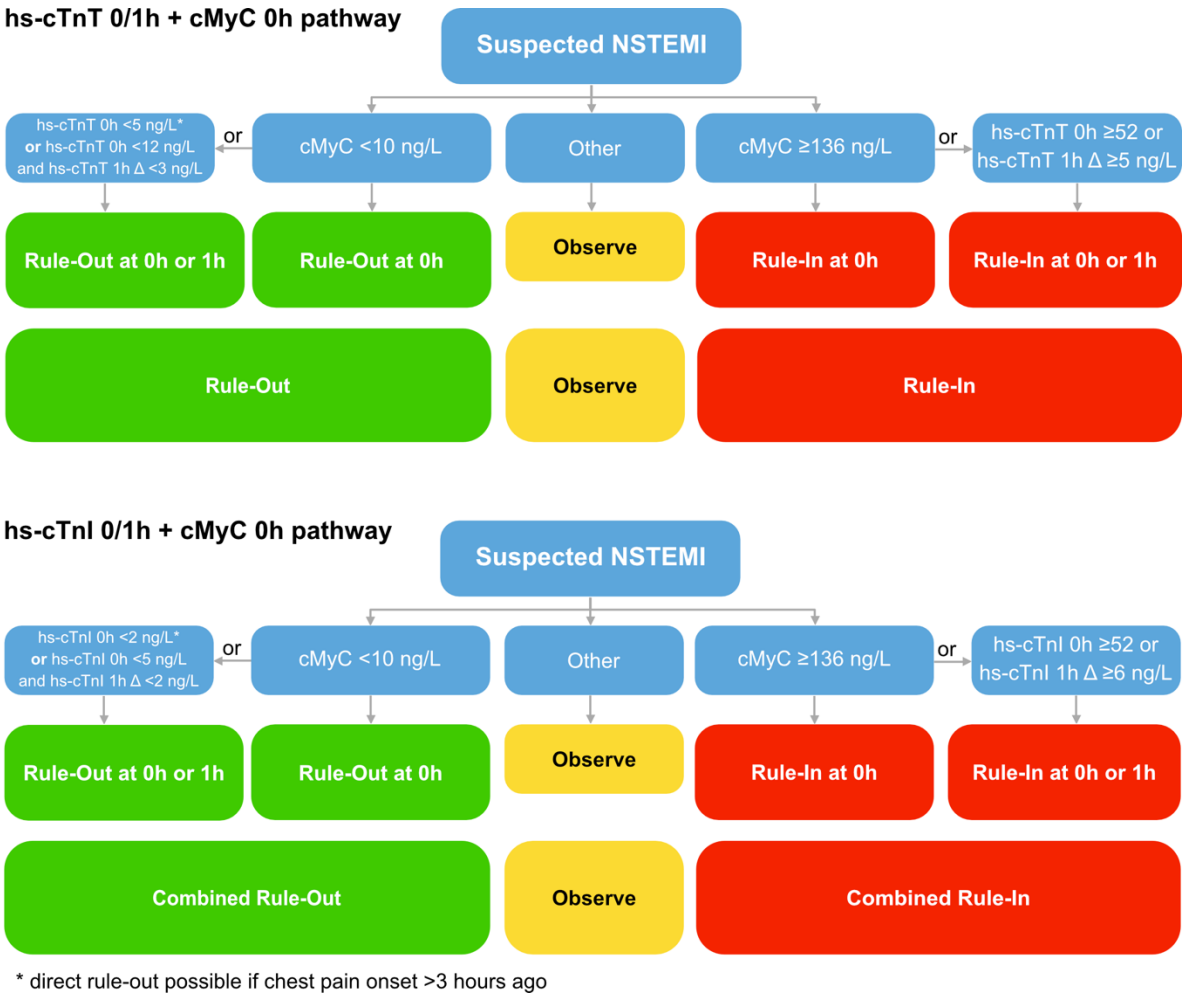
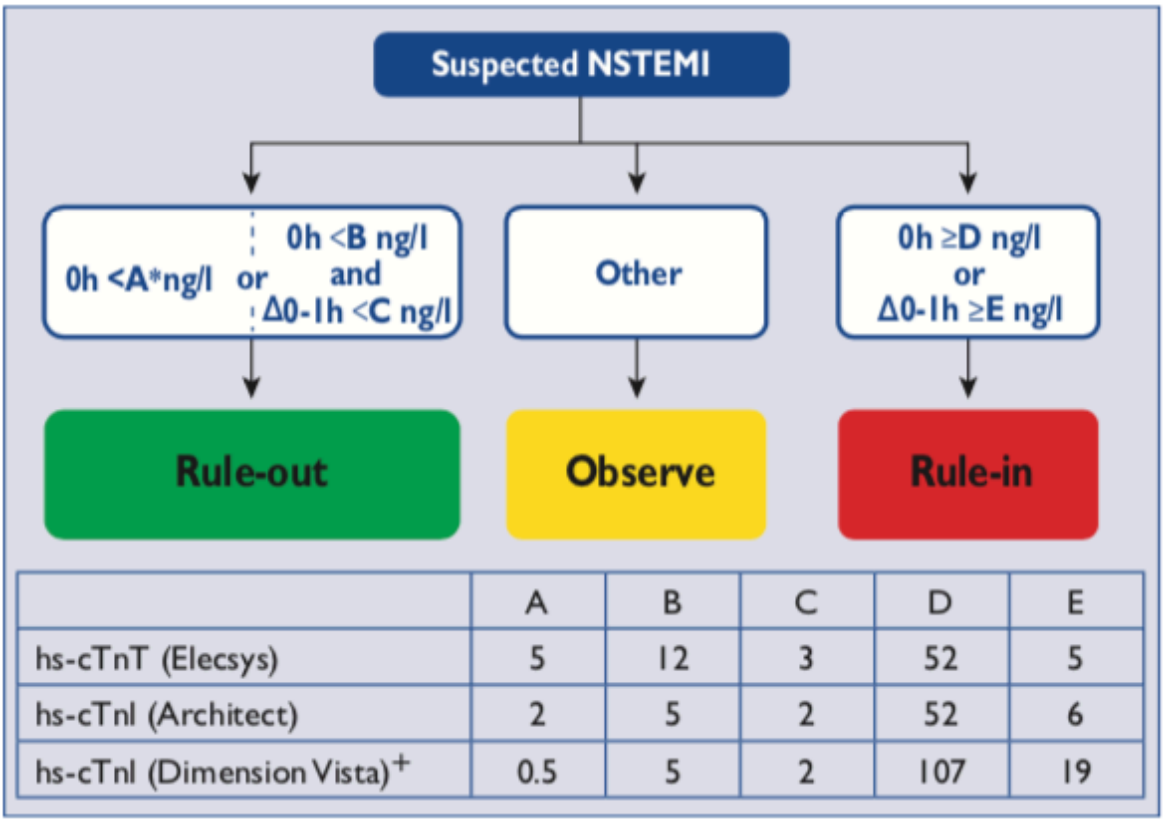


Figure 30 – Dual-marker strategy incorporating the 0h cMyC concentration as a triage-booster into the established ESC 0/1h-algorithm



	A	B	C	D	E
hs-cTnT (Elecsys)	5	12	3	52	5
hs-cTnI (Architect)	2	5	2	52	6
hs-cTnI (Dimension Vista)+	0.5	5	2	107	19

Figure 31 – ESC 2015 pathway for rapid rule-out/rule-in of AMI in patients with suspected Non ST-elevation MI, using hs-cTn; from Roffi et al.[12]

6.2.5 Benefit of delta-change values over static thresholds

Two different approaches to combining the hs-cTn & cMyC algorithms were tested. Version 1) includes only 0h cMyC cut-off values for risk stratification in addition to the established ESC hs-cTnI algorithm. Version 2) uses the entire cMyC 0/1h algorithm in addition to the ESC hs-cTnI algorithm. The different approaches are displayed below in a step-wise approach – Table 9, Table 10.

Rule-Out	Observe	Rule-In
cMyC <A ng/L		
hs-cTnI <2 ng/L		
hs-cTnI <5 ng/L & hs-cTnT delta <2 ng/L		
		cMyC ≥D
		hs-cTnI ≥52 ng/L
		hs-cTnI delta ≥6 ng/L
	Remainder	

Table 9 – Version 1) Combined algorithm using hs-cTnI 0/1h and cMyC 0h samples

Rule-Out	Observe	Rule-In
cMyC <A ng/L		
hs-cTnI <2 ng/L		
cMyC <B & cMyC delta <C		
hs-cTnI <5 ng/L & hs-cTnT delta <2 ng/L		
		cMyC ≥D
		hs-cTnI ≥52 ng/L
		cMyC delta ≥E
		hs-cTnI delta ≥6 ng/L
	Remainder	

Table 10 – Version 2) Combined algorithm using hs-cTnI 0/1h and cMyC 0/1h samples

6.2.6 Statistical analysis

All data are expressed as medians [1st quartile; 3rd quartile] or means (standard deviation) for continuous variables (compared with the Mann-Whitney-U test or student's t-test), and for categorical variables as numbers and percentages (compared with Pearson chi-square). Sensitivity and specificity values were compared using McNemar testing[166]; NPV and PPV were compared testing for differences in (positive and negative) predictive values of two binary diagnostic tests in a paired study design as proposed by Moskowitz and Pepe.[167] Hypothesis

testing was two-tailed, and p values <0.05 were considered statistically significant. No adjustment for multiple comparisons was performed.

Discrimination power was quantified by the area under the receiver-operating characteristics curve (AUC [Confidence intervals 2.5-97.5%]) for each biomarker with all cases available. The AUC was calculated for hs-cTnT/I and cMyC results at presentation and the combination with delta-change values. Comparison of the areas under the ROC curves was performed as recommended by DeLong et al.[168]

Logistic regression was used to combine cMyC levels with hs-cTnT, hs-cTnI or s-cTnI values for the assessment of an incremental value using two biomarkers or delta-change values. Sub-group analysis was performed for patients presenting early, defined as chest pain onset within 3 hours of presentation to the Emergency Department. This is a particular limitation of the published ESC guidance on the use of hs-cTn for risk-stratification, as the rapid rule-out/rule-in algorithms are only applicable to patients with chest pain onset >3 hours.

Predictive value of the biomarkers during follow-up was assessed two-fold: We calculated 1) Harrell's C statistic for each biomarker at presentation for endpoints AMI, death or the composite of AMI and all-cause mortality during follow-up – a higher C index indicates a higher probability of an event occurring during follow-up with higher biomarker values[131]; and 2) Kaplan-Meier survival curves. The formula for obtaining Harrel's C statistic is C = (mean(rank(x)[y == 1]) – (n1 + 1)/2)/(n – n1), with n1 being the frequency of y=1, and y being a binary outcome variable (usually, 0 = survival, 1 = death during follow-up). All statistical analyses were performed using R, version 3.3.0 GUI 1.68 (The R Foundation for Statistical Computing), including packages ggplot2, the tidyverse, RMarkdown, RStudio, survival, Hmisc, compareC and pROC.

6.3. Results

6.3.1 Baseline demographics

Of all 2829 patients recruited, 1431 had both hs-cTnT and cMyC results available at 0/1hr time points available for analysis; 1390 had both hs-cTnI and cMyC results. AMI was the final diagnosis in 17% of all recruited patients. Across the entire cohort, median age was 61 [49; 74], 68% of patients were male and 36% had a history of smoking. Demographics are displayed in Table 11 for all patients, stratified by the diagnosis of Acute Myocardial Infarction; in Table 12 for all patients with complete data on hs-cTnT and cMyC; in Table 13 for all patients with complete data on hs-cTnI and cMyC.

	No AMI (n=2335)	AMI (n=494)	p*	n**
cMyC at presentation	13 [7;28]	237 [72;876]	<0.001	1954
hs-cTnI at presentation	4 [2;8]	109 [22;562]	<0.001	2536
hs-cTnT at presentation	7 [5;12]	60 [27;142]	<0.001	2726
Gender: male	1564 (67%)	364 (74%)	0.004	2829
Age (years)	59 [47;72]	72 [59;80]	<0.001	2829
Hypertension	1352 (58%)	386 (78%)	<0.001	2829
Hyperlipidaemia	1077 (46%)	336 (68%)	<0.001	2829
Diabetes mellitus	382 (16%)	133 (27%)	<0.001	2829
Current smoking	600 (26%)	120 (24%)	0.552	2829
History of smoking	826 (35%)	201 (41%)	0.029	2829
Previous revascularisation (CABG or PCI)	604 (26%)	186 (38%)	<0.001	2829
Coronary artery disease	737 (32%)	245 (50%)	<0.001	2829
eGFR	87 [71;102]	74 [56;94]	<0.001	2810
Heart rate, beats/min	76 [66;89]	78 [67;91]	0.119	2818
Systolic blood pressure, mmHg	141 [126;158]	142 [126;160]	0.201	2824
Diastolic blood pressure, mmHg	82 [73;92]	80 [70;92]	0.075	2823

Table 11 – Demographics for all patients without (left) and with (right) AMI; * p values for comparison validation to derivation cohort; ** n denotes the number of available data points; data are expressed as medians [1st quartile, 3rd quartile] or means ± standard deviation, for categorical variables as numbers (percentages); AMI = Acute Myocardial Infarction; CABG = Coronary Artery Bypass Graft; PCI = Percutaneous Coronary Intervention; † glomerular filtration rate was estimated using the Modification of Diet in Renal Disease (MDRD) formula

	Derivation (n=715)	Validation (n=716)	p*	n**
AMI	122 (17%)	122 (17%)	1.000	1431
cMyC at presentation	17 [8;49]	16 [7;49]	0.310	1431
hs-cTnT at presentation	9 [5;19]	9 [5;21]	0.274	1431
Gender: male	507 (71%)	476 (66%)	0.080	1431
Age (years)	64 [51;75]	61 [49;74]	0.026	1431
Hypertension	463 (65%)	444 (62%)	0.307	1431
Hyperlipidaemia	403 (56%)	346 (48%)	0.003	1431
Diabetes mellitus	145 (20%)	129 (18%)	0.307	1431
Current smoking	175 (24%)	162 (23%)	0.446	1431
History of smoking	274 (38%)	269 (38%)	0.811	1431
Previous revascularisation (CABG or PCI)	231 (32%)	180 (25%)	0.003	1431
Coronary artery disease	295 (41%)	229 (32%)	<0.001	1431
eGFR†	83 [68;98]	85 [68;102]	0.047	1422
Heart rate, beats/min	73 [65;88]	77 [67;89]	0.012	1427
Systolic blood pressure, mmHg	142 [126;160]	142 [128;159]	0.993	1429
Diastolic blood pressure, mmHg	82 [72;92]	82 [71;91]	0.636	1428

Table 12 – Demographics for all patients with complete data for hs-cTnT and cMyC; * p values for comparison validation to derivation cohort; ** n denotes the number of available data points; data are expressed as medians [1st quartile, 3rd quartile] or means ± standard deviation, for categorical variables as numbers (percentages); AMI = Acute Myocardial Infarction; CABG = Coronary Artery Bypass Graft; PCI = Percutaneous Coronary Intervention; † glomerular filtration rate was estimated using the Modification of Diet in Renal Disease (MDRD) formula

	Derivation (n=695)	Validation (n=695)	p*	n**
AMI	121 (17%)	120 (17%)	1.000	1390
cMyC at presentation	17 [8;49]	15 [7;47]	0.209	1390
hs-cTnI at presentation	5 [2;14]	4 [2;14]	0.367	1390
Gender: male	476 (68%)	479 (69%)	0.908	1390
Age (years)	63 [51;75]	62 [49;75]	0.702	1390
Hypertension	440 (63%)	430 (62%)	0.618	1390
Hyperlipidaemia	366 (53%)	349 (50%)	0.391	1390
Diabetes mellitus	152 (22%)	115 (17%)	0.014	1390
Current smoking	170 (24%)	167 (24%)	0.900	1390
History of smoking	264 (38%)	257 (37%)	0.740	1390
Previous revascularisation (CABG or PCI)	210 (30%)	188 (27%)	0.213	1390
Coronary artery disease	260 (37%)	246 (35%)	0.469	1390
eGFR†	84 [68;100]	86 [68;102]	0.216	1381
Heart rate, beats/min	75 [66;89]	76 [65;88]	0.441	1386
Systolic blood pressure, mmHg	142 [128;160]	142 [126;159]	0.523	1388
Diastolic blood pressure, mmHg	82 [73;91]	81 [70;92]	0.281	1387

Table 13 – Demographics for all patients with complete data for hs-cTnI and cMyC; * p values for comparison validation to derivation cohort; ** N denotes the number of available data points; data are expressed as medians [1st quartile, 3rd quartile] or means ± standard deviation, for categorical variables as numbers (percentages); AMI = Acute Myocardial Infarction; CABG = Coronary Artery Bypass Graft; PCI = Percutaneous Coronary Intervention; † glomerular filtration rate was estimated using the Modification of Diet in Renal Disease (MDRD) formula

6.3.2 Diagnostic accuracy of 0/1hr sampling

A dual-marker strategy, combining baseline levels of cMyC and hs-cTn, increased the diagnostic accuracy based on the Area under the Receiver-Operating Curve with hs-cTnI (0.925 [0.909-0.941], p=0.008) and with hs-cTnT (0.930 [0.914-0.946], p=0.006). For the combination of baseline levels and 1h-changes, the diagnostic performance of cMyC was comparable to hs-cTnI (AUC 0.924 [0.908-0.940] vs 0.917 [0.899-0.935], p=0.194), and inferior to hs-cTnT (AUC 0.925 [0.907-0.942] vs 0.945 [0.931-0.958], p=0.003). When baseline cMyC was added to baseline levels and 1h-changes of hs-cTn, the diagnostic accuracy further improved with hs-cTnI (AUC 0.926 [0.909-0.942], p=0.012), but not significantly with hs-cTnT (AUC 0.947 [0.935-0.960], p=0.186) – see Table 14, Table 15, Table 16, Table 17.

AUC values for cMyC vs hs-cTnT comparisons							
	AUC1	CI1	AUC2	CI2	cases	controls	p
cMyC | 1h delta	0.919	0.901-0.937	0.904	0.882-0.926	244	1187	0.1959
cMyC | cMyC + 1h delta	0.919	0.901-0.937	0.925	0.907-0.942	244	1187	1e-04
cTnT | cMyC	0.921	0.903-0.939	0.919	0.901-0.937	244	1187	0.8097
cTnI | cMyC	0.916	0.898-0.934	0.919	0.902-0.936	241	1149	0.6158

Table 14 – Comparison cMyC and hs-cTnT – 0h samples and, for cMyC, 1h-delta sample; p for comparison AUC1 and AUC2

AUC values for cMyC vs hs-cTnT comparisons							
	AUC1	CI1	AUC2	CI2	cases	controls	p
cMyC + 1h delta | cTnT + delta	0.925	0.907-0.942	0.945	0.931-0.958	244	1187	0.003
cTnT + cMyC at 0h | cTnT at 0h	0.93	0.914-0.946	0.921	0.903-0.939	244	1187	0.0059
cTnT + delta | cTnT + 1h delta + cMyC + 1h delta	0.945	0.931-0.958	0.947	0.935-0.96	244	1187	0.1779
cTnT + delta | cTnT + 1h delta + cMyC at 0h	0.945	0.931-0.958	0.947	0.935-0.96	244	1187	0.1863

Table 15 – Comparison cMyC and hs-cTnT using 0h sample + 1h-delta; p for comparison AUC1 and AUC2

AUC values for cMyC vs hs-cTnI comparisons							
	AUC1	CI1	AUC2	CI2	cases	controls	p
cTnI | 1h absolute delta	0.916	0.898-0.934	0.921	0.9-0.943	241	1149	0.5941
cTnI | cTnI + delta	0.916	0.898-0.934	0.917	0.899-0.935	241	1149	0.0013

Table 16 – Performance of hs-cTnI at baseline (0h) and after 1h-delta; p for comparison AUC1 and AUC2

AUC values for cMyC vs hs-cTnI comparisons							
	AUC1	**CI1**	**AUC2**	**CI2**	**cases**	**controls**	**p**
cMyC + 1h delta | cTnI + 1h delta	0.924	0.908-0.94	0.917	0.899-0.935	241	1149	0.1943
cTnI + cMyC at 0h | cTnI at 0h	0.925	0.909-0.941	0.916	0.898-0.934	241	1149	0.008
cTnI + 1h delta | cTnI + 1h delta + cMyC at 0h	0.917	0.899-0.935	0.926	0.909-0.942	241	1149	0.0121
cTnI + 1h delta | cTnI + 1h delta + cMyC + 1h delta	0.917	0.899-0.935	0.924	0.908-0.941	241	1149	0

Table 17 – Comparison cMyC and hs-cTnI using 0h sample + 1h-delta; p for comparison AUC1 and AUC2

For early presenters (patients with presentation samples ≤3 hours since chest pain onset), the addition of cMyC did significantly increase the AUC of hs-cTnI at baseline plus 1h-changes (0.926 [0.900-0.951] vs 0.920 [0.893-0.948], p=0.017); but not significantly with hs-cTnT ((0.933 [0.911-0.954] vs 0.931 [0.908-0.953], p=0.500) – see Table 18, Table 19, Table 20, Table 21, Table 22.

AUC values for cMyC vs hs-cTnT comparisons – early presenters							
	AUC1	CI1	AUC2	CI2	cases	controls	p
cMyC | 1h delta	0.91	0.881-0.938	0.885	0.852-0.918	117	580	0.1446
cMyC | cMyC + 1h delta	0.91	0.881-0.938	0.913	0.885-0.941	117	580	0.0549

Table 18 – Performance of cMyC at baseline (0h) and with 1h-delta in early presenters; p for comparison AUC1 and AUC2

AUC values for cMyC vs hs-cTnT comparisons – early presenters							
	AUC1	CI1	AUC2	CI2	cases	controls	p
cTnT | 1h delta	0.92	0.895-0.946	0.907	0.873-0.941	117	580	0.4939
cTnT | cMyC	0.92	0.895-0.946	0.91	0.881-0.938	117	580	0.3813
cTnT | cTnT + 1h delta	0.92	0.895-0.946	0.931	0.908-0.953	117	580	2e-04

Table 19 – Comparison cMyC and hs-cTnT using 0h sample + 1h-delta in early presenters; p for comparison AUC1 and AUC2

AUC values for cMyC vs hs-cTnT comparisons – early presenters							
	AUC1	**CI1**	**AUC2**	**CI2**	**cases**	**controls**	**p**
cTnI | 1h delta	0.911	0.882-0.941	0.923	0.892-0.954	109	571	0.4034
cTnI | cMyC	0.911	0.882-0.941	0.91	0.883-0.936	109	571	0.8562
cTnI | cTnI + 1h delta	0.911	0.882-0.941	0.92	0.893-0.948	109	571	0.0015

Table 20 – Comparison cMyC and hs-cTnI using 0h sample + 1h-delta in early presenters; p for comparison AUC1 and AUC2

AUC values for cMyC vs hs-cTnT comparisons – early presenters							
	AUC1	CI1	AUC2	CI2	cases	controls	p
cMyC + 1h delta | cTnT + 1h 1delta	0.913	0.885-0.941	0.931	0.908-0.953	117	580	0.1287
cTnT + 1h delta | cTnT + 1h delta + cMyC + 1h delta	0.931	0.908-0.953	0.933	0.911-0.954	117	580	0.4999
cTnT + 1h delta | cTnT + 1h delta + cMyC at 0h	0.931	0.908-0.953	0.933	0.912-0.955	117	580	0.3551

Table 21 – Comparison cMyC and hs-cTnT using 0h sample + 1h-delta in early presenters; p for comparison AUC1 and AUC2

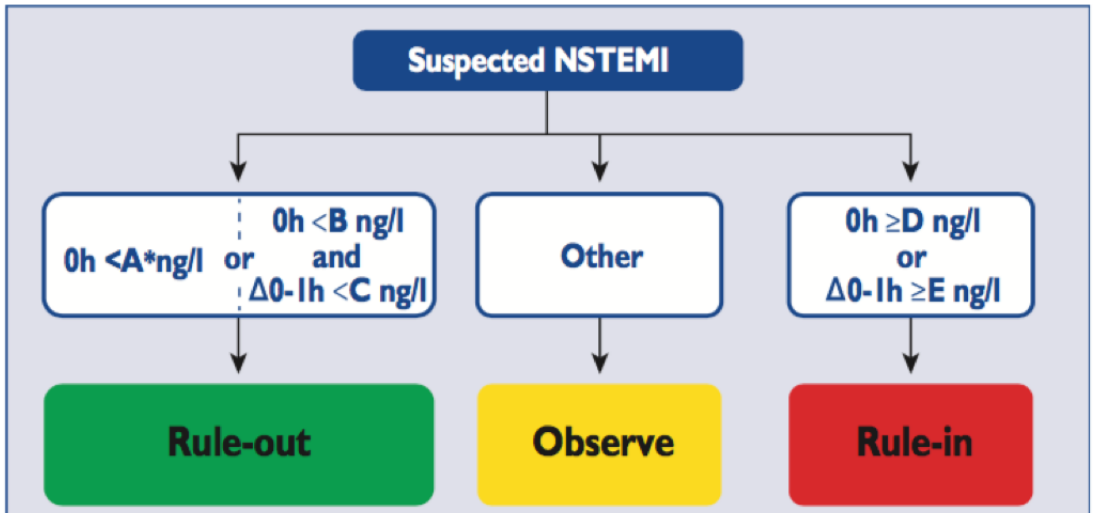
AUC values for cMyC vs hs-cTnI comparisons – early presenters							
	AUC1	CI1	AUC2	CI2	cases	controls	p
cMyC + 1h delta | cTnI + 1h delta	0.921	0.896-0.946	0.92	0.893-0.948	109	571	0.9607
cTnI + delta | cTnI + 1h delta + cMyC + delta	0.92	0.893-0.948	0.926	0.9-0.951	109	571	0.0166
cTnI + 1h delta | cTnI + 1h delta + cMyC at 0h	0.92	0.893-0.948	0.929	0.905-0.952	109	571	0.1009

Table 22 – Comparison cMyC and hs-cTnI using 0h sample + 1h-delta in early presenters; p for comparison AUC1 and AUC2

6.3.3 Derivation and Validation of the cMyC rule-out/rule-in algorithm

The best performing cMyC cut-off combination [0h <10 (A) or 0h <26 (B) AND Δ0-1h <8 (C) for rule-out; ≥136 (D) OR Δ0-1h ≥15 (E) for rule-in (all values ng/L); see Figure 32] was

selected in the derivation cohort and applied to the validation cohort (n=716 for hs-cTnT, n=695 for hs-cTnI). The results of cMyC performance in the two validation sets are presented separately below.



	A	B	C	D	E
hs-cTnT (Elecsys)	5	12	3	52	5
hs-cTnI (Architect)	2	5	2	52	6
cMyC	10	26	8	136	15

Figure 32 – ESC 0/1h rule-out/rule-in pathway adjusted with proposed cMyC cut-offs; adapted from Roffi et al.[12]

6.3.4 cMyC in comparison to hs-cTnT

Accuracy for rule-out by the best performing cMyC 0/1h-algorithm was high in the hs-cTnT validation cohort: NPV 99.3% [95% CI, 98.5-99.9%]; sensitivity 97.6% [94.7-100%]; and comparable to the ESC hs-cTnT 0/1h-algorithm: NPV 99.7% [99.2-100%; p=0.332]; sensitivity 99.2% [97.3-100%, p=0.317). Accuracy for rule-in was high: PPV 70% [62.2-77.6%]; specificity 93.3% [91.1-95.1%]; but inferior to the ESC hs-cTnT 0/1h-algorithm: PPV 78.9%

[71.7-85.87%; p=0.007]; specificity 95.6% [93.9-97.2%; p=0.011]. Proportion of patients assigned to either rule-out or rule-in based on the 0/1h-samples significantly increased from 75.28% using hs-cTnT to 79.19% using cMyC (p<0.001). Direct rule-out (feasible in patients presenting >3 hours after chest pain onset) or rule-in based on a single blood draw at ED presentation increased from 35.75% using hs-cTnT to 47.21% using cMyC (p<0.001) – see Figure 33, Table 23, Table 24 for exact distributions.

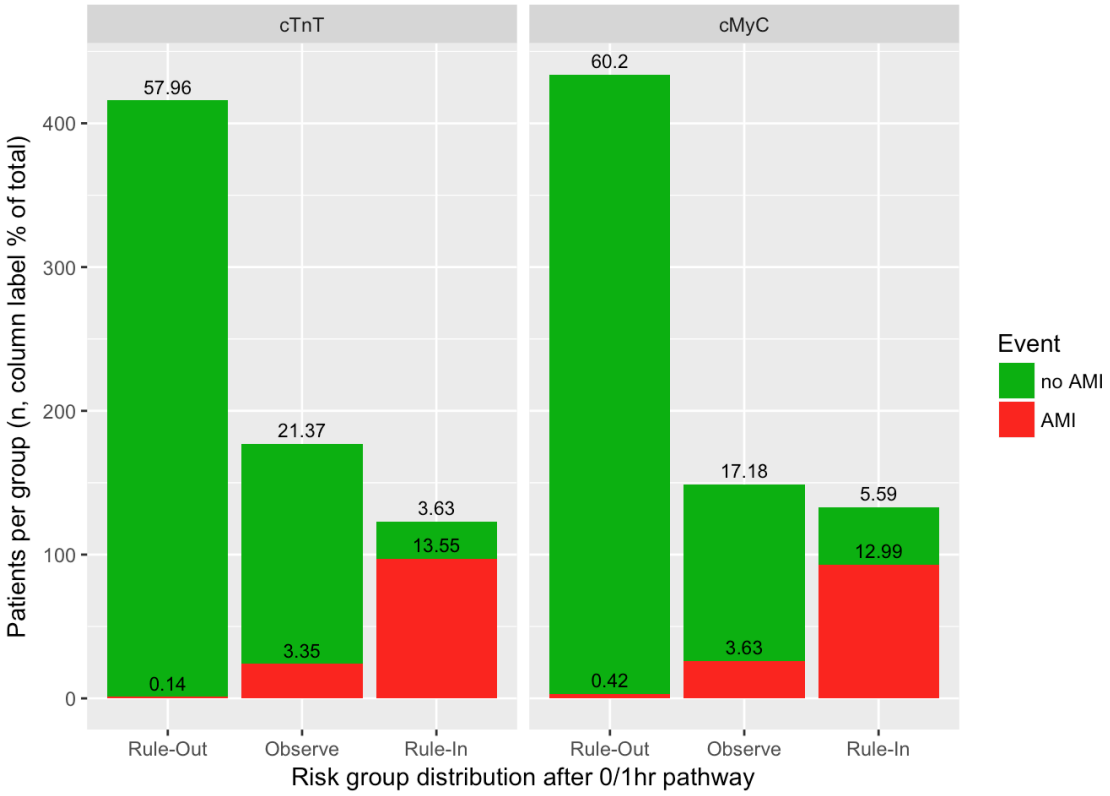


Figure 33 – Risk group distribution following application of 0/1h rule-out/rule-in pathways for either hs-cTnT (left panel) or cMyC (right panel)

	hs-cTnT			cMyC		
AMI	Rule-Out	Observe	Rule-In	Rule-Out	Observe	Rule-In
No	181	399	14	252	324	18
Yes	1	61	60	0	54	68
Sum	182	460	74	252	378	86
Percent	25.42%	64.25%	10.34%	35.2%	52.79%	12.01%

Table 23 – 0h triage of hs-cTnT vs cMyC

	hs-cTnT			cMyC		
AMI	Rule-Out	Observe	Rule-In	Rule-Out	Observe	Rule-In
No	415	153	26	431	123	40
Yes	1	24	97	3	26	93
Sum	416	177	123	434	149	133
Percent	58.1%	24.72%	17.18%	60.61%	20.81%	18.58%

Table 24 – 0/1h triage of hs-cTnT vs cMyC

6.3.5 cMyC in comparison to hs-cTnI

Accuracy for rule-out by the novel cMyC 0/1h-algorithm was high in the hs-cTnI cohort: NPV 99.3% [95% CI, 98.5-99.9%]; sensitivity 97.5% [94.1-100%]; and comparable to the ESC hs-cTnI 0/1h-algorithm: NPV 99.1% [98.2-99.9%; p=0.785]; sensitivity 97.5% [94.6-100%; p=1]. Similarly, accuracy for rule-in was high: PPV 70.9% [63.1-78.5%]; specificity 93.4% [91.2-95.3%]; and comparable to the ESC hs-cTnI 0/1h-algorithm: PPV 71.2% [63.3-78.9%; p=0.914]; specificity 93.6% [91.5-95.4%; p=0.847].

Proportion of patients assigned to either rule-out or rule-in based on the 0/1h-samples significantly increased from 70.1% using hs-cTnI to 80.7 % using cMyC ($p<0.05$). Direct rule-out (feasible in patients presenting >3 hours after chest pain onset) or rule-in based on a single blood draw at ED presentation increased from 28.8% using hs-cTnI to 48.5% using cMyC ($p<0.05$) – see Figure 34, Table 25, Table 26 for exact distributions.

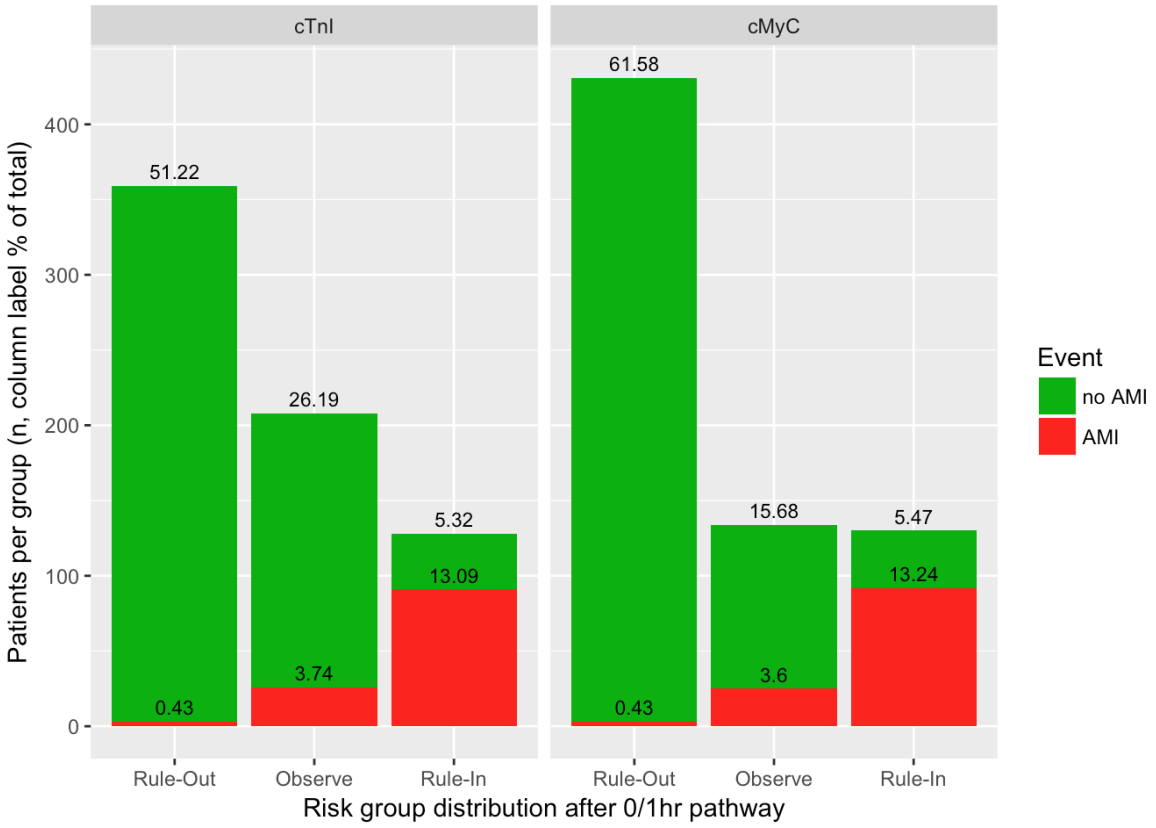


Figure 34 – Risk group distribution following application of 0/1h rule-out/rule-in pathways for either hs-cTnI (left panel) or cMyC (right panel)

	hs-cTnI			cMyC		
AMI	Rule-Out	Observe	Rule-In	Rule-Out	Observe	Rule-In
No	109	442	24	248	303	24
Yes	0	53	67	0	55	65
Sum	109	495	91	248	358	89
Percent	15.68%	71.22%	13.09%	35.68%	51.51%	12.81%

Table 25 – 0h triage of hs-cTnI vs cMyC

	hs-cTnI			cMyC		
AMI	Rule-Out	Observe	Rule-In	Rule-Out	Observe	Rule-In
No	356	182	37	428	109	38
Yes	3	26	91	3	25	92
Sum	359	208	128	431	134	130
Percent	51.65%	29.93%	18.42%	62.01%	19.28%	18.71%

Table 26 – 0/1h triage of hs-cTnI vs cMyC

6.3.6 cMyC in addition to hs-cTnT

The established hs-cTnT 0/1h rule-out/rule-in algorithm was then combined with the novel cMyC algorithm. Accuracy for rule-out by the combined hs-cTnT + cMyC 0/1h-algorithm was high in the validation cohort: NPV 99.13% [95% CI, 98.3-99.83%]; sensitivity 96.7% [93.1-99.3%]; and comparable to the ESC hs-cTnT 0/1h-algorithm: NPV 99.7% [99.2-100%; p=0.332]; sensitivity 99.2% [97.3-100%, p=0.317). Accuracy for rule-in was high: PPV 78.4% [71.0-85.5%]; specificity 95.6% [94.0-97.2%]; and comparable to the ESC hs-cTnT 0/1h-algorithm: PPV 78.9% [71.7-85.87%; p=0.007]; specificity 95.6% [93.9-97.2%; p=0.011]. Proportion of patients assigned to either rule-out or rule-in based on the 0/1h-samples

significantly increased from 75.28% using hs-cTnT alone to 83.66 % using the combination of hs-cTnT and cMyC (p<0.001). Direct rule-out (feasible in patients presenting >3 hours after chest pain onset) or rule-in based on a single blood draw at ED presentation increased from 35.75% using hs-cTnT alone to 51.54% using the combination of hs-cTnT and cMyC (p<0.001) – see Figure 35, Table 27, Table 28 for exact distributions.

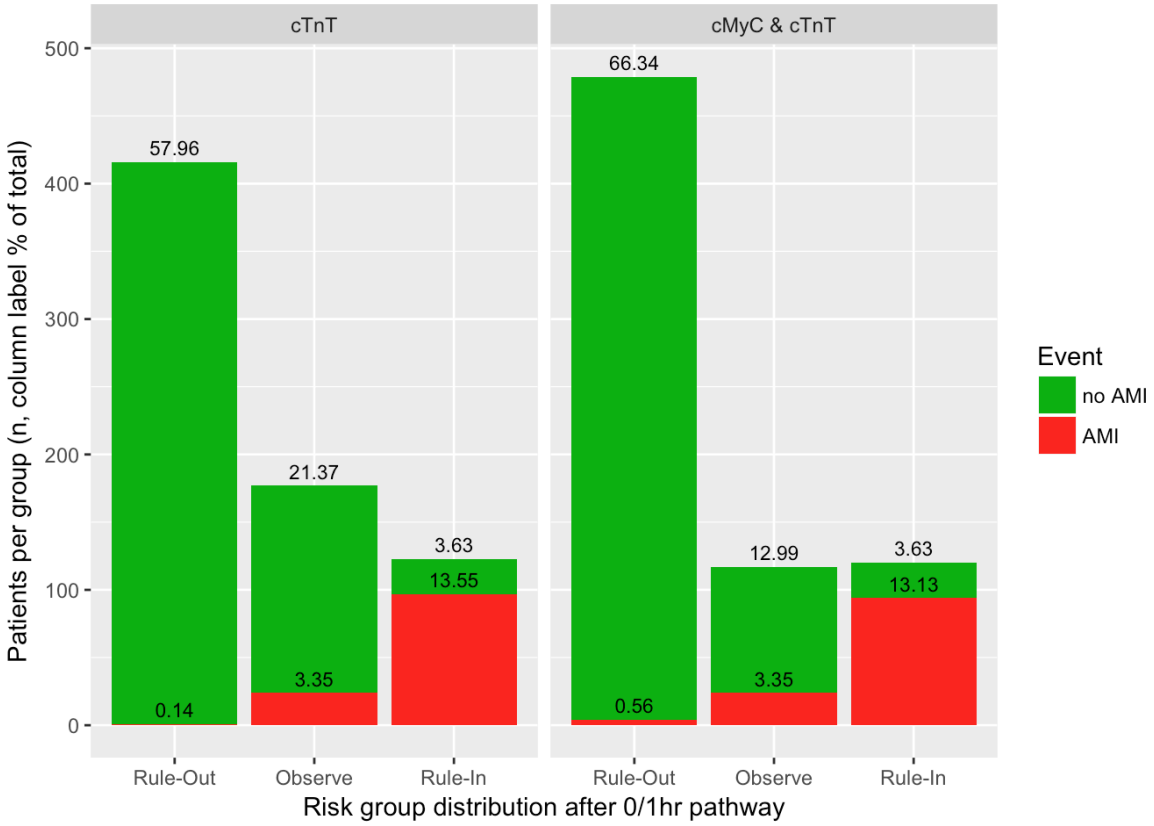


Figure 35 – Risk group distribution following application of 0/1h rule-out/rule-in pathways for either hs-cTnT alone (left panel) or the combination of hs-cTnT and cMyC (right panel)

AMI	hs-cTnT			hs-cTnT + cMyC		
	Rule-Out	Observe	Rule-In	Rule-Out	Observe	Rule-In
No	181	399	14	294	286	14
Yes	1	61	60	1	61	60
Sum	182	460	74	295	347	74
Percent	25.42%	64.25%	10.34%	41.2%	48.46%	10.34%

Table 27 – 0h triage of hs-cTnT vs combination of hs-cTnT and cMyC

AMI	hs-cTnT			hs-cTnT + cMyC		
	Rule-Out	Observe	Rule-In	Rule-Out	Observe	Rule-In
No	415	153	26	475	93	26
Yes	1	24	97	4	24	94
Sum	416	177	123	479	117	120
Percent	58.1%	24.72%	17.18%	66.9%	16.34%	16.76%

Table 28 – 0/1h triage of hs-cTnT vs combination of hs-cTnT and cMyC

6.3.7 cMyC in addition to hs-cTnI

The established hs-cTnI 0/1h rule-out/rule-in algorithm was then combined with the novel cMyC algorithm. Accuracy for rule-out by the combined hs-cTnI + cMyC 0/1h-algorithm was high in the validation cohort: NPV 98.9% [95% CI, 97.9-99.7%]; sensitivity 95.8% [91.9-99.1%]; and statistically non-inferior to the ESC hs-cTnI 0/1h-algorithm: NPV 99.1% [98.2-99.9%; p=0.406]; sensitivity 97.5% [94.6-100%; p=0.157]. As can be seen from table 30, the patients with AMI misclassified into the rule-out group (n=5 for the combined algorithm, n=3 for hs-cTnI alone) are different individuals depending on which biomarker is used – for a comparison on the missed patients, see supplemental Table 38. It would be interesting to

compare the performance of both in a cohort adjudicated with hs-cTnI, but the overall, combined accuracy for rule-out would unlikely be acceptable in daily clinical practice.

Accuracy for rule-in was high: PPV 73.5% [65.7-81.0%]; specificity 94.3% [92.3-96.1%]; and statistically superior to the ESC hs-cTnI 0/1h-algorithm: PPV 71.2% [63.3-78.9%; p=0.046]; specificity 93.6% [95.4-95.4%; p=0.046].

Proportion of patients assigned to either rule-out or rule-in based on the 0/1h-samples significantly increased from 70.07% using hs-cTnI alone to 82.88% using the combination of hs-cTnI and cMyC (p<0.001). Direct rule-out (feasible in patients presenting >3 hours after chest pain onset) or rule-in based on a single blood draw at ED presentation increased from 28.78% using hs-cTnI alone to 51.22% using the combination of hs-cTnI and cMyC (p<0.001) – see Figure 36, Table 29, Table 30 for exact distributions.

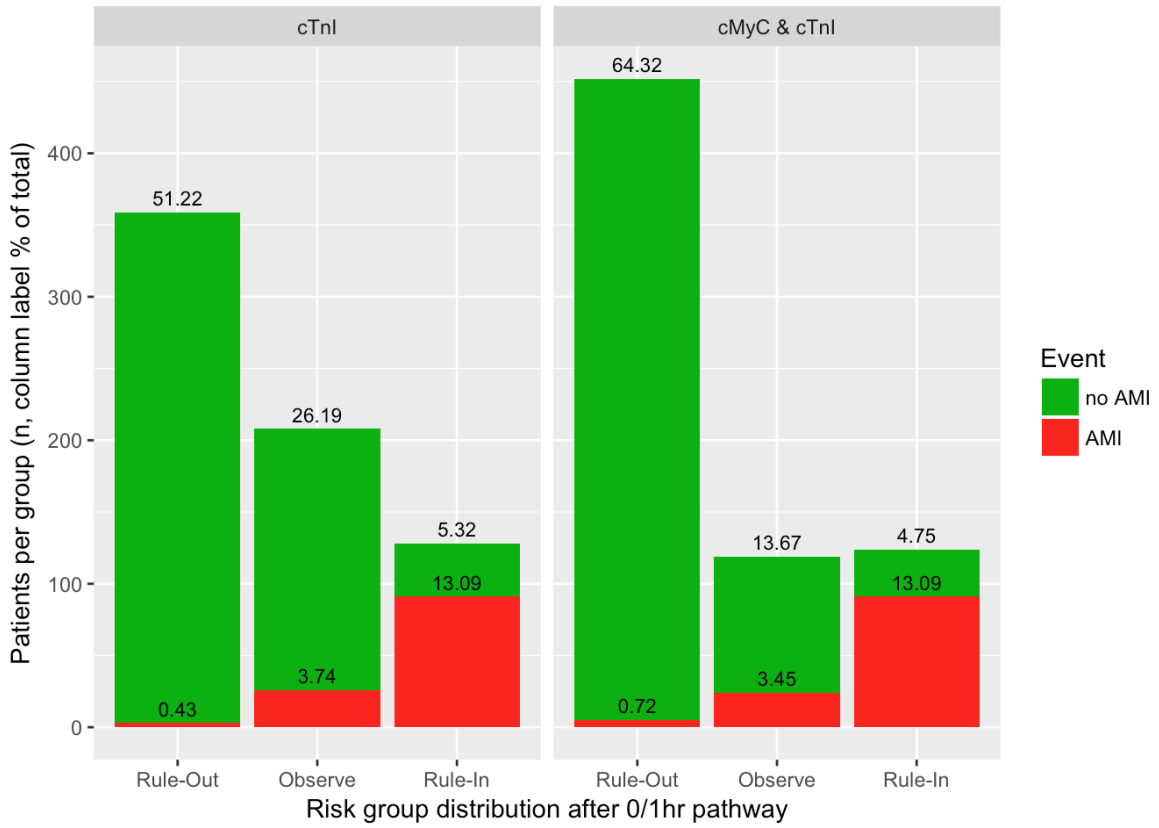


Figure 36 – Risk group distribution following application of 0/1h rule-out/rule-in pathways for either hs-cTnI alone (left panel) or the combination of hs-cTnI and cMyC (right panel)

	hs-cTnI			hs-cTnI + cMyC		
AMI	Rule-Out	Observe	Rule-In	Rule-Out	Observe	Rule-In
No	109	442	24	265	286	24
Yes	0	53	67	0	53	67
Sum	109	495	91	265	339	91
Percent	15.68%	71.22%	13.09%	38.13%	48.78%	13.09%

Table 29 – 0h triage of hs-cTnI vs combination of hs-cTnI and cMyC

	hs-cTnI			hs-cTnI + cMyC		
AMI	Rule-Out	Observe	Rule-In	Rule-Out	Observe	Rule-In
No	356	182	37	447	95	33
Yes	3	26	91	5	24	91
Sum	359	208	128	452	119	124
Percent	51.65%	29.93%	18.42%	65.04%	17.12%	17.84%

Table 30 – 0/1h triage of hs-cTnI vs combination of hs-cTnI and cMyC

6.3.8 Benefit of delta-change values over static thresholds

In a subgroup analysis, we investigated whether delta-change values are better than static cut-offs for rule-out/rule-in of AMI. Different approaches were tested: 1) Using only the 0h cut-offs of the cMyC algorithm in addition, or 2) using the entire cMyC 0/1h algorithm in addition to the established ESC hs-cTnI 0/1h algorithm. In version 1), the addition of cMyC significantly increases the amount of patients qualifying for direct rule-out (38.1% vs 15.7% with hs-cTnI alone, $p<0.001$; Table 31, Table 32), however this benefit diminishes as the remaining patients undergo a second blood-draw – with the observe-zone marginally smaller with a dual-marker strategy than with hs-cTnI alone (26.6% vs 29.9%, $p<0.001$) – Table 33, Table 34.

	hs-cTnI Triage			hs-cTnI + cMyC 0h		
AMI	Rule-Out	Observe	Rule-In	Rule-Out	Observe	Rule-In
No	109	442	24	265	277	33
Yes	0	53	67	0	45	75
Sum	109	495	91	265	322	108
Percent	15.68%	71.22%	13.09%	38.13%	46.33%	15.54%

Table 31 – 0h triage of hs-cTnI vs combination of hs-cTnI and cMyC 0h; p<0.001 for comparison of group size

	hs-cTnI	**hs-cTnI + cMyC**	**p value**
NPV (%)	100.00	100.00	n/a
Sensitivity (%)	100.00	100.00	n/a
PPV (%)	73.63	69.44	0.061
Specificity (%)	95.83	94.26	0.003

Table 32 – Performance of 0h triage hs-cTnI vs hs-cTnI + cMyC 0h

	hs-cTnI Triage			hs-cTnI + cMyC 0h		
AMI	Rule-Out	Observe	Rule-In	Rule-Out	Observe	Rule-In
No	356	182	37	371	162	42
Yes	3	26	91	3	23	94
Sum	359	208	128	374	185	136
Percent	51.65%	29.93%	18.42%	53.81%	26.62%	19.57%

Table 33 – 0/1h triage of hs-cTnI vs combination of hs-cTnI and cMyC 0h; p<0.001 for comparison of group size

	hs-cTnI	hs-cTnI + cMyC	p value
NPV (%)	99.16	99.20	0.114
Sensitivity (%)	97.50	97.50	n/a
PPV (%)	71.09	69.12	0.172
Specificity (%)	93.57	92.70	0.059

Table 34 – Performance of 0/1h triage hs-cTnI vs hs-cTnI + cMyC 0h

In version 2), the addition of cMyC would naturally only affect triage allocation after incorporation of cMyC and hs-cTnI deltas. Here, the addition of cMyC deltas increases the amount of patients qualifying for rule-out after two blood draws (65.0% vs 51.7% with hs-cTnI alone, p<0.001; Table 35), with the observe-zone significantly smaller with the dual-marker strategy than with hs-cTnI alone (14.2% vs 29.9%, p<0.001) – Table 33, Table 34. Of note, all performance metrics suffer from this approach – as cMyC deltas incorrectly rule-out different patients to hs-cTnI, NPV and sensitivity decrease numerically (albeit not statistically significant) – Table 36.

	hs-cTnI Triage			hs-cTnI + cMyC		
AMI	Rule-Out	Observe	Rule-In	Rule-Out	Observe	Rule-In
No	356	182	37	447	83	45
Yes	3	26	91	5	16	99
Sum	359	208	128	452	99	144
Percent	51.65%	29.93%	18.42%	65.04%	14.24%	20.72%

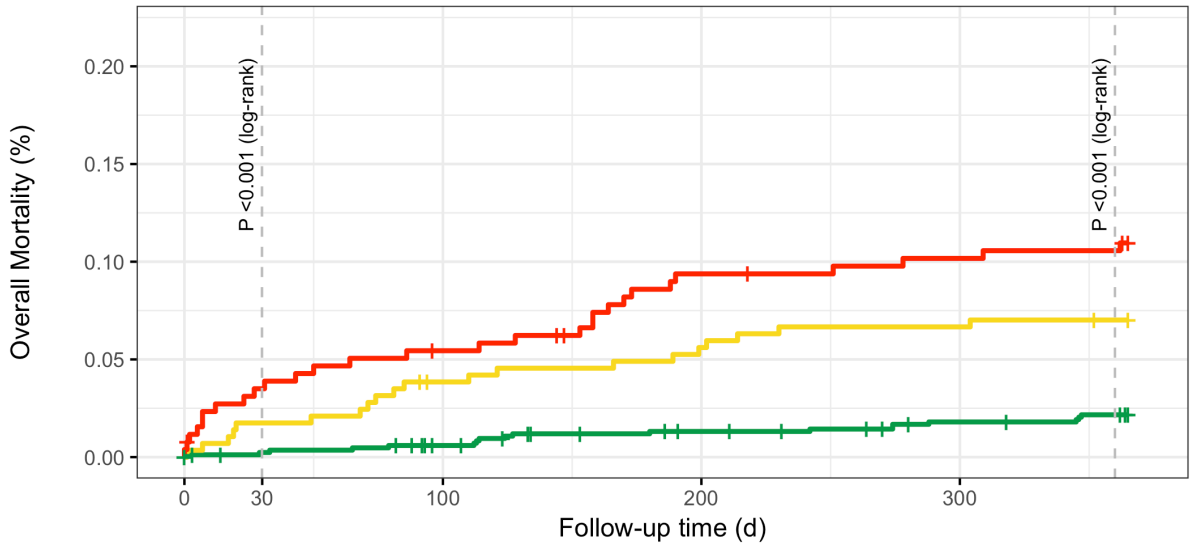
Table 35 – 0/1h triage of hs-cTnI vs combination of hs-cTnI and cMyC 0h; p<0.001 for comparison of group size

	cTnI	hs-cTnI + cMyC	p value
NPV (%)	99.16	98.89	0.406
Sensitivity (%)	97.50	95.83	0.157
PPV (%)	71.09	68.75	0.271
Specificity (%)	93.57	92.17	0.046

Table 36 – Performance of 0/1h triage hs-cTnI vs hs-cTnI + cMyC

6.3.9 Prognostic impact: cMyC – 30d and 1y mortality

The cMyC 0/1h-triage algorithm distinguishes significantly between patients at low, intermediate and high risk of overall at 30-day and 1-year follow-up - Figure 37. Hazard ratios are 3.35 ($p<0.001$) for observe, and 5.36 ($p<0.001$) for rule-in categories (Wald test 31.38 on 2 df, $p<0.001$) – see Table 37 for full details. Further survival analysis of combined pathways can be found in the supplement.



cMyC 0/1h-triage algorithm - Number at risk (events)

Rule-Out	846 (0)	840 (2)	830 (5)	817 (11)	808 (15)	0 (18)
Observe	286 (0)	281 (5)	273 (11)	268 (16)	265 (19)	0 (20)
Rule-In	258 (1)	248 (9)	242 (14)	230 (24)	227 (26)	0 (28)

Figure 37 – Cumulative event (mortality) plot for the cMyC 0/1h-triage algorithm, with statistical comparison of event curves at 30d and 365d with log-rank tests; table displays number-at-risk and absolute number of events

Category	*coef*	*HR*	*SE*	*z*	*p value*
Observe	1.2098	3.35 (95% CI, 1.77-6.34)	0.3249	3.724	<0.001
Rule-In	1.6791	5.36 (95% CI, 2.97-9.69)	0.3021	5.558	<0.001
Concordance	0.69	SE = 0.031			
R^2	0.025	max possible = 0.495			
Likelihood ratio test	34.72	on 2 df, p=3e-08			
Wald test	31.38	on 2 df, p=2e-07			
Score (logrank) test	37.99	on 2 df, p=6e-09			

Table 37 – Cox regression statistics for model using a 3-risk-group distribution for modelling 1-year survival; coef = regression coefficient, HR = Hazard ratio, SE = standard error, z = Wald statistic value

6.4. Discussion

In this analysis of >1,300 patients presenting with suspected myocardial infarction, cMyC has been shown to significantly increase the diagnostic accuracy when added to the cardiac biomarker not used for adjudication (hs-cTnI), for all patients as well as early presenters. Through the use of an internal derivation/validation split, we were able to closely examine the performance of a newly developed cMyC 0/1h rule-out/rule-in algorithm and the effects of using a more dynamic biomarker[50] in comparison or in addition to established cardiac biomarkers. As opposed to our first study investigating the use of cMyC as a candidate-biomarker for chest pain triage[169], we focussed on NPV, PPV and triage efficacy (determined

by the size of the observe-zone after applying the 0/1h-pathway) as the main metrics to select and validate the 0/1h-triage pathway. Pragmatic reasons underpin the choice of performance metrics: from a computational perspective, we had to carefully select clinically relevant, but as few outcome measures as possible; from a clinical point-of-view, the novel biomarker seemed a good fit as a add-on triage-tool – to optimise rapid triage, and enable discharge or admit decisions at an earlier time-point. For this reason, it appeared more appropriate to focus on risk for diagnosis (or likelihood of absence of a diagnosis of AMI) within the populations assigned to either triage category. As an interesting side-effect, whilst there is a numerical difference in the sensitivity values quoted between cMyC and hs-cTnT algorithms, the p-values do not reach statistical significance. Therefore, maintaining equivalent safety (based on NPV, and 30-day mortality), cMyC is both able to directly rule-out AMI in more patients at presentation and maintain an absolute reduction of 4-10% in the observe-zone (equivalent to a 20-50% relative reduction).

We have previously published on the use of cardiac Troponins in a busy central London hospital[170], demonstrating that around 7,800 patients undergo hs-cTn testing annually. Based on pragmatic assumptions, 300-900 patients would benefit from earlier rule-out and rule-in if the cMyC algorithm was used for triage in a single hospital. If a dual-marker strategy employing hs-cTn plus cMyC was used for triage, the observe-zone would shrink further – by 8-12% following a completed 0/1h triage protocol. If one was to choose the optimal time point for a dual-marker strategy, the biggest incremental benefit occurs at 0h-testing – where immediate rule-out with hs-cTnI is more than doubled through the addition of cMyC. Intriguingly, it appears almost irrelevant whether hs-cTnI or cMyC are used first-line – phrased

differently, the addition of hs-cTnI leads to a 3% absolute increase in rule-out achievable with cMyC alone, at no incremental benefit in terms of safety.

From this analysis, it is further evident that delta-change values act as a remarkably good discriminator of acute versus chronic injury, and are an invaluable tool in the arsenal of chest pain triage. An approach using two markers (hs-cTnI and cMyC) and both delta cut-offs failed to reach our a-priori minimal performance threshold of an NPV <99% and PPV <70%, albeit not statistically significantly when compared to hs-cTnI alone. This decrement in classification performance is interesting from a number of perspectives: hs-cTnI and cMyC have 'missed' different patients with AMI (adjudicated by hs-cTnT), thus resulting in a total of 5 missed events (and a lower NPV) in the dual-marker arm. The survival curves for death during 30-day follow-up demonstrate that 5/9 deaths occur in the hs-cTnI observe-zone, but 5/9 in the dual-marker rule-in zone. Does that mean in turn, that the addition of cMyC results in better risk-stratification, shifting more patients *at risk* into a higher-risk category? A 3-tiered risk-stratification is only useful as long as treatment strategies applied to each category clearly differ – recommendations as to the ideal work-up and treatment of patients in the observe-zone is, however, lacking to this date. This is partly driven by the profound heterogeneity of diagnoses identified amongst this group of patients.[133] The benefit of more effective rule-out of low-risk cases is a 'concentration' of high-risk cases in smaller non-'low-risk' categories. But if one novel and one established cardiac biomarker used for risk-stratification (and diagnosis of AMI[11,127]) fail to identify the same patients at risk, does that not question the 'gold-standard'? The gold-standard adjudication in the entire cohort was based, solely, on the use of (hs-)cTnT. This is further reflected in the variable rule-in performance – the biomarkers not used for adjudication (hs-cTnI and cMyC) share comparable performance characteristics regarding

specificity and PPV, which are statistically not different between the groups. One might consider an independent judge for comparison: a 'hard' endpoint such as cardiac death during 30-day follow-up – where, according to the plotted Kaplan-Meier curves, neither hs-cTnT, hs-cTnI nor cMyC inappropriately rule-out.

The study has several limitations: 1) cMyC analysis was performed retrospectively on stored patients' samples, and all considerations regarding performance in real-life are speculative. 2) cMyC analysis was performed on a research assay and requires migration onto a clinical laboratory platform for automated use as part of chest pain triage. 3) Derivation/validation was performed using an internal split in a cohort adjudicated with hs-cTnT – this is likely to lead to bias in favour of the adjudicating biomarker and requires external validation. Ideally, this external validation should be performed in a hs-cTnI adjudicated cohort. 4) The internal split limits the sample size – and thus the number of events. However, derivation of cut-offs recommended in guidelines[1] stems from even smaller sample-sets.[87,113]

It is now well established that many different paths lead to the same goal – the use of various algorithms can achieve safety of rule-out with equal performance.[34] Renal dysfunction does not appear to impair safety[171,172], and at least for hs-cTnI the evidence base is shifting such that patients presenting early after symptom onset are unlikely to be missed by rule-out strategies.[35] It proves challenging to increase the efficiency of algorithms, i.e. the successful triage into rule-out and rule-in categories to leave a minimal number of patients in an indeterminate grey-zone (such as the ESC observe zone) at the end of the path. Strategies include raising the rule-out threshold (for hs-cTnI)[107], employing computer models which incorporate the biomarker[173], or using two different hs-cTn assays (of competing manufacturers)[174]. Maybe the answer lies in using cardiac-specific biomarkers, which do not originate from the same compartment within

the sarcomere? This also has the likely benefit of providing an additional biological signal, rather than amplifying the injury occurring within one compartment – with proven benefit in very early presenters[123,126], through greater abundance[50,122] and a more dynamic release profile[50]. The next step ought to prove the utility of cMyC in a parallel setup, measuring the biomarker on a clinical platform alongside hs-cTn to demonstrate stability in a laboratory assay. Subsequently, only a cluster-randomised cross-over trial design can answer the question of whether the difference in NPV and PPV is artefactually introduced through bias in favour of an adjudicating biomarker – or a true biological signal. The survival curves favour the former hypothesis. In summary, a newly developed cMyC AMI rule-in/rule-out pathway identifies a greater proportion of patients suitable for safe rule-out as compared with the ESC 0/1h-algorithm using hs-cTnI or hs-cTnT and thus reduces the number of patients in a diagnostic grey zone.

6.5. Supplement to Chapter 6

6.5.1 Supplemental results

hs-cTnT validation cohort																		
										Triage			cMyC		hs-cTnT		hs-cTnI	
Age	Gender	Chol	DM	Smoking history	Previous MI	CAD	GFR	Chest pain to blood draw (h)	Adjudication	hs-cTnT	hs-cTnI	cMyC	0h	1h	0h	1h	0h	1h
68	female	no	No	no	no	no	106	5	Type 1 MI	Rule-In	Observe	Rule-Out	21	19	70	66	8	7
63	female	yes	diet	no	yes	yes	75	2	Type 1 MI	Rule-In	Rule-In	Rule-Out	12	15	16	5	12	25
64	male	yes	no	no	yes	yes	78	0	Type 1 MI	Rule-In	Observe	Rule-Out	12	17	68	67	6	6

hs-cTnI validation cohort																		
										Triage								
Age	Gender	Chol	DM	Smoking history	Previous MI	CAD	GFR	Chest pain to blood draw (h)	Adjudication	hs-cTnT	hs-cTnI	cMyC	0h	1h	0h	1h	0h	1h
73	male	yes	no	no	yes	yes	124	4	Type 2 MI	Observe	Rule-Out	Rule-Out	18	17	33	32	3	4
65	male	yes	no	no	yes	yes	54	1	Type 1 MI	NA	Observe	Rule-Out	23	27	NA	NA	7	7
77	male	yes	no	no	no	no	50	6	Type 1 MI	Observe	Observe	Rule-Out	21	21	20	20	8	7
75	male	yes	no	yes	no	yes	72	1	Type 1 MI	Observe	Rule-Out	Rule-In	29	49	6	11	3	5
93	female	yes	no	no	no	yes	34	9	Type 1 MI	Observe	Rule-Out	Rule-In	47	67	41	38	4	2

Table 38 – Overview of patients missed by any biomarker during triage process; Chol = Hypercholesterolaemia; DM = Diabetes mellitus; MI = Myocardial Infarction; GFR = glomerular filtration rate was estimated using the Modification of Diet in Renal Disease (MDRD) formula

Outcomes for patients assigned rule-out, observation or rule-in categories – hs-cTnT and cMyC

Harrell's C and Somers' D statistics demonstrate comparable risk prediction for cardiac death during a 30-days follow-up period: hs-cTnT 0.874 (Somers D 0.749±0.075), hs-TnT + cMyC 0.868 (Somers D 0.737±0.060, p=0.493; **Error! Reference source not found.**, **Error! Reference source not found.**). For the composite endpoint of AMI and death at 30 days, the C-statistics for hs-cTnT are 0.819 (Somers D 0.639±0.053), for hs-cTnT + cMyC 0.821 (Somers D 0.642±0.069, p=0.942; **Error! Reference source not found.**, **Error! Reference source not found.**). For the composite endpoint of AMI and death at 1 year, the C-statistics for hs-cTnT are 0.725 (Somers D 0.451±0.057), for hs-cTnT + cMyC 0.704 (Somers D 0.408±0.066, p=0.296; **Error! Reference source not found.**, **Error! Reference source not found.**). Kaplan-Meier curves for hs-cTnT and the combination with cMyC are displayed below, including tables for the C-statistics.

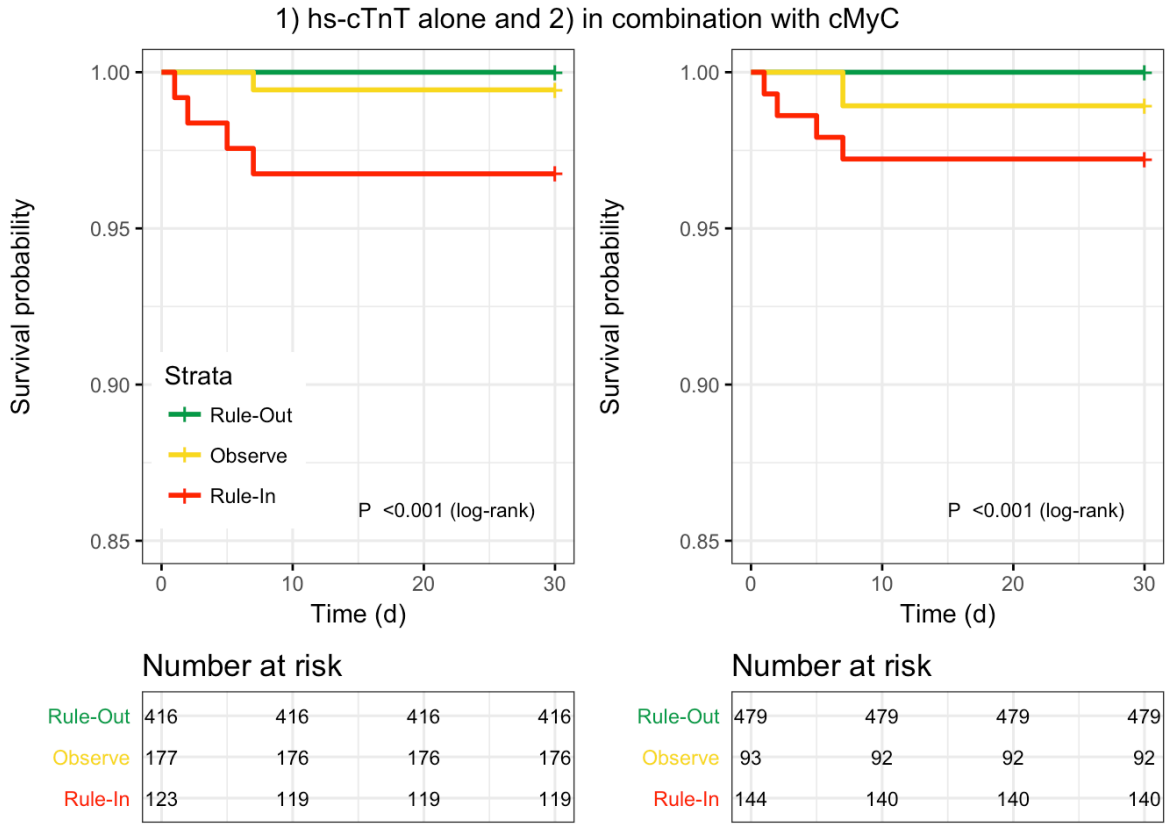


Figure 38 – Kaplan-Meier curves for endpoint cardiac death during 30-day follow-up; left – risk-stratification using hs-cTnT alone, right – risk-stratification using hs-cTnT + cMyC; p value obtained using the log-rank test; Number at risk displayed below and stratified per assigned risk-category

Variables	C-statistics	Somers D	SD
hs-cTnT	0.874	0.749	0.075
hs-cTnT + cMyC	0.868	0.737	0.060
p value	0.493		

Table 39 – Harrell's C and Somers D statistics for endpoint cardiac death during 30-day follow-up, for hs-cTnT triage alone and for the combination of hs-cTnT with cMyC; p value obtained comparing the C-statistics; SD = standard deviation of Somers D

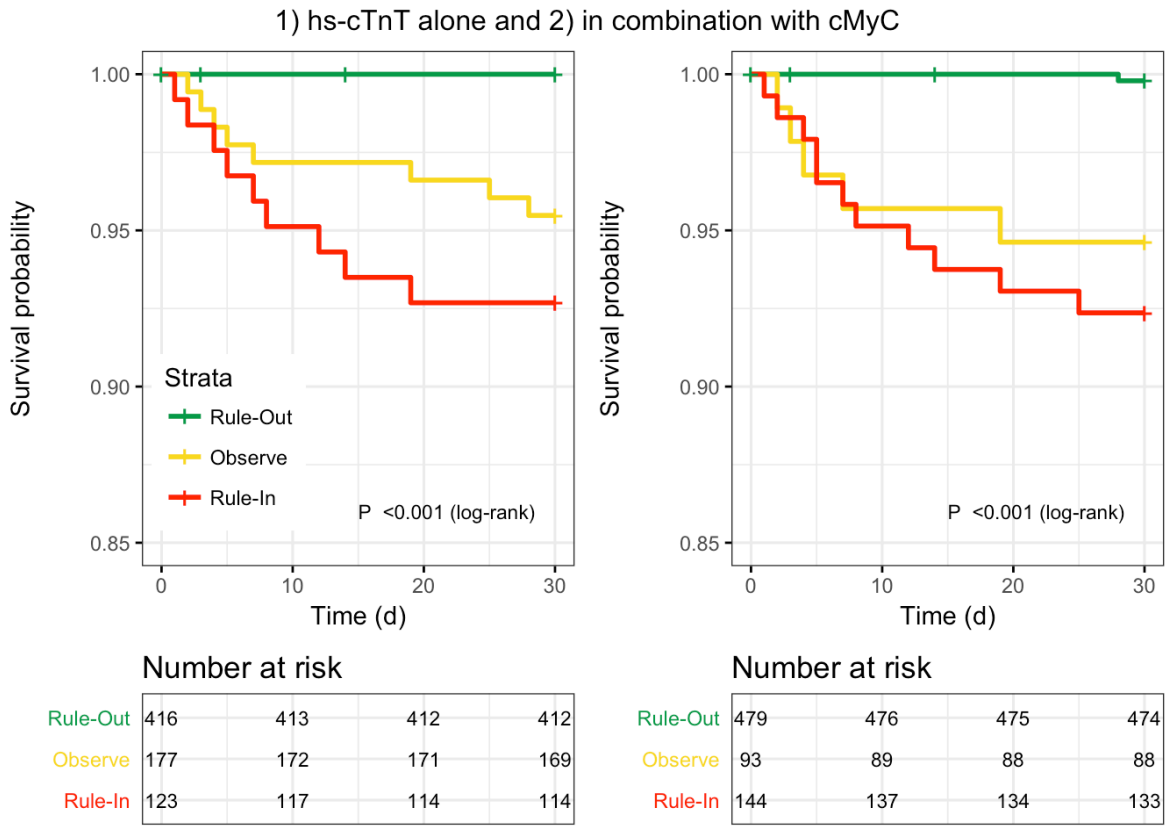


Figure 39 – Kaplan-Meier curves for endpoint death or AMI during 30-day follow-up; left – risk-stratification using hs-cTnT alone, right – risk-stratification using hs-cTnT + cMyC; p value obtained using the log-rank test; Number at risk displayed below and stratified per assigned risk-category

Variables	C-statistics	Somers D	SD
hs-cTnT	0.819	0.639	0.053
hs-cTnT + cMyC	0.821	0.642	0.069
p value	0.942		

Table 40 – Harrell's C and Somers D statistics for endpoint death or AMI during 30-day follow-up, for hs-cTnT triage alone and for the combination of hs-cTnT with cMyC; p value obtained comparing the C-statistics; SD = standard deviation of Somers D

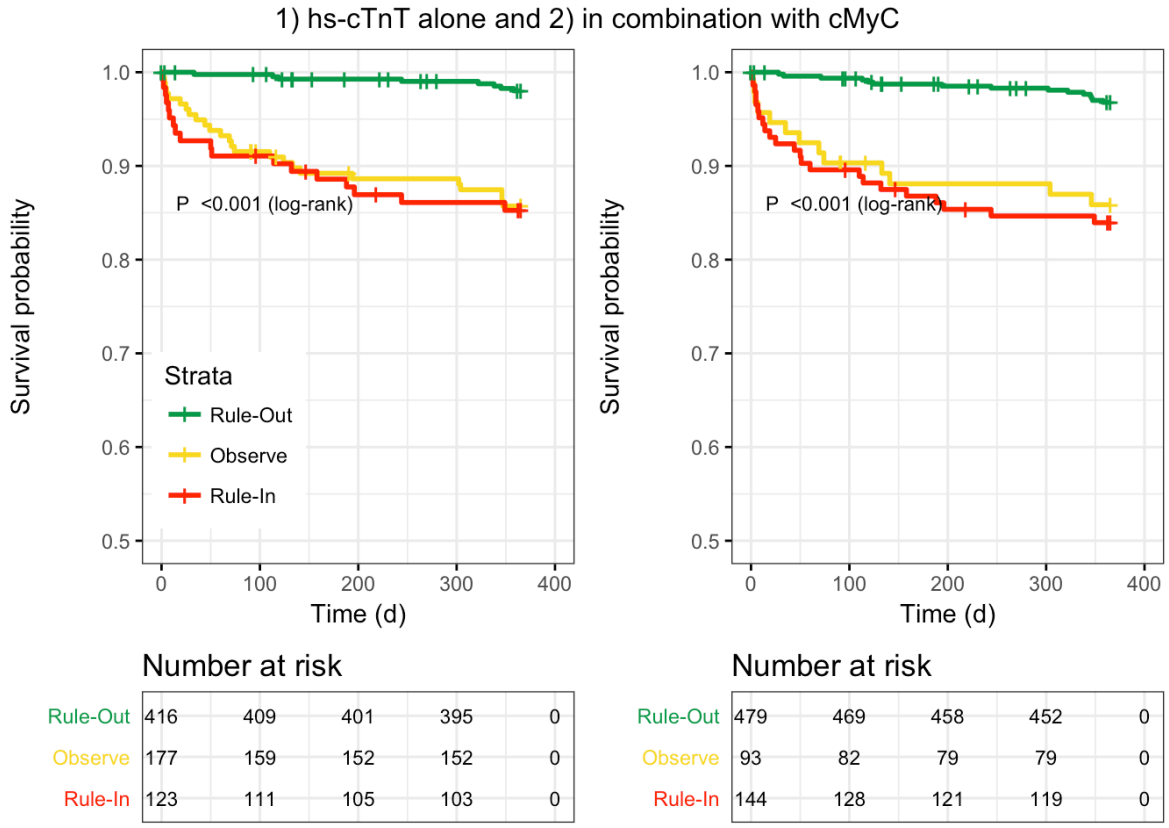


Figure 40 – Kaplan-Meier curves for endpoint death or AMI during 1-year follow-up; left – risk-stratification using hs-cTnT alone, right – risk-stratification using hs-cTnT + cMyC; p value obtained using the log-rank test; Number at risk displayed below and stratified per assigned risk-category

Variables	C-statistics	Somers D	SD
hs-cTnT	0.725	0.451	0.057
hs-cTnT + cMyC	0.704	0.408	0.066
p value	0.296		

Table 41 – Harrell's C and Somers D statistics for endpoint death or AMI during 1-year follow-up, for hs-cTnT triage alone and for the combination of hs-cTnT with cMyC; p value obtained comparing the C-statistics; SD = standard deviation of Somers D

Outcomes for patients assigned rule-out, observation or rule-in categories – hs-cTnI and cMyC

Harrell's C and Somers' D statistics demonstrate comparable risk prediction for cardiac death during a 30-days follow-up period: hs-cTnI 0.775 (Somers D 0.550±0.081), hs-TnI + cMyC 0.776 (Somers D 0.553±0.120, p=0.974; **Error! Reference source not found.**, **Error! Reference source not found.**). For the composite endpoint of AMI and death at 30 days, the C-statistics for hs-cTnI are 0.791 (Somers D 0.581±0.059), for hs-cTnI + cMyC 0.811 (Somers D 0.622±0.069, p=0.457; **Error! Reference source not found.**, **Error! Reference source not found.**). For the composite endpoint of AMI and death at 1 year, the C-statistics for hs-cTnI are 0.741 (Somers D 0.483±0.053), for hs-cTnI + cMyC 0.747 (Somers D 0.494±0.062, p=0.783; Figure 36, **Error! Reference source not found.**). Kaplan-Meier curves for hs-cTnI and the combination with cMyC are displayed below, including tables for the C-statistics.

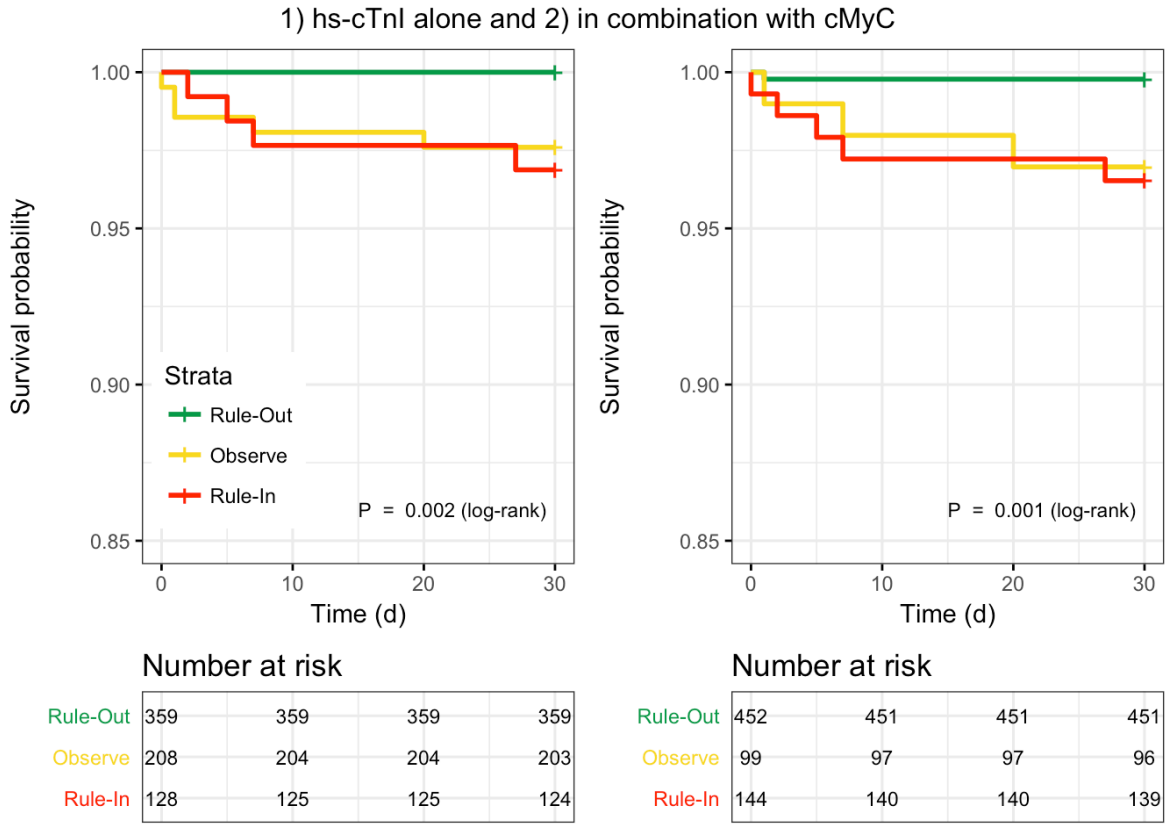


Figure 41 – Kaplan-Meier curves for endpoint cardiac death during 30-day follow-up; left – risk-stratification using hs-cTnI alone, right – risk-stratification using hs-cTnI + cMyC; p value obtained using the log-rank test; Number at risk displayed below and stratified per assigned risk-category

Variables	C-statistics	Somers D	SD
hs-cTnI	0.775	0.550	0.081
hs-cTnI + cMyC	0.776	0.553	0.12
p value	0.974		

Table 42 – Harrell's C and Somers D statistics for endpoint cardiac death during 30-day follow-up, for hs-cTnI triage alone and for the combination of hs-cTnI with cMyC; p value obtained comparing the C-statistics; SD = standard deviation of Somers D

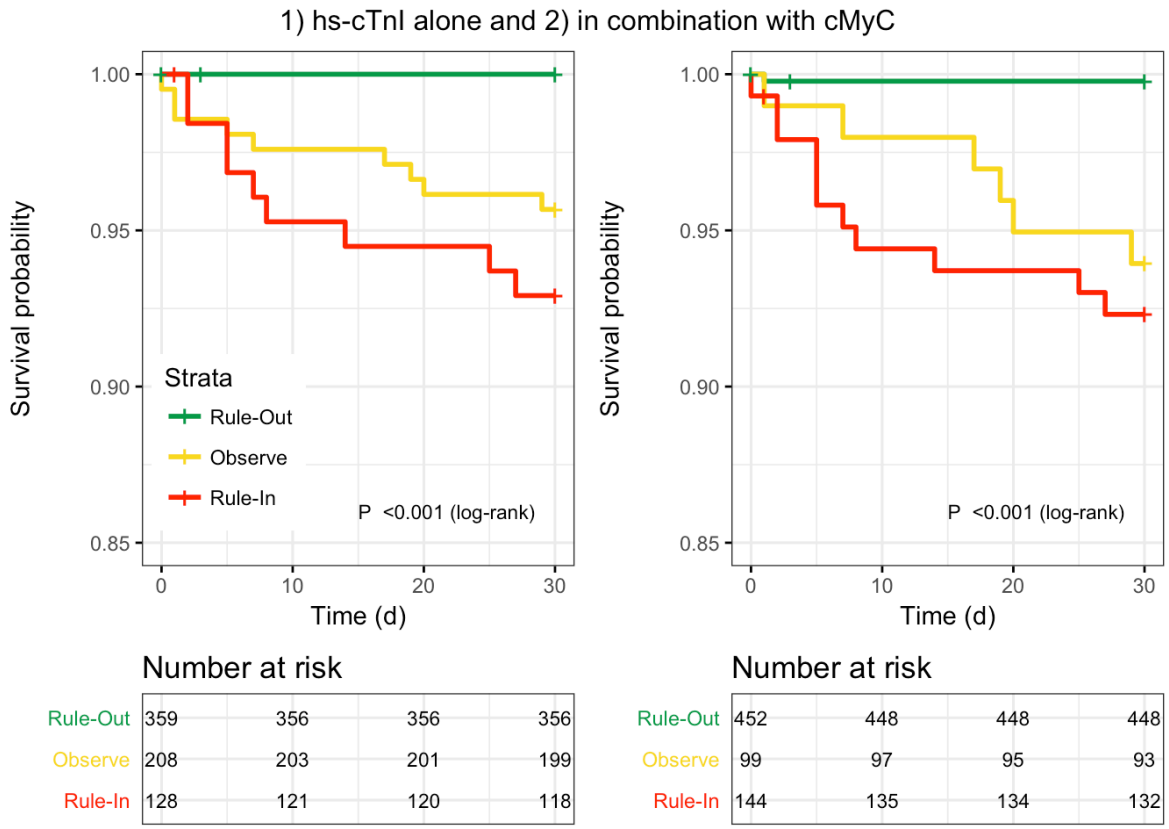


Figure 42 – Kaplan-Meier curves for endpoint death or AMI during 30-day follow-up; left – risk-stratification using hs-cTnI alone, right – risk-stratification using hs-cTnI + cMyC; p value obtained using the log-rank test; Number at risk displayed below and stratified per assigned risk-category

Variables	C-statistics	Somers D	SD
hs-cTnI	0.791	0.581	0.059
hs-cTnI + cMyC	0.811	0.622	0.069
p value	0.457	695.000	

Table 43 – Harrell's C and Somers D statistics for endpoint death or AMI during 30-day follow-up, for hs-cTnI triage alone and for the combination of hs-cTnI with cMyC; p value obtained comparing the C-statistics; SD = standard deviation of Somers D

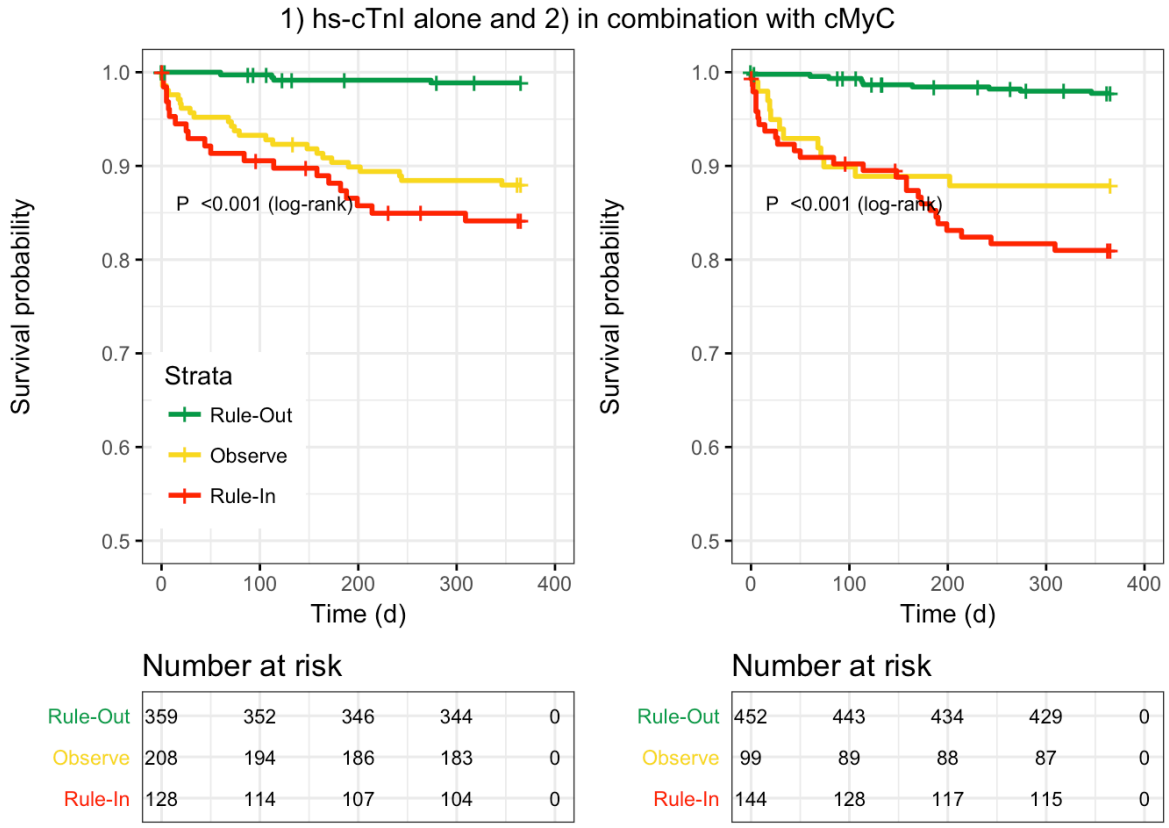


Figure 43 – Kaplan-Meier curves for endpoint death or AMI during 1-year follow-up; left – risk-stratification using hs-cTnI alone, right – risk-stratification using hs-cTnI + cMyC; p value obtained using the log-rank test; Number at risk displayed below and stratified per assigned risk-category

Variables	C-statistics	Somers D	SD
hs-cTnI	0.741	0.483	0.053
hs-cTnI + cMyC	0.747	0.494	0.062
p value	0.783		

Table 44 – Harrell's C and Somers D statistics for endpoint death or AMI during 1-year follow-up, for hs-cTnI triage alone and for the combination of hs-cTnI with cMyC; p value obtained comparing the C-statistics; SD = standard deviation of Somers D

Prelude to Chapter 7

Findings in chapters 5 & 6 investigate the potential of cMyC in the diagnosis of AMI, and for the use of cMyC as a triage-tool to accelerate rule-out and rule-in of AMI in patients presenting with chest pain. Notably, the cohort used for this analysis consists of relative late-presenters – the median chest pain time to first blood draw is 5 hours, whereas the greater advantage of cMyC appears to lie with the earliest time-points after symptom onset (evident in the sub-group analysis of early presenters with <3 hours of symptoms). To build on the experience from this subgroup analysis, and the (small) cohort tested from the HighSTEACS early-presenters (see Chapter 4), we partnered with colleagues in Denmark (Hans Erik Bøtker et al.) who provided us with access to their pre-hospital cohort – a study conducted on patients with suspected AMI, who underwent blood draws in the ambulance for the evaluation of POCT copeptin and cTnT analysers. This cohort has a markedly shorter time-to-blood-test (median 70 mins). In parallel, we worked on migrating the cMyC assay onto a POCT-platform (provided by AgPlus) and tested the hypothesis that we could reach a limit of detection on POCT that would allow rule-out with cMyC (around 10 ng/L). Preliminary results are included in the data presented below, but the analysis of cMyC in the ambulance cohort has been conducted on the established research assay (Erenna), with cut-offs calibrated to what a POCT can feasibly achieve.

The manuscript presented in Chapter 7 represents a secondary analysis of the pre-hospital study – the candidate forged the collaborations with colleagues at Aarhus University Hospital, interpreted all cMyC concentrations, wrote the analysis plan, performed the statistical analysis and wrote the manuscript. The findings are reproduced with amendments for inclusion in the thesis.

Chapter 7. Cardiac Myosin-Binding Protein C to diagnose Acute Myocardial Infarction in the pre-hospital setting – identifying the high-risk patient

Submitted to peer-reviewed journal

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Total Word count: 4,986 (text, references, tables, and figure legends)

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7.1. Abstract

Aims: Early triage is essential to improve outcome in patients with suspected Acute Myocardial Infarction (AMI). This study investigated whether cardiac myosin-binding protein C (cMyC), a novel biomarker of myocardial necrosis, can aid early diagnosis of AMI and risk stratification.

Methods: 776 patients with chest pain had blood taken by ambulance-based paramedics. cMyC and high-sensitivity cardiac troponin T (hs-cTnT) were analysed retrospectively: The area under the curve (AUC [95% confidence interval]) determined discrimination power against adjudicated AMI. All biomarker analysis has been conducted on laboratory analysers achieving high-sensitivity for the respective biomarkers, however, sensitivity & specificity were calculated including a real and realistic Limit of Detection (LoD) on a point-of-care testing (POCT) device for cTnT and cMyC, respectively.

Results: Median time from chest pain onset to blood sampling was 70 minutes. cMyC concentration in patients with AMI was significantly higher than with other diagnoses: 98 [43;855] vs 17 [9;42] ng/L. Discrimination power of cMyC was better than hs-cTnT: AUC 0.839 (0.803-0.871) vs 0.813 (0.777-0.847; p=0.005). The POCT threshold of cTnT (50 ng/L, 10-fold LoD of laboratory assay) achieved a sensitivity of 40.5% (33.3-47.6%); and cMyC (12 ng/L, 30-fold LoD of laboratory assay) achieved a sensitivity of 94.8% (91.2-97.7%). Risk prediction was superior for cMyC at the POCT-detection limit.

Conclusions: cMyC identifies a larger proportion of patients with AMI and at future risk of death than cTnT in a cohort presenting early after symptom onset. This distinction is likely related to the documented abundance and rapid release of cMyC. If used on a point-of-care platform, cMyC could significantly improve the early triage of patients with suspected AMI.

Keywords: Cardiac myosin-binding protein C; cMyC; Troponin T; myocardial infarction; pre-hospital triage; point of care testing

7.2. Translational Perspective

Early and accurate triage is essential to improve outcome in patients with suspected acute myocardial infarction. As only a small proportion of patients have diagnostic ECG changes, diagnosis has become reliant on the use of cardiac-specific biomarkers such as high-sensitivity cardiac Troponin. Due to slow release kinetics and comparably insensitive point-of-care applications of the gold-standard test, clinicians face a sensitivity-gap at the earliest time points after chest pain onset. As demonstrated in this retrospective study, cardiac myosin-binding protein C (cMyC) is a novel biomarker of cardiac injury and has superior biological characteristics that could bridge this gap.

7.2.1 Outlook

cMyC can be reliably quantified on a research platform, however it requires migration onto a random-access laboratory analyser or a point-of-care platform to facilitate prospective clinical trials. Owing to the biomarker's relative abundance and release kinetics, it is likely better suited for reliable near-patient testing and early rule-out of AMI.

7.3. Introduction

Rapid triage to the appropriate treatment is the cornerstone of improving outcome for patients presenting with suspected Acute Myocardial Infarction (AMI).[11,12,114] Physicians at Aarhus University Hospital evaluate over 6,000 pre-hospital electrocardiograms (ECG) per year: transmitted from paramedics in the field. This system allows the team in the regional tertiary-care interventional centre to select the cases for priority transfer; bypassing the nearest secondary-care facility.[175] To date, the diagnosis of AMI in the pre-hospital setting mostly relies on detecting ECG abnormalities, which identify only a minority of cases of AMI, do not allow risk-stratification[10] and are compounded by bundle branch block (BBB) and other longstanding abnormalities. Only few healthcare environments support the use of pre-hospital point-of-care testing (POCT) of biomarkers such as copeptin or cTnT – the former limited by its lack of specificity, the latter by (insufficient) sensitivity when compared to the laboratory equivalent.

A recent study investigating the precision with which emergency staff interpret ECGs (including ST-elevation) has demonstrated a mean accuracy of 81% across all study groups (such as paramedics, residents and cardiologists).[176] Notably, even amongst cardiologists, the rate of false positive ECG diagnoses exceeded 40%. Since the majority of patients with AMIs lack hallmark-features such as ST-elevation; most patients are admitted for further clinical and biochemical evaluation.[10] For patients with high-risk non ST-elevation MI (NSTEMI), the inherent diagnostic challenges lead to delayed appropriate treatment and may be associated with worse outcomes: In a recent study, the endpoint committee re-adjudicated 9-14% of NSTEMI patients as STEMI, challenging the perception that ECG-based triage by a hospital physician is sufficient to identify all high-risk patients.[177]

The team in Denmark have previously studied the performance of cTnT and Copeptin point-of-care testing (POCT) devices to aid triage in the pre-hospital setting. Both approaches have faced challenges. Whilst cardiac-specific[142], the cTnT POCT assay has a Limit of Quantification (LoQ) of 50 ng/L, with a 99th centile, defined by laboratory platforms, of 14 ng/L. Copeptin, on the other hand, is released early after acute illness, but low specificity limits its use in guiding patients towards regional interventional cardiology centres.[38]

This study investigated whether cardiac myosin-binding protein C (cMyC), a novel biomarker of myocardial necrosis, can aid the early diagnosis of AMI and identify patients at high risk of death. cMyC is a more abundant analyte than cardiac Troponins (cTn)[122,126], this translates into an enhanced early rule-in/rule-out of myocardial infarction in the setting of the emergency room. In smaller studies investigating patients early after chest pain onset or timed cardiac injury, cMyC rises more rapidly than cTn[50,123] – at equal, absolute tissue-specificity. In combination, these features make cMyC an attractive biomarker for POCT. The aim of this study was to investigate the diagnostic and prognostic properties of cMyC in comparison with high-sensitivity cardiac troponin T in the prehospital setting. Further, we examined the performance of cMyC modelled with cut-off concentration thresholds calibrated to the capabilities of the best cTnT point-of-care platform and likely to be feasible based on preliminary data.

7.4. Methods

7.4.1 Study design and population

In an observational, prospective, quality-control study, paramedics routinely performed point-of-care cTnT measurements in patients with suspected AMI.[178] The point-of-care cTnT measurements were performed in 25 ambulances in the eastern part of the Central Denmark

Region with a population of approximately 600,000 inhabitants from 26 May 2010 to 16 May 2011. Each patient in whom the standard operating procedure (SOP) instructed the recording of a prehospital ECG qualified for blood testing. The SOP criteria included ongoing or prolonged periods of chest discomfort within the past 12 hours, acute dyspnoea in the absence of known pulmonary disease, or clinical suspicion of AMI. The ECG was transmitted to the invasive cardiology centre at Aarhus University Hospital, Denmark, and interpreted by the cardiologist on call. Subsequently, a telephone interview was conducted with the patient. Thereafter, a tentative cardiac or a non-cardiac diagnosis was established and the patient underwent triage to either the PCI centre or a local hospital for further assessment.[175]

Following point-of-care cTnT analysis, the paramedics saved the remaining blood sample obtained in the ambulance. A participant flow-chart in shown in Figure 47.

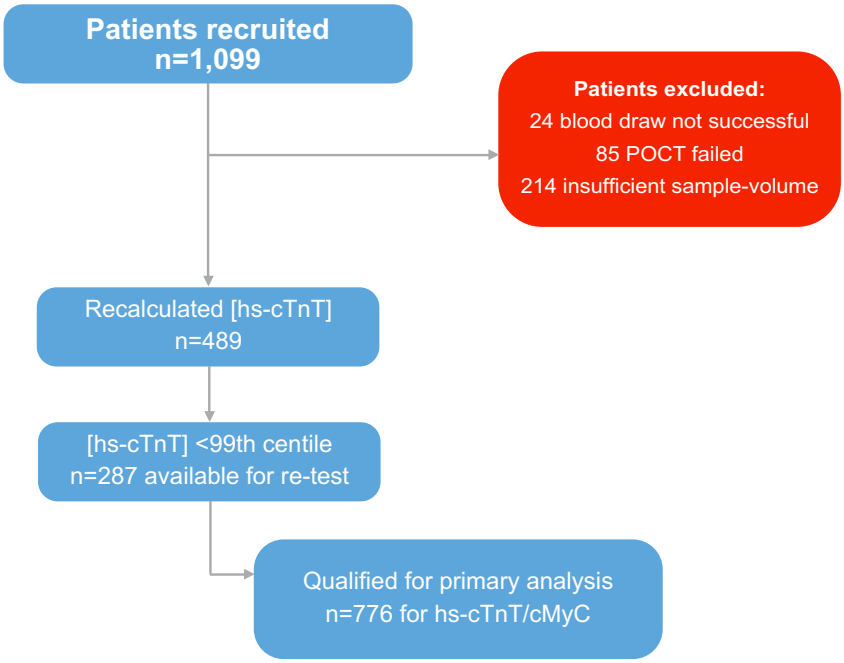

Figure 44 – Study flow diagram demonstrating original recruitment, excluded patients, and samples with recalculated hs-cTnT values, as well as re-analysed hs-cTnT concentrations

7.4.2 Sample storage and analysis

The sample was initially stored at 4°C in the ambulance and later stored in refrigerators at Aarhus University Hospital. Laboratory personnel collected the blood samples from the refrigerators periodically at intervals of a maximum of 12h, centrifuged the samples, and stored the plasma at -80°C. The Central Denmark Region Committees on Biomedical Research Ethics reviewed the protocol and approved the study as a biological registry study. Handling of patient data and storage of the blood samples were reported to the Danish Data Protection agency. Clinical data were reviewed with permission from the Danish National Board of Health. Both high-sensitivity assays, hs-cTnT and cMyC, were performed using laboratory analysers on stored plasma samples. The POCT cTn readings are not included in our analysis.

cMyC was measured in a secondary analysis using the previously established high-sensitivity assay on the Erenna platform and was performed by Millipore Sigma (Hayward, California) on a fee-per-sample basis.[84] All samples with sufficient remaining volume available at time of analysis (mid 2016) qualified for inclusion in this study. The assay has a lower Limit of Detection (LoD) of 0.4 ng/L and a lower Limit of Quantification (LoQ) of 1.2 ng/L with a ≤20% coefficient of variation at LoQ, and ≤10% CV at 99th centile. The estimated 99th percentile cut-off point (URL) determined previously is 87 ng/L.[84] The precision profile is displayed below (Figure 45, Table 45) and remains ≤10% above 4.6 ng/L. We have recently contracted a POCT diagnostics device manufacturer to migrate cMyC onto their platform. As demonstrated in Figure 46, our proposed threshold of 10 ng/L is realistic with a CV ≤10%.

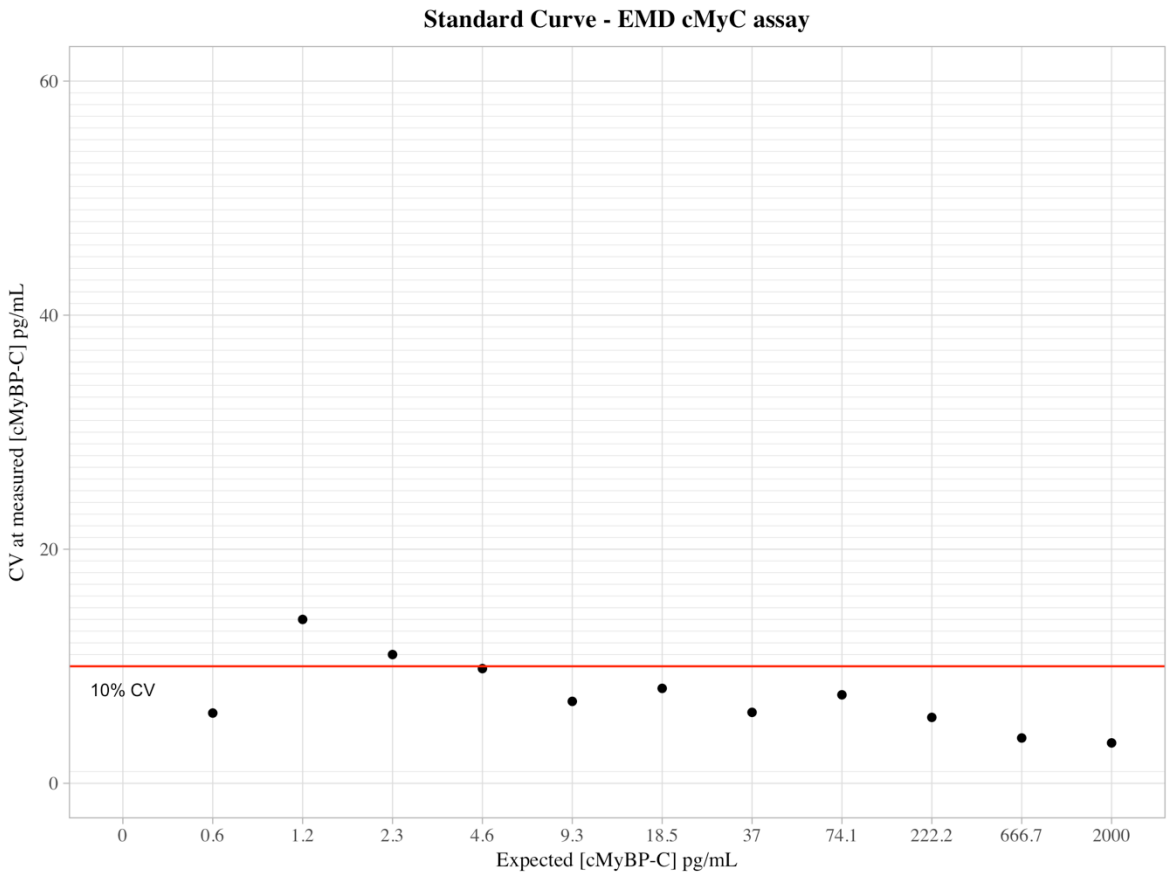

Figure 45 – Coefficient of Variation (CV) of cMyC assay as performed on EMD Erenna platform, across the standard curve

Expected (pg/mL)	Mean (pg/mL)	SD	CV (%)
0	0	0.02	0
0.6	1	0.06	6
1.2	1	0.15	15
2.3	2	0.22	11
4.6	5	0.4	8
9.3	9	0.76	8.44
18.5	19	1.2	6.32
37	35	2.04	5.83
74.1	71	5.03	7.08
222.2	236	12.92	5.47
666.7	703	33.3	4.74
2000	1998	67.87	3.4

Table 45 – Precision profile across standard curve; SD = Standard Deviation; CV = Coefficient of Variation

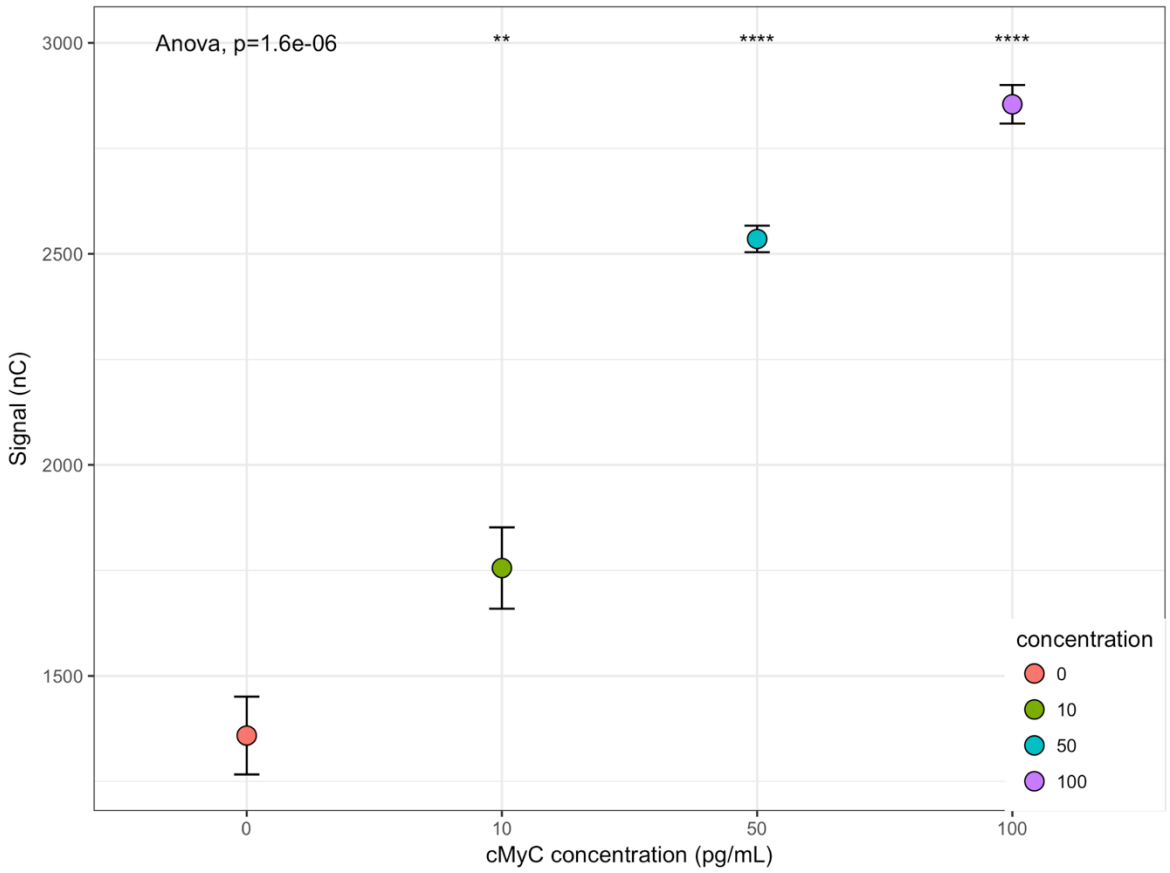

Figure 46 – Point-of-Care Testing for cMyC – preliminary results; signal differentiation has been achieved for 10, 50 and 100 pg/mL of recombinant cMyC (C0C2 region). A combination of our antibodies 235-3H8 and 259-1A4 were used on para-magnetic and metal nano-particles (AgC and MgC) to achieve the signal (nanocoulomb) as demonstrated. Signal obtained for AgC (235-3H8) against MgC (259-1A4) for varying concentrations of C0C2 analyte. Points represent mean concentration, error bars the standard error of the mean. Significance tests have been performed comparing all groups (Anova, as printed) and as unpaired T-test against concentration 0: **: $p \leq 0.01$; ****: $p \leq 0.0001$; CV: 10% at 10 pg/mL; 2% at 50 pg/mL, 3% at 100 pg/mL

For hs-cTnT, the samples were thawed and analysed as one batch in a "thaw-freeze" cycle at the central laboratory of Aarhus University Hospital, using the high-sensitivity cardiac Troponin T assay (Roche Diagnostics GmbH, Mannheim, Germany). The assay has a LoD of 5 ng/L, the lowest concentration with a coefficient of variation below 10% of 13 ng/L, and a 99th percentile URL of 14 ng/L.[95] Roche Diagnostics has previously released a technical

bulletin regarding a calibration issue affecting all lots used in this study and for routine hs-cTnT measurements made during hospital admission.[134] The manufacturer recommended a method for recalculating the reported values using combined calibration information, reagent lot number information and instrument details if the original signal data was not available.[179] Initially, most hs-cTnT values presented in this study were recalculated (n=489). The hs-cTnT recovery rate and the 99th centile comply with those found in the original studies.[95,134,179] Where available, hs-cTnT samples below the 99th centile were subsequently re-analysed using reagent lots unaffected by the calibration issue to avoid ambiguities due to recalculation (n=287).

7.4.3 Data sources

The cardiologist on call used a web-based telemedicine database to record clinical, baseline demographic and timing data, as well as the tentative diagnosis, ECG changes and triage decision. Timings were obtained from the Central Denmark Region's Prehospital Emergency Medical Services. Clinical details and demographic data were acquired using hard copies of patient files and from the National Patient Registry. Symptom duration was calculated using the difference between recorded symptom onset to prehospital blood sampling time point. Follow-up data to assess survival was obtained from The Danish Civil Registration System. electrocardiogram recorded. The study was reviewed by the Regional Ethical Committee and accepted as a quality control study. Oral informed consent for participation in the study was obtained in the ambulance. The study was approved by the Danish Data Protection Agency and the Danish National Board of Health.

7.4.4 Adjudicated final diagnosis

As previously described, all admissions were reviewed by an endpoint committee for adjudication of the final diagnosis.[178] This was performed according to the 1st Universal

Definition of MI.[127] For the diagnosis of myocardial damage, the hs-cTnT URL was used. hs-cTnT values obtained from prehospital samples were not disclosed or used in clinical decision making, nor used in the gold-standard adjudication – only the clinically used samples (from first contact in hospital) were used for adjudication of events. The endpoint committee had access to all patient file material including the discharge file, with the diagnoses determined by the clinicians. AMI patients were classified as ST-elevation Myocardial Infarction (STEMI) or Non-ST-elevation Myocardial Infarction (NSTEMI); unstable angina (UA) was diagnosed in patients with a significant episode of chest pain thought to be of ischemic origin who did not fulfil AMI criteria.

7.4.5 Diagnostic proportions of hs-cTnT and cMyC

Classification power of both biomarkers was assessed by calculating sensitivity, negative predictive value (NPV), specificity and positive predictive value (PPV) for each cut-off threshold. The 99th centile of hs-cTnT is 14 ng/L, and the currently available POCT platform (Roche Cobas h323 handheld instrument) can detect a laboratory-equivalent value of 50 ng/L (POCT LoD, correct at date of submission) – about 3-fold the LoQ or 10-fold the LoD of the laboratory assay.[36] The result is reported as 'negative' <50 ng/L, 'positive' at 50-100 ng/L, and quantitatively positive with a numerical value >100 ng/L. Assuming a conservative but similar signal-loss for cMyC if migrated from the current laboratory platform to POCT, we defined a LoD of cMyC POCT at 30-fold LoD (or 10-fold LoQ; in line with results from feasibility testing, see below). Using established hs-cTnT and realistic cMyC cut-off thresholds, we used 1,000 bootstrap replicates to determine the classification power for each biomarker with 95% confidence intervals (95% CI).

7.4.6 Statistical analysis

All data are expressed as medians [1st quartile; 3rd quartile] or means (standard deviation) for continuous variables (compared with t-test or ANOVA for continuous normal distributed variables, and Kruskal-Wallis test if continuous non-normal distributed); categorical variables are expressed as absolute and relative frequencies (compared with Pearson chi-square). Hypothesis testing was two-tailed and p values <0.05 were considered statistically significant. Where bootstrap techniques were used, the calculations were performed using 1,000 stratified replicates.

Diagnostic accuracy was quantified by the area under the receiver-operating curve (AUC (95% confidence interval)) against adjudicated AMI. Bootstrapping was used to calculate Confidence Intervals (CI), compare the AUC between biomarkers and calculate the classification function. Logistic regression was used to combine cMyC with hs-cTnT values for the assessment of an incremental value using the two biomarker concentrations at presentation. Correlation was assessed with Spearman's rho (r_s) and adjusted R^2 by fitting a linear regression model. Prognostic performance was assessed as follows: We calculated 1) Harrell's C statistics[131] for each biomarker for cumulative long-term mortality, 2) an adjusted multivariable Cox proportional hazards model and 3) displayed Kaplan-Meier survival curves. The Cox models were tested for violation of the proportional hazards assumption by calculating correlation coefficients between transformed survival time and the scaled Schoenfeld residuals and testing the former with chi-square comparisons. All available variables were tested in a univariate regression model; significant variables (pre-defined as Wald test p<0.1) were selected for the final Cox multivariate regression model. The biomarkers were entered log-transformed.

All statistical analyses were performed using R, version 3.3.0 GUI 1.68 (The R Foundation for Statistical Computing), including packages ggplot2, RMarkdown, the tidyverse, survival, survminer and pROC.

7.5. Results

7.5.1 Baseline characteristics

A total of 776 patients were recruited during the study period. Median age was 68 years [58; 78], 303 patients (39%) were women, and 232 (30%) had a prior history of myocardial infarction (Table 46). Sixty-six patients (9%) had a final diagnosis of STEMI, 107 (14%) NSTEMI. Median time since onset of chest pain was 70 minutes [35; 173]. There was considerable discrepancy between telemedicine-triage and final diagnosis: 107 patients (14%) presented with BBB on ECG; only 59% of patients with a final adjudicated diagnosis of STEMI had clear ST-elevation identified during telemedicine assessment. Sensitivity for NSTEMI during telemedicine assessment was 33%.

	All	STEMI	NSTEMI	UA	p	N
	N=776	N=66	N=107	N=27		
Gender male	473 (61%)	54 (82%)	75 (70%)	24 (89%)	<0.001	776
Age (years)	68 [58;78]	66 [58;75]	74 [65;81]	63 [53;68]	<0.001	776
Hypertension	439 (57%)	31 (47%)	71 (66%)	17 (63%)	0.062	776
Hyperlipidemia	622 (80%)	49 (74%)	93 (87%)	24 (89%)	0.103	776
Diabetes mellitus	147 (19%)	4 (6%)	19 (18%)	6 (22%)	0.04	776
Current smoking	230 (30%)	30 (45%)	35 (33%)	10 (37%)	0.003	776
History of smoking	217 (28%)	16 (24%)	34 (32%)	8 (30%)	0.264	776
Previous myocardial infarction	232 (30%)	11 (17%)	47 (44%)	13 (48%)	<0.001	776
Previous percutaneous intervention	200 (26%)	10 (15%)	39 (36%)	14 (52%)	<0.001	776
Systolic blood pressure (mmHg)	146 [130; 166]	141 [123; 168]	150 [132; 177]	154 [142; 169]	0.152	764
Diastolic blood pressure (mmHg)	87 [75; 99]	84 [72; 105]	91 [75; 104]	90 [84; 99]	0.208	764
Heart rate (beats/min)	84 [70; 100]	81 [62; 95]	88 [74; 102]	84 [70; 100]	0.084	765
eGFR	71 [56;86]	66 [61; 84]	70 [56; 82]	77 [66; 82]	0.455	605
Time since chest pain onset (minutes)	70 [35; 173]	71 [35; 140]	73 [39; 162]	44 [27; 125]	0.48	726

Table 46 – Baseline characteristics; STEMI = ST elevation myocardial infarction; NSTEMI = Non-ST elevation myocardial infarction; UA = Unstable Angina; eGFR = Estimated glomerular filtration rate, ml/min/1.73m2 (estimated using the Modification of Diet in Renal Disease (MDRD) formula)

7.5.2 Distribution of biomarker concentrations

All blood samples were obtained in the ambulance but measured in a laboratory. In ambulance concentrations of cMyC at 0h were significantly higher in patients with AMI (median 98 ng/L [43; 855]) than in patients with other diagnoses (17 ng/L [9; 42], p<0.001). Median

concentrations of cMyC were 88 ng/L [42; 253] for NSTEMI, 306 ng/L [49; 1706] for STEMI, 19 ng/L [11; 25] for UA. The corresponding concentrations for hs-cTnT, were 33 ng/L [18; 72], 58 ng/L [15; 295] and 9 ng/L [7; 14], respectively (Figure 47, Table 47). An overview of the distribution of all markers measured is shown in table 2. Overall, when comparing blood concentrations of biomarkers to assay-specifics (LoQ, LoD), cMyC levels were higher than those of hs-cTnT in all diagnostic categories.

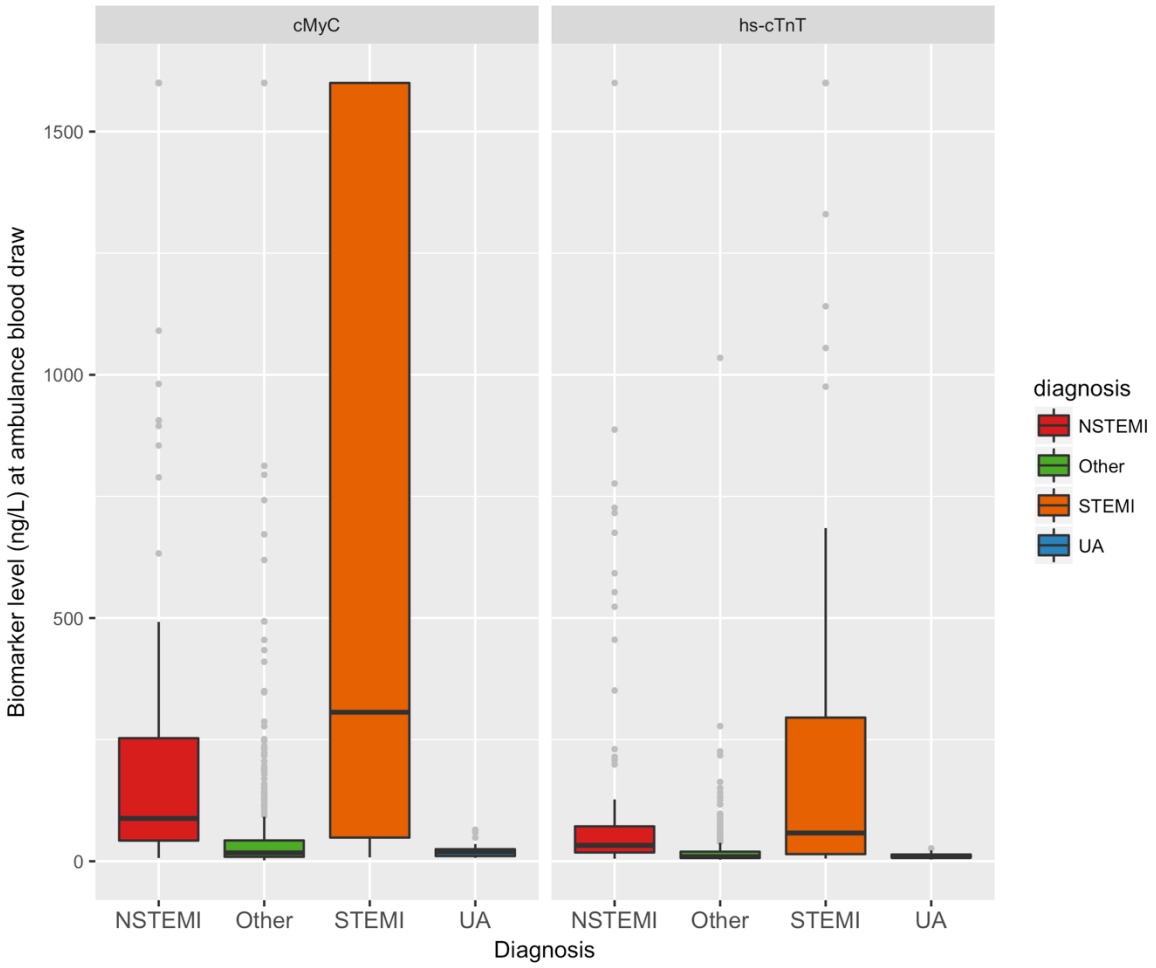

Figure 47 – Distribution of cMyC and hs-cTnT concentrations in samples obtained in the ambulance, based on adjudicated final diagnosis. Boxes represent interquartile ranges; whiskers extend to 1.5 * IQR from the hinges; light grey bullets are outliers. NSTEMI = Non-ST elevation Myocardial Infarction; STEMI = ST-elevation Myocardial Infarction; UA = Unstable Angina

	Minimum	1st Q	Median	Mean	3rd Q	Maximum
cMyC (ambulance, ng/L)						
NSTEMI	6.6	42.4	88.0	554.1	253.1	11430
Other	1.9	9.1	17.4	62.8	42.7	6362
STEMI	7.9	48.6	306.3	1525.0	1706.0	19720
UA	6.8	10.7	19.4	21.6	24.8	64.72
hs-cTnT (ambulance, ng/L)						
NSTEMI	5.2	18.0	32.6	122.3	71.8	2493.9
Other	3.0	6.7	9.6	20.2	19.7	1035.0
STEMI	5.5	14.7	58.1	375.6	295.3	4023.7
UA	3.4	7.3	9.3	11.3	13.8	26.5

Table 47 – Distribution of biomarker concentration by final adjudicated diagnostic category; STEMI = ST-elevation Myocardial Infarction; NSTEMI = Non ST-elevation Myocardial Infarction; UA = Unstable Angina

7.5.3 Discrimination power

In blood draws performed in the ambulance, the discrimination power against ultimate diagnosis (AMI) as quantified by the AUC was higher for cMyC than for hs-cTnT: 0.839 (95% CI, 0.803-0.871) vs 0.813 (0.777-0.847; p=0.005 for direct comparison; Figure 48, Table 48). The discrimination power of cMyC for the individual diagnoses was: AUC 0.816 (0.761-0.866) for STEMI, AUC 0.787 (0.741-0.829) for NSTEMI, AUC 0.599 (0.531-0.67) for UA.

The discrimination power for hs-cTnT for the individual diagnoses was: AUC 0.766 (0.701-0.828; p<0.001 for direct comparison to cMyC) for STEMI, AUC 0.781 (0.737-0.820; p=0.595) for NSTEMI, AUC 0.608 (0.529-0.692; p=0.711) for UA.

The combination of both markers provided incremental value for STEMI (AUC 0.780; 0.719-0.84; p<0.001) and NSTEMI (0.786; 0.745-0.824; p=0.037) compared to using hs-cTnT alone.

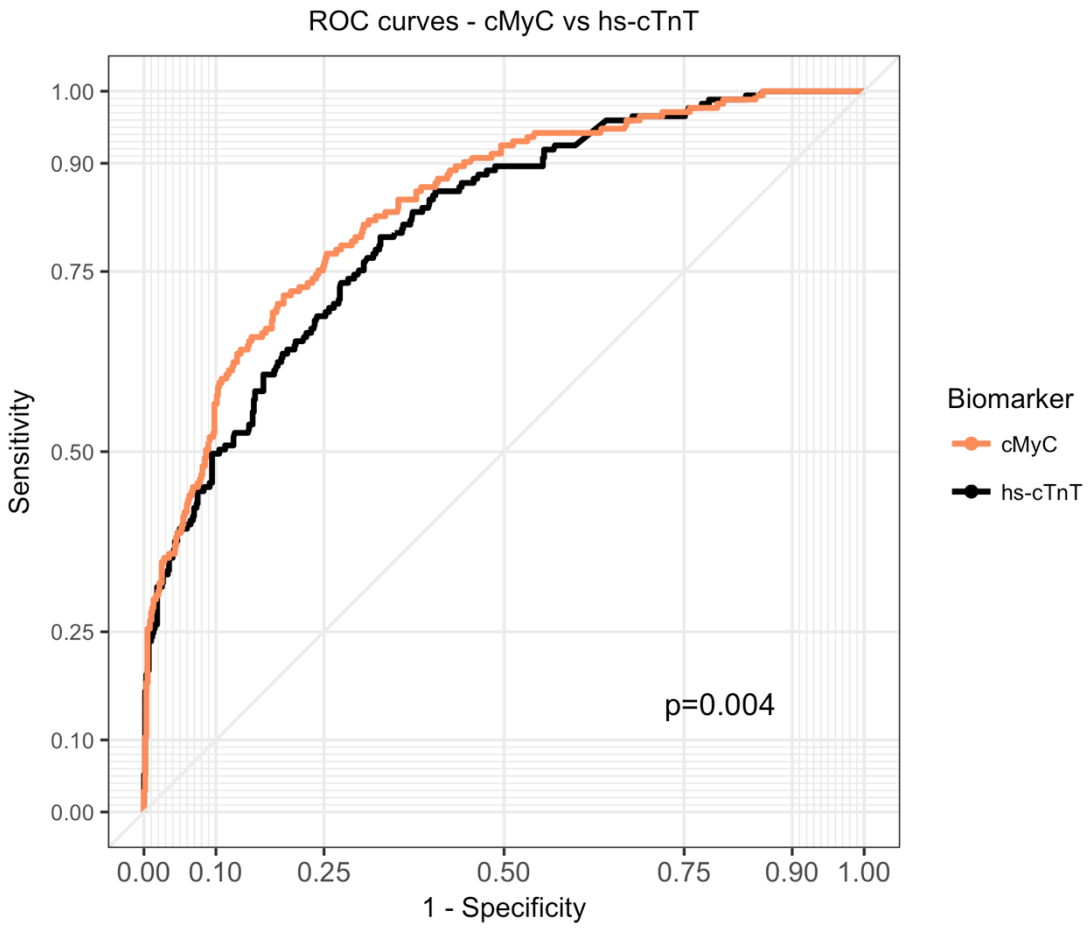

Figure 48 – Receiver-operating characteristics (ROC) curves for cMyC (ambulance) and hs-cTnT (ambulance) for the diagnosis of acute myocardial infarction. The AUC for cMyC was 0.839 (95% CI, 0.804-0.87), for hs-cTnT 0.813 (0.777-0.847).

Outcome	AUC	95% CI	AUC	95% CI	Cases	Controls	p.value
Biomarker	cMyC		hs-cTnT				
AMI	0.839	0.805-0.873	0.813	0.777-0.847	173	603	0.005
STEMI	0.816	0.759-0.865	0.766	0.695-0.831	66	710	<0.001
NSTEMI	0.787	0.742-0.828	0.781	0.737-0.821	107	669	0.599
UA	0.599	0.524-0.670	0.608	0.531-0.690	27	749	0.715
Biomarker	cMyC + hs-cTnT		hs-cTnT				
AMI	0.822	0.791-0.856	0.813	0.775-0.847	173	603	<0.001
STEMI	0.780	0.716-0.836	0.766	0.699-0.834	66	710	<0.001
NSTEMI	0.786	0.744-0.830	0.781	0.738-0.823	107	669	0.041
UA	0.613	0.535-0.695	0.608	0.530-0.693	27	749	0.377

Table 48 – Area under the Receiver-operating Characteristics Curve for cMyC and hs-cTnT; AMI = Acute Myocardial Infarction; STEMI = ST-elevation Myocardial Infarction; NSTEMI = Non ST-elevation Myocardial Infarction; UA = Unstable Angina; AUC = Area under the Curve; CI = Confidence Interval

7.5.4 Correlation

The biomarkers correlated positively across all patient groups (R^2=0.730, r_s=0.855) and for all patients with AMI (R^2=0.699, r_s=0.836).

Figure 49 and Table 49 show the relationships between the biomarkers for each individual final adjudicated diagnosis. Serum concentrations of cMyC and hs-cTnT are positively correlated throughout, with strongest correlations observed in the non-cardiac and NSTEMI groups.

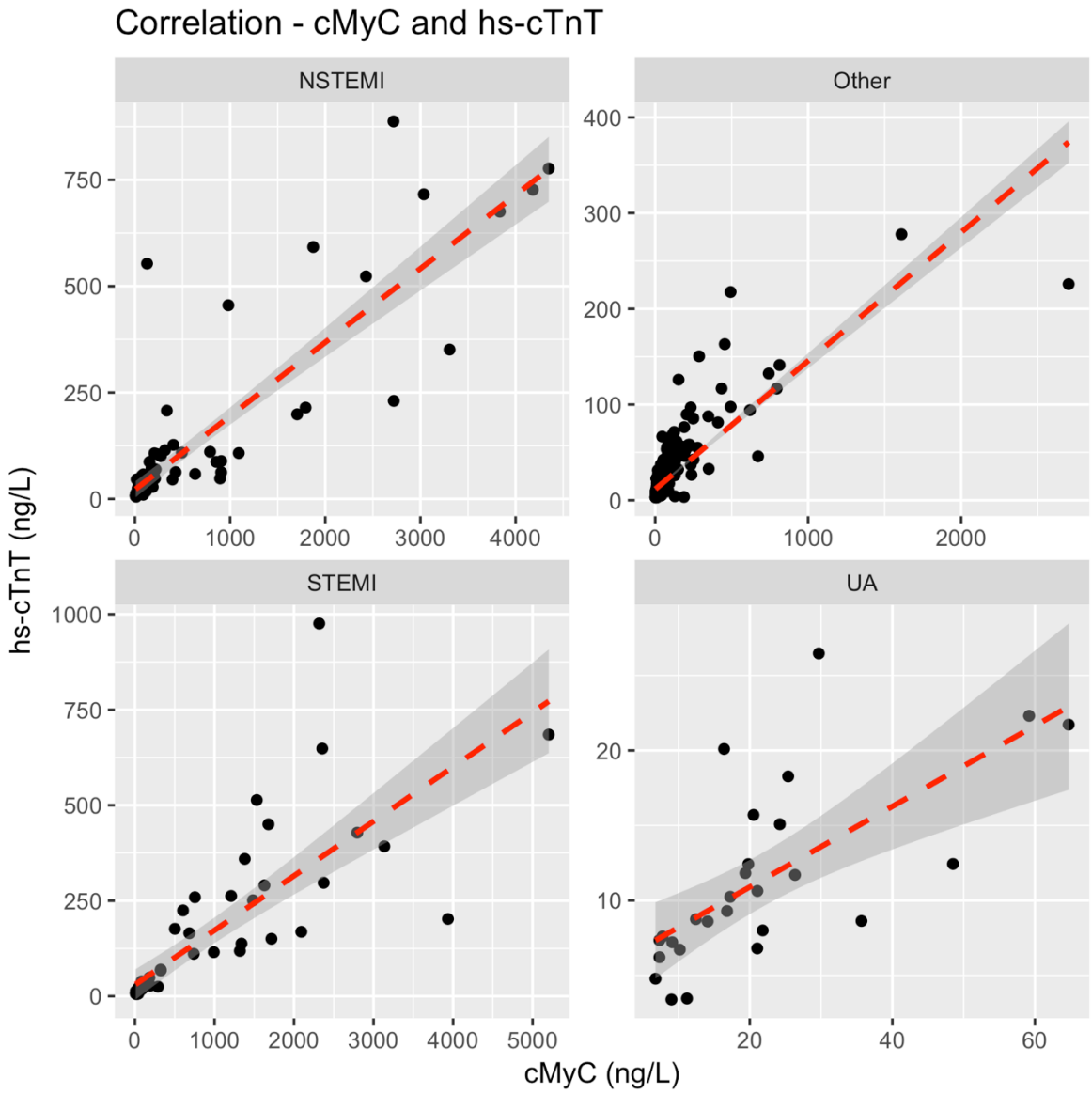

Figure 49 – Scatter plots outlining correlation between cMyC and hs-cTnT concentrations (ng/L both) in samples obtained in the ambulance for each diagnostic group. Light grey shading depicts the boundaries of the 95% confidence intervals, line of best fit indicated in red. NSTEMI = Non-ST elevation Myocardial Infarction; STEMI = ST-elevation Myocardial Infarction; UA = Unstable Angina

Diagnosis	R^2	f	Spearman's rho	n
NSTEMI	0.897	913.56	0.947	107
Other	0.897	5000.05	0.947	576
STEMI	0.631	109.61	0.795	66
UAP	0.453	20.73	0.673	27

Table 49 – Correlations between cMyC and hs-cTnT concentrations by diagnostic group. R^2 = correlation coefficient

7.5.5 Diagnostic proportions of hs-cTnT and cMyC

Initially we compared the 99th centile of hs-cTnT (14 ng/L) with the 99th centile of cMyC (87 ng/L). hs-cTnT achieved a negative predictive value (NPV) of 92.1% (89.3-94.4%), and positive predictive value (PPV) of 39.6% (34.6-44.9%), vs cMyC of 87.5% (84.8-90%) and 61.6% (54.2-69.3%), respectively. hs-cTnT at a threshold calibrated to the performance of the POCT platform (LoD 50 ng/L) achieved NPV 84.6% (81.7-87.1%) and PPV 63.9% (54.5-72.3%); a POCT device modelled for cMyC (LoD 12 ng/L) achieves 96% (93.1-98.2%) and 29.4% (25.7-33.6%) – see Table 50.

Number of cases	**776**	
AMI	173	
Biomarker	hs-cTnT (LoQ 13 ng/L)	
Threshold	99th centile	POCT (10x LoD)
Value	14 ng/L	50 ng/L
Sensitivity	80.5% (95% CI, 74.3-86.1%)	40.5% (95% CI, 33.3-47.6%)
Specificity	65% (95% CI, 61.4-68.8%)	93.4% (95% CI, 91.3-95.3%)
NPV	92.1% (95% CI, 89.3-94.4%)	84.6% (95% CI, 81.7-87.1%)
PPV	39.6% (95% CI, 34.6-44.9%)	63.9% (95% CI, 54.5-72.3%)
Biomarker	cMyC (LoQ 1.2 ng/L)	
Threshold	99th centile	POCT (estimated 30x LoD, 10x LoQ)
Value	87 ng/L	12 ng/L
Sensitivity	54.9% (95% CI, 47.2-62.2%)	94.8% (95% CI, 91.2-97.7%)
Specificity	90.2% (95% CI, 88.1-92.6%)	35.1% (95% CI, 31.5-39.2%)
NPV	87.5% (95% CI, 84.8-90%)	96% (95% CI, 93.1-98.2%)
PPV	61.6% (95% CI, 54.2-69.3%)	29.4% (95% CI, 25.7-33.6%)

Table 50 – Discriminatory power of cMyC v cTnT at 99th centile and POCT thresholds; AMI = Acute Myocardial Infarction; POCT = Point-of-Care Testing; LoQ = Limit of Quantification; LoD = Limit of Detection; NPV = Negative Predictive Value; PPV = Positive Predictive Value

The changes between early- (<60 mins of chest pain), intermediate- (60-120 mins) and late-, presenting cohorts (>120 mins) are displayed in Table 51. Notably, the sensitivity for AMI is higher with cMyC (at 12 ng/L) than at the cTnT POCT threshold (≥50 ng/L): 92.6% vs 26.9% in the very early presenters with chest pain onset <60 minutes prior to blood draw,

respectively. The sensitivity of cTnT improves in the later cohorts, however, it never reaches a sensitivity >60%. This would translate into a 15% higher detection of AMI if cMyC was to be used instead of the most accurate cTnT assay on a POCT device.

Early cohort (0-60 mins)		
Number of cases	321	
AMI	66	
	hs-cTnT (POC)	cMyC (12 ng/L)
Sensitivity	26.9% (95% CI, 16.7-37.5%)	93% (95% CI, 85.3-98.3%)
Specificity	94.2% (95% CI, 90.9-96.9%)	37.5% (95% CI, 31.8-43.9%)
NPV	83.3% (95% CI, 78.7-87.5%)	95.4% (95% CI, 90.3-98.9%)
PPV	54.5% (95% CI, 36.7-71.1%)	27.5% (95% CI, 22.3-33.7%)
Intermediate cohort (60-120 mins)		
Number of cases	156	
AMI	51	
Sensitivity	39.6% (95% CI, 25.6-53.5%)	96.2% (95% CI, 89.4-100%)
Specificity	93.4% (95% CI, 88.3-97.4%)	28.3% (95% CI, 19.6-36.5%)
NPV	76.2% (95% CI, 68.8-83.2%)	93.9% (95% CI, 83.9-100%)
PPV	74.2% (95% CI, 56.5-89.7%)	39.2% (95% CI, 31.7-47.6%)
Late cohort (>120 mins)		
Number of cases	249	
AMI	52	
Sensitivity	58.2% (95% CI, 45-71.2%)	98.1% (95% CI, 93.5-100%)
Specificity	92.5% (95% CI, 88.8-95.8%)	35.1% (95% CI, 28.6-42.1%)
NPV	89.3% (95% CI, 84.9-93.2%)	98.6% (95% CI, 95.2-100%)
PPV	67.4% (95% CI, 53.5-80%)	28.5% (95% CI, 22.5-34.9%)

Table 51 – Discriminatory power of cMyC v cTnT at POCT thresholds by symptom duration; AMI = Acute Myocardial Infarction; POCT = Point-of-Care Testing; LoQ = Limit of Quantification; LoD = Limit of Detection; NPV = Negative Predictive Value; PPV = Positive Predictive Value

7.5.6 Prognostic value of hs-cTnT and cMyC

C Statistics:

Harrell's C statistics demonstrate comparable risk prediction for subsequent death during median follow-up of 557 days [493; 618] for both markers: cMyC 0.767, hs-cTnT 0.775 (p=0.422; Table 52). Comparing C statistics for cut-offs achievable with POCT platforms, cMyC is better able to predict subsequent death than hs-cTnT: 0.762 vs 0.656 (p<0.001).

n=769	cMyC	hs-cTnT	p value*	est.cov
FU death – whole spectrum				
Harrell's C Statistic	0.767	0.775	0.422	0.000
Somers' D ± SD	0.535 ±0.047	0.550 ±0.046		
FU death – POCT				
Harrell's C Statistic	0.762	0.656	<0.001	0.000
Somers' D ± SD	0.525±0.050	0.312 ±0.054		

Table 52 – Harrell's C statistics and Somers' D for cMyC and hs-cTnT across the entire analytic bandwidth and stratified according to point-of-care testing thresholds; FU = Follow-up; POCT = Point-of-Care Testing; SD = Standard Deviation; * p for comparison of C statistics between cMyC and hs-cTnT

Cox regression models:

The final Cox regression model consisted of risk factors diabetes, prior myocardial infarction and biomarkers cMyC or hs-cTnT (no other covariates reached a significance level p<0.1 in univariate analysis). Notably, the hazard ratios for cMyC and hs-cTnT are similar when the entire spectrum of the biomarkers (available only on high-sensitivity platforms) is used. When assessing possible risk prediction in the region of POCT device capabilities, cMyC identifies a higher-risk cohort at a cut-off of 12 ng/L – HR 7.23 vs. 4.99 with hs-cTnT at 50 ng/L (Table

53). This was subsequently used to create survival curves with adjustment for the stratified Cox model (Figure 50). Separated Kaplan-Meier curves for cMyC and hs-cTnT using a 3-tiered risk stratification are displayed below (Figure 51, Figure 52).

	Beta	SE (Beta)	HR (95% CI)	Wald test (z)	p value
cMyC					
Diabetes mellitus	0.23	0.26	1.26 (0.76-2.1)	0.91	0.364
Prior AMI	0.85	0.23	2.34 (1.49-3.68)	3.67	<0.001
cMyC (log)	0.36	0.05	1.44 (1.3-1.59)	6.95	<0.001
cMyC POCT					
Diabetes mellitus	0.30	0.25	1.35 (0.82-2.22)	1.19	0.234
Prior AMI	0.82	0.23	2.25 (1.44-3.52)	3.59	<0.001
cMyC ≥12 ng/L	1.56	0.47	7.23 (2.26-23.14)	3.34	<0.001
hs-cTnT					
Diabetes mellitus	0.19	0.26	1.21 (0.73-2.00)	0.76	0.446
Prior AMI	0.90	0.23	2.46 (1.57-3.87)	4.03	<0.001
hs-cTnT (log)	0.47	0.06	1.60 (1.41-1.81)	7.26	<0.001
hs-cTnT POCT					
Diabetes mellitus	0.30	0.25	1.35 (0.82-2.22)	1.19	0.234
Prior AMI	0.96	0.23	2.61 (1.67-4.09)	4.21	<0.001
hs-cTnT >50 ng/L	1.61	0.23	4.99 (3.19-7.79)	7.07	<0.001

Table 53 – Hazard ratios for cMyC and hs-cTnT values in samples obtained in the ambulance; with statistically significant confounders based on prior univariate regression analysis

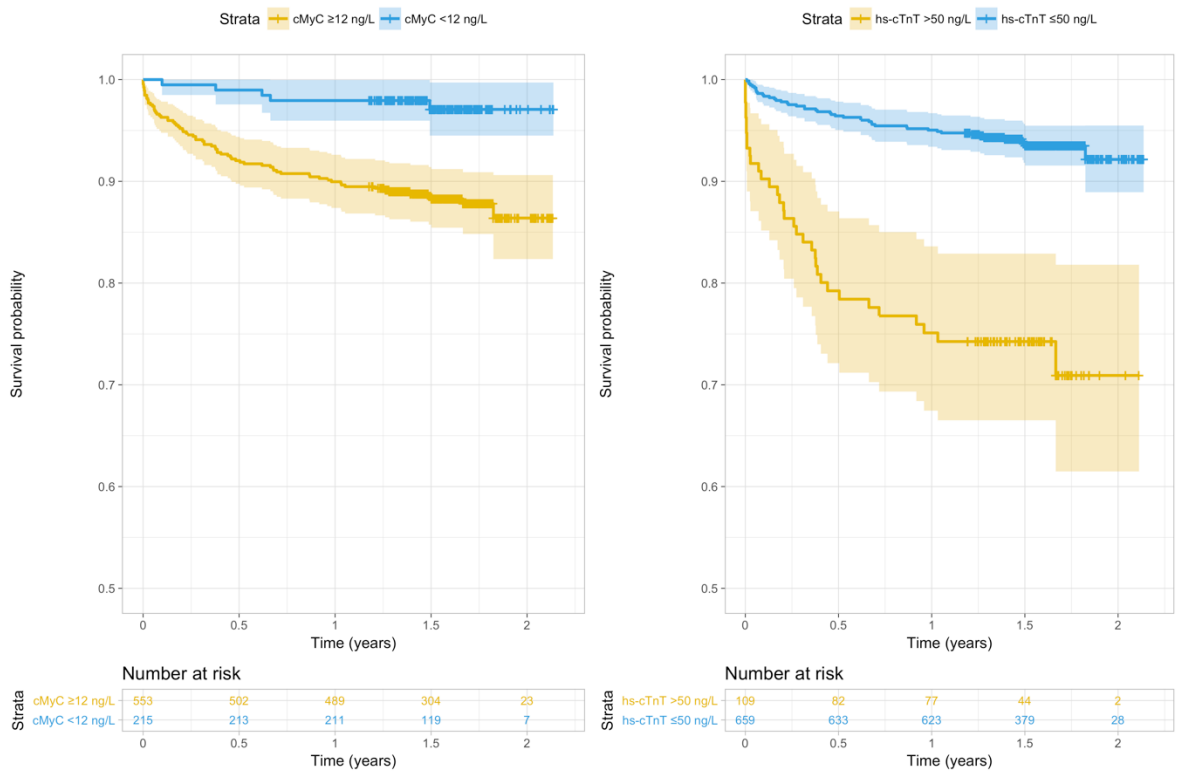

Figure 50 – Survival curves for all patients over a 2-year follow-up for cMyC (left) and hs-cTnT (right) from samples obtained in the ambulance. These are adjusted for the Cox model (using presence of baseline diabetes mellitus and prior myocardial infarction as significant covariates) and stratified for the following cut-offs: cMyC 12 ng/L; hs-cTnT 50 ng/L

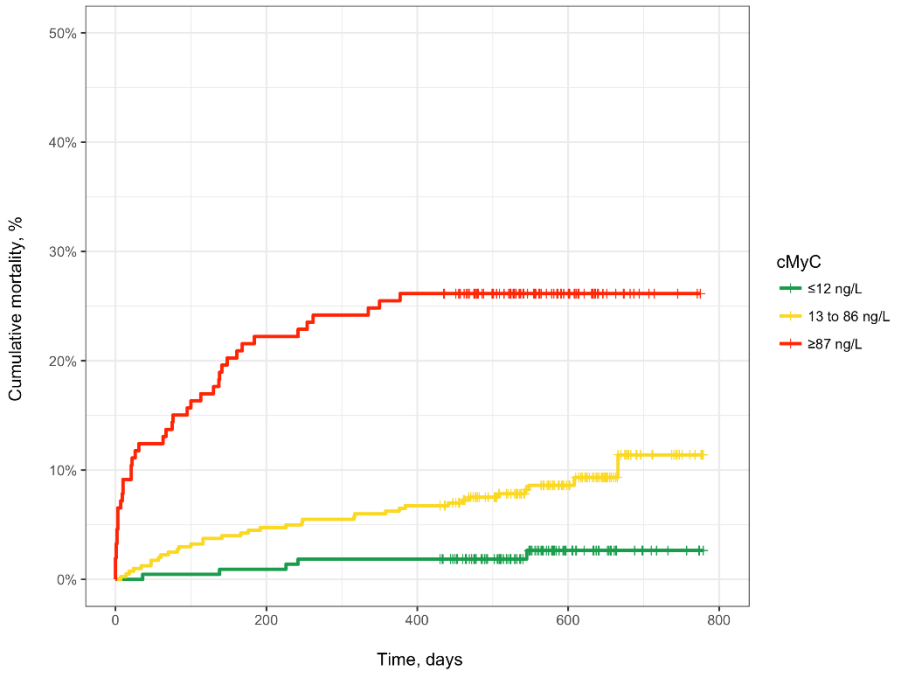

Figure 51 – Kaplan-Meier survival curves using cMyC for risk stratification (tiers: ≤12 ng/L for POCT threshold, 13-86 ng/L, ≥87 ng/L as 99th centile)

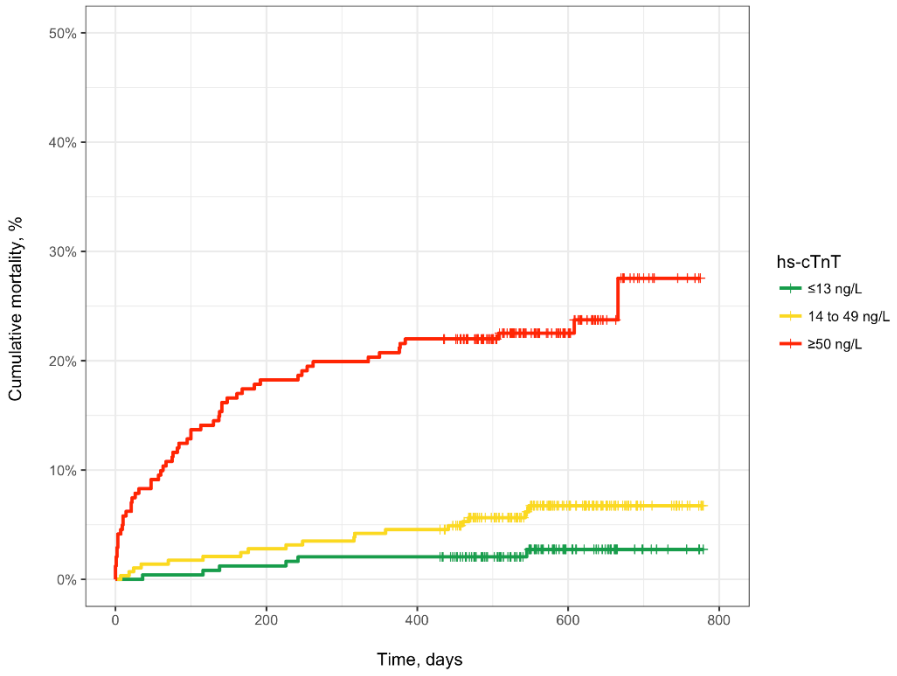

Figure 52 – Kaplan-Meier survival curves using hs-cTnT for risk stratification (tiers: ≤13 ng/L for 99th centile threshold, 14-49 ng/L, ≥50 ng/L as POCT threshold). While the highest-risk group with a cMyC level ≥87 ng/L

(99th centile, 200-fold Limit of Detection (LoD)) performs similarly to hs-cTnT ≥50 ng/L (the POCT LoD), the spectrum for precise risk-stratification is significantly broader using the novel marker.

7.6. Discussion

Cardiac myosin-binding protein C (cMyC) is a myocardial protein that is released into the circulation after injury in a similar manner to the cardiac troponins (cTn). A prior publication has suggested that the concentration of cMyC rises more rapidly than cTn based on an analysis of 26 patients with AMI, who presented to hospital within 180 mins of symptom onset.[123] This finding is in keeping with an *in vitro* analysis of human heart that shows cMyC is more abundant than cTn.[122] A recent investigation has further shown superiority in early triage of >1,900 patients presenting with chest pain and suspected AMI – particularly in subjects presenting early after symptom onset.[126] The current study extends these findings to unselected patients in the prehospital setting and shows that cMyC is better than hs-cTnT at diagnosing AMI; based on an analysis of receiver operator characteristics. Furthermore, using a conservative estimate of signal loss together with early experience of assay migration – we propose that a point-of-care test for cMyC would outperform those for cTn. Our direct observations and hypothetical models suggest that cMyC may have distinct advantages as a point-of-care biomarker for AMI.

Our results underline the potential of cMyC in the assessment of patients presenting very early after chest pain onset. The median chest pain duration before first blood draw is typically 3-5 hours in large cohort studies undertaken in the secondary-care setting.[105,165] In contrast, we studied patients with a median time of just 70 mins between symptom onset and blood draw in the ambulance. Reliably, cMyC levels were higher in patients with AMI compared to any other diagnoses. Despite a strong positive correlation between cMyC and cTnT, in keeping with

previously presented findings[126], cMyC appears to have an advantage when compared to hs-cTnT in discriminating between patient with and without AMI: ROC analysis demonstrates a higher AUC value for cMyC. This translates into higher sensitivity and NPV at concentrations of cMyC likely to be achieved on a POCT device. The real benefit of a highly-sensitive assay for an abundant marker such as cMyC would be in enabling risk-stratification at the earliest time-points possible – the first encounter between the patient and a healthcare professional. Survival analysis demonstrates that cMyC and hs-cTnT are comparable in terms of risk prediction. This is to be expected since the greater abundance of cMyC is unlikely to offer further discriminative power in the low to intermediate risk patient cohorts.

Currently, on-call physicians in Aarhus evaluate approximately 6,000 ECGs annually – this facilitates rapid triage of patients with clearly abnormal ECG, however in approximately 50% of patients with an ultimate diagnosis of STEMI, the ECG was non-diagnostic at the time of blood draw. ECGs yield particularly low sensitivity in the context of (more common[10]) NSTEMI presentations, not only because 1 in 7 patients displays a bundle branch block – thus triage would be improved if a highly-sensitive cardiac marker was available to the paramedic.

The best commercially available POCT cTn platforms have limits of quantification that are well above the population 99th centile defined using a laboratory assay. While this is useful in selecting the cases with the greatest mortality risk, these platforms lack the sensitivity to achieve rapid rule-in for most patients with AMI – this is reflected in the comparably low sensitivity values quoted in Table 50 and Table 51. Due to the inability to measure below the 99th centile, it is not possible to triage according to thresholds endorsed in international guidelines[12] for rule-out, or indeed make the diagnosis of AMI.[11] Furthermore, the latest ESC guidelines[12] specifically warn against the use of high-sensitivity Troponin assays in early-

presenters (<3 hours of chest pain). A protein which is much more abundant than cTn following myocardial injury would allow careful titration to individual requirements: whether the goalpost is maximum specificity/PPV, or maximum sensitivity/NPV, such as in rapid rule-in and rule-out pathways – the greater the 'detectable' spectrum of concentrations of an equally cardiac-specific marker, the greater the possibility to choose cut-offs to achieve local objectives.

This study has several limitations: 1) cMyC is currently only available on a high-sensitivity research platform and the migration onto POCT has not been completed. 2) Any cut-offs investigated are subject to cohort-specific calibration. These are not to be interpreted as a recommendation but would require validation in a separate cohort. As is evident in this study, the sensitivity achieved at 12 ng/L would not be acceptable for clinical use as part of a rule-out pathway unless used in patients presenting >2 hours following chest pain onset. This also requires validation, as the subgroup in which this was tested was comparably small (naturally, only 249 patients presented later than 2 hours following symptom onset) and might overestimate the sensitivity and NPV for rule-out. This is further reflected in the comparably wide confidence intervals. However, we argue that the ability to detect lower volumes of myocardial injury earlier is of particular use in a cohort such as the one studied, where the median time since onset of chest pain is substantially lower than in other, diagnostic chest pain studies, and rule-in of high-risk cases is of much greater importance to both the clinician and the patient. 3) As in most studies of this type there is an inherent bias against the new biomarker since hs-TnT was measured during the in-hospital course and used in the clinical adjudication of AMI.

In summary, we have demonstrated that 1) cMyC achieves improved diagnostic discrimination at earlier time points compared to hs-cTnT; 2) the addition of cMyC to hs-cTnT would provide additional diagnostic information; 3) cMyC achieves greater sensitivity and NPV at a threshold comparable to a TnT POCT device and is superior in predicting risk of subsequent mortality at a 2-year follow-up.

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The remaining authors have no conflicts of interest.

Disclosures:

Millipore Sigma was contracted to undertake the analyses of cMyC on a fee-for-service basis and holds no commercial interest. Prof Marber is named as an inventor on a patent held by King's College London for the detection of cardiac myosin–binding protein C as a biomarker of myocardial injury. The remaining authors have nothing to disclose.

Chapter 8. Conclusions and future direction

Cardiac myosin-binding protein C is a novel biomarker of myocardial injury with great potential – other groups[118,119,121] have investigated the use of cMyC in the diagnosis of myocardial infarction with confirmatory findings[48,50], however, were limited by poor assay-sensitivity. Despite careful selection of monoclonal antibodies and initially promising results on our electrochemiluminescence platform, cMyC sensitivity was outperformed by the increasingly available high-sensitivity Troponin assays. Kuster et al.[118] independently reached a comparable LoD on the same device (MesoScale Discovery), making the translation of the assay onto a platform with greater sensitivity the natural next step. Our work in migrating onto the Singulex Erenna enabled – for the first time – reliable cMyC quantification in stable outpatients. As demonstrated[84], this assay enabled two leaps in the translational phase: (i) quantify the cMyC level in all but one of 360 individuals without acute cardiovascular disease, thus allowing (ii) the derivation of a 99th centile (87 ng/L, as published[84]). The assay, performed by a contract research organisation, achieved a LoD 200-times lower than our in-house assay and laid the foundation for the studies described in this thesis.

8.1. Summary of findings

8.1.1 Quantifying the release of biomarkers of myocardial necrosis from cardiac myocytes and intact myocardium

The purpose of this study was two-fold: (i) establish the amount of cTn and cMyC release from cardiomyocytes and human cardiac tissue undergoing simulated necrosis, (ii) examine if dietary troponin can confound the laboratory results.

Serum from healthy volunteers was obtained and used as reference. Rat cardiomyocytes and human cardiac tissue were subjected to ultrasonication to simulate complete necrosis and

spiked into the healthy reference serum. For the dietary troponin consumption, a healthy volunteer had a 200 g dietary load of ovine left ventricular myocardium (boiled for 3 hours) and underwent frequent venesection. Samples were measured with hs-cTnI, hs-cTnT and cMyC assays (human cardiac tissue spikes only).

It was possible to detect the cTn release from the equivalent of a single cardiomyocyte with both hs-cTn assays, resulting in a slope of 19 $ng.L^{-1}$/cell [95% CI 16.8–21.2]) for hs-cTnT, and 18.9 $ng.L^{-1}$/cell [95% CI 14.7–23.1] for hs-cTnI. Similarly, each μg of myocardial tissue resulted in an increase in measured hs-cTn values: 3.9 $ng.L^{-1}$/μg [95% CI 3.6-4.3] for hs-cTnT, 4.3 $ng.L^{-1}$/μg [95% CI 3.8-4.7] for hs-cTnI. cMyC generated a much greater response on the Erenna assay, with a slope coefficient of 41.0 $ng.L^{-1}$/μg [95% CI 38.0-44.0]. This demonstrated the exquisite sensitivity of contemporary cardiac biomarker assays, and we concluded that necrosis of only 40 mg of myocardium is sufficient to breach the respective 99th centiles – too little to be detected by modern cardiac tissue imaging.

Whilst cooked ovine myocardium could be detected by both hs-cTn assays (with a much greater troponin content than human myocardium – possibly due to a relatively 'non-diseased' heart in comparison to the donated human tissue), none of the serial samples obtained from the healthy volunteer demonstrated a cTn response after oral load; suggesting the human gastrointestinal tract is impervious to a large polypeptide such as cardiac Troponin.

8.1.2 A single centre prospective cohort study addressing the effect of a rule-in / rule-out troponin algorithm on routine clinical practice

Cardiac Troponins are well established as valuable diagnostic tools for chest pain triage in the Emergency Department.[12,35,107,114] To quantify the number of patients undergoing cTn testing annually in the ED of a central London hospital, we performed a single-centre, prospective

cohort study investigating the use of hs-cTnT at our local centre. We collected data from all patients undergoing hs-cTnT testing in the months preceding and following the introduction of a new guideline for the management of patients with suspected ACS, modelled on the ESC 0/1h rule-out/rule-in algorithm.[12] Over the course of 7 months, 4,644 individual patients underwent hs-cTnT testing, and 40.4% were eligible for direct rule-out at presentation with a single, undetectable hs-cTnT result (< 5 ng/L); 7.6% were ruled-in. Therefore, the triage algorithm was successful in assigning 48% of patients to a definitive triage category, leaving 52% of patients in the ESC observe zone after first blood draw. Whilst the introduction of the novel pathway at our institution led to a shorter time-to-repeat testing, it was also used in a significant proportion of patients without clear symptoms suggestive of ACS (as presenting complaints ranged from 'chest pain' in 45.7%, to 'collapse' and 'unwell adult').

These findings are, however, relevant in a number of ways: triage using hs-cTnT assigns almost 50% to a definitive triage category within the first blood draw. The other 50% of patients, assigned to the ESC observe zone, have to rely on the use of delta-change values for further risk stratification. The study further demonstrated that time intervals between first and second blood draw decreased during the implementation (with 40.8% instead of only 3.3% receiving a repeat blood draw within 1.5 hours). There was, however, no significant reduction in length-of-stay.

One can only speculate why length-of-stay failed to show an improvement, despite a decent sample size (n=946 for the period following introduction of the new clinical pathway) and a marked increase in repeat blood testing. A possible explanation is of a logistical nature: stakeholders in the ED frequently highlighted the challenge of facilitating a second blood draw within an hour of the first test. Many patients undergo the initial blood draw at triage, but may

not see a physician until at least 90 mins later – by which point, the 'clock' towards the UK-wide 4-hour performance target requiring an admit or discharge decision has been ticking for about 2 hours. Chemical pathology at St Thomas' Hospital is contracted to provide a time-to-result (TTR) of 60 mins for cardiac Troponin in 80% of cases. Thus, the repeat result would often come very close to the 4-hour target, encouraging clinicians to make admit/discharge decisions based on the first blood test alone – to then pursue further workup during the inpatient stay. Once admitted, patients frequently await a Consultant-led ward round for a final decision, which inevitably occurs far later than the reporting of a second cTn result.

This is only to highlight that healthcare environments are complex and pathways, while clinically and biologically compelling, might not yield the expected result in the institution. It further highlights the importance placed upon the first initial blood draw – any definitive triage decisions made upon this result can only benefit the clinical course and expedite care provision (where required).

8.1.3 Temporal relationship between cardiac myosin-binding protein C and cardiac troponin I in type 1 myocardial infarction

In this pilot study, we investigated the performance of our novel cMyC assay (Erenna) in 174 patients presenting with suspected AMI. All patients were part of a subgroup of individuals recruited in the HighSTEACS[107] study, presenting with symptoms of less than 3 hours duration prior to first blood draw – all underwent blood draws at 0h, 3h and 6-12h (late); 26 were adjudicated with type 1 myocardial infarction.

We calculated a cMyC/hs-cTnI ratio for each of the 3 sampling time points. This demonstrated a positive linear correlation between the two biomarkers. However, mean and median ratios in patients with AMI were much greater at presentation than in the later

timepoints (median 2.72 at 0h, 1.83 at 3h, 0.63 at 6-12h), suggestive of a more dynamic rise of cMyC in the early stages of myocardial infarction than hs-cTnI. We hypothesised that this could enable more rapid and/or accurate triage – but clearly, a more in-depth evaluation of the diagnostic performance of cMyC was required in a larger study.

8.1.4 Direct comparison of cardiac myosin-binding protein C with cardiac troponins for the early diagnosis of acute myocardial infarction

We analysed cMyC in 1,954 unselected patients presenting with symptoms suggestive of AMI to Emergency Departments in a prospective, diagnostic multi-centre study based in Europe. We focussed on studying the diagnostic properties of the presentation blood test alone and compared cMyC performance to that of hs-cTnT and hs-cTnI. The study was adjudicated using hs-cTnT and the Universal Definition of MI[127], the prognostic endpoint being long-term mortality at 3-year follow-up.

AMI was the final diagnosis in 340 patients (17%), and we observed a much greater dynamic range of cMyC in AMI vs non-AMI patients, and in comparison to both hs-cTn assays. The diagnostic performance was investigated by calculating the area under the receiver-operating characteristics curve, and cMyC matched the performance of both hs-cTn assays (cMyC AUC 0.924 vs 0.927 hs-cTnT and 0.922 hs-cTnI). We used an internal derivation/validation split of the cohort to obtain optimal cut-offs for cMyC-guided rule-out and rule-in of AMI at presentation – 10 ng/L for rule-out, 120 ng/L for rule-in. These were used to calculate a Net Reclassification Improvement, based on re-classification of patients to rule-out or rule-in categories, where cMyC was substantially more effective than either hs-cTn assay (NRI +0.149 vs hs-cTnT, +0.235 vs hs-cTnI). A remarkable signal was the higher AUC in early presenters (chest pain <3h) when compared to the adjudicating biomarker hs-cTnT (AUC 0.915 vs 0.892,

p=0.022), also reflected in an even higher NRI in this subgroup. Based on Harrell's C statistics, cMyC was superior to hs-cTnI but not hs-cTnT at predicting death at 3-year follow-up.

This was the first study to comprehensively study cMyC performance in comparison to the best available biological signals for the diagnosis of AMI. Notably, the study was adjudicated using hs-cTnT and yet triage classification was more efficient (based on smaller observe-zone) and as accurate using cMyC. Furthermore, the patients recruited overall represent a cohort of late presenters, with a median chest pain time of 5 hours prior to admission. Findings including subgroup analysis corroborate our previous observations in the HighSTEACS subgroup – a marked advantage in early presenters, with an at least as good diagnostic performance but better triage capability.

Subsequently, we investigated two aspects further: (i) derivation of optimal cut-offs for delta-change values, to enable a rule-out/rule-in algorithm matching (or improving upon) the performance of the 2015 ESC NSTEMI pathways; (ii) study performance of cMyC in very early presenters (pre-hospital).

8.1.5 Derivation and Validation of a 0/1h-algorithm to diagnose Myocardial Infarction using Cardiac Myosin-binding Protein C

Extrapolating from our prospective study at St Thomas' Hospital and the European multi-centre study, delta-change values were likely to play a significant role in the successful management of patients presenting with suspected AMI. Given our perception of cMyC as a more dynamic marker, we expected to see an at least equivalent benefit of using delta-change values after second blood draw, both in diagnostic accuracy quantified by AUC, and the rapid triage into rule-out and rule-in of AMI. In >1,300 complete datasets, we established that cMyC was beneficial in addition to 0h hs-cTnI and hs-cTnT results (AUC from 0.916 to 0.925 with

hs-cTnI, 0.921 to 0.930 with hs-cTnT). It remains unclear as to why the combined AUC for cMyC, incorporating both 0h and delta-change, is inferior to hs-cTnT but equivalent to hs-cTnI – it is possible that this reflects bias from the use of hs-cTnT as the adjudicating biomarker. Further, we saw an improvement of AUC in early presenters with hs-cTnI 0h+delta, but this did not apply to hs-cTnT.

The derivation of a cMyC rule-out/rule-in algorithm was performed in a split-sample set of hs-cTnT, aiming for NPV ≥99% and PPV ≥70%. Meeting these primary endpoints, the cMyC algorithm is inferior to hs-cTnT with respect to specificity, but superior with respect to complete triage after 0/1h blood testing (4% absolute increase of patients assigned to rule-out and rule-in zones). When compared to hs-cTnI, cMyC is comparable on all performance metrics but increases complete triage by 10%. Subgroup analyses demonstrate that the overwhelming advantage of cMyC lies with direct rule-out based on a presentation sample, with diminishing (yet still superior) efficiency after a completed 0/1h protocol. This is also evident when adding cMyC to either hs-cTn pathway at presentation: direct triage at presentation, as well as triage after a completed 0/1h protocol benefit significantly from the additional biomarker and leave a maximum of 17% of patients in the observe zone, with matching NPV and PPV. This has not been done before and calls for a real-life, real-time diagnostic trial.

8.1.6 Cardiac Myosin-Binding Protein C to diagnose Acute Myocardial Infarction in the pre-hospital setting – identifying the high-risk patient

As established in the large, multi-centre trial investigating patients presenting to emergency departments[126], and previously in our subgroup analysis of HighSTEACS[123], cMyC has a biological advantage in very early presenters. However, many secondary and tertiary care trials

enrol patients relatively late in their clinic course, often with a median symptom onset time of 5 hours prior to presentation.[87,124,126,180] Hence we investigated the diagnostic value of cMyC in patients undergoing blood draws in a pre-hospital setting: Seven-hundred and seventy-six patients with a median time of 70 minutes from chest pain onset to blood sampling were enrolled by paramedics in Denmark. The study was originally used to assess the value of pre-hospital triage using a cTnT-based POCT device in the Danish country-side, where patients with suspected AMI face ambulance-transfer times of up to 2 hours to reach the nearest hospital offering percutaneous coronary intervention. Currently, triage is reliant on ECGs transmitted to and interpreted by cardiology residents at Aarhus University hospital – a test with, even for ST-elevation MI, only moderate diagnostic accuracy.

We demonstrated a similar discrepancy between telemedicine-triage and final diagnosis, where only 59% of patients with a final adjudicated diagnosis of STEMI had their ST-elevation clearly identified pre-hospital. Median concentrations for cMyC were, even within this short time frame, higher than hs-cTnT when compared to the respective LoD of the assay – reflecting a higher AUC (0.839 vs 0.813), as well as incremental benefit when both markers are used together. The changes between early, intermediate and late timepoints in this cohort are notable. We have shown that a POCT threshold of 10-12 ng/L for cMyC is feasible with a preliminary foray on an OEM platform – at this cut-off, the sensitivity for AMI in patients with <60 mins of chest pain is already 92.6% (vs 26.9% with hs-cTnT), and rises throughout the later stages – whereas the hs-cTnT POCT threshold, due its low (analytic) sensitivity (50 ng/L), never reaches a diagnostic sensitivity ≥60%. The clear benefit transpires when incorporating the biomarker into a risk-model, where the C statistic for cMyC is markedly higher than hs-cTnT at the POCT threshold. To date, most POCT devices have failed to

achieve a Limit of Detection that allows such risk stratification – in particular in a pre-hospital setting. The investigated device testing hs-cTnT achieves 50 ng/L (comparable to values derived from the laboratory analyser) in semi-quantitative detection <100 ng/L. There is, however, great potential in the testing for cardiac biomarkers in point-of-care – it might just require a more abundant biomarker.[122] Our results translate into a successful rule-in of almost all AMIs, and would – owing to the greater abundance of cMyC – enable risk-stratification in a pre-hospital setting: with a cMyC-level based judgement on who will likely benefit from medical care in a tertiary cardiac care facility and who can be safely managed in a hospital not providing invasive cardiac investigations and treatments.

8.2. Future direction

8.2.1 cMyC and renal function

Several unanswered questions remain that warrant further studies. Based on the study introducing the high-sensitivity assay for cMyC[84], the novel biomarker appears to be affected by decreased renal function similarly as cTn. We were unable to test this hypothesis in greater detail in the cohorts studied to date – one reason being the enrolment of relatively 'healthy' individuals with regards to underlying renal function. The European multi-centre study (APACE) precluded patients with end-stage renal failure from participation (thus the mean eGFR was 84 ±26). Underlying renal disease would likely only significantly alter cMyC values of patients without AMI, as the dynamic range of cMyC in response to myocardial injury would likely trump any interference from abnormal renal function at baseline. This would potentially reduce the number of patients with renal dysfunction that are eligible for direct rule-out. An effect on the safety of rule-out based on our current cut-off values, or indeed specificity for rule-in is unlikely given widely spaced decision limits.

8.2.2 cMyC sources other than cardiac muscle

We have not identified any evidence to suggest that the cardiac isoform of myosin-binding protein C is expressed in tissue other than cardiac, from neonatal through adult tissue development[116,117] Nevertheless, similar assumptions previously applied to cTn – absent in healthy adult skeletal muscle, cTnT is present in foetal skeletal muscle[181]. Muscle regeneration in adult life, due to muscle injury or neuromuscular disease, can lead to re-expression of cTnT (but not cTnI) according to work published by Rittoo et al.[182] The authors compared sensitive cTnT and cTnI assays (Roche, Siemens) in patients with skeletal muscle diseases and demonstrated persistent cTnT elevation, but no signal using cTnI assays. Jaffe et al. had previously demonstrated immunoreactivity between the antibodies used for (sensitive and high-sensitivity) cTnT assays and a 37- to 39-kDa protein in 4 diseased (skeletal) muscle biopsies;[183] findings which were confirmed recently in a publication by Schmid et al.[24] As the targets of our monoclonal antibodies are located in an N-terminal region, cross-reactivity with fast- or slow-skeletal muscle protein is even less likely.[50,83,84] Therefore, it is worth noting that cMyBP-C re-expression might occur and has not been specifically investigated.

8.2.3 Biological variation in healthy individuals

We have performed the derivation of cMyC delta-change values in a retrospective sample set, focussing on identifying the patients most likely to benefit from rule-out and rule-in through the use of biomarker changes between first and second blood draw. There is mounting evidence that cTnT is subject to a diurnal rhythm, with one study describing peak concentrations during morning hours followed by gradual decrease during daytime hours and rising concentrations during the night – quoting concentration changes of up to 24%.[28] Counter-intuitively, cTnI concentrations appear to vary randomly over a 24h period, with a

constant CV_I (coefficient of variation within-subject) of 8-9% for all time intervals, irrespective of underlying renal function.[27,184] We should study day-to-day biological variation as well as diurnal variation for cMyC values using the following approach:

Study proposal for biological variation study

The study should include healthy, adult, gender-matched volunteers.[185] Participants should be free of diabetes, cardiovascular disease, renal disease, chronic lung disease or cancer. In one group of participants, samples should be obtained once a week for ten weeks. Samplings should be done between 08.00 and 10.00 after the participants have rested in a sitting position for at least 15 minutes and at the same weekday ±1 day each week. In a second group, samples should be collected at hourly intervals throughout a 24-hour period to investigate diurnal variation.

Samples should be collected in serum-separating and EDTA tubes, centrifuged within 30 minutes and 8 vials of 1ml serum and 8 vials of 1 ml plasma should be frozen at -80 ºC in cryovial tubes, within 1 hour. The laboratory will measure glucose, eGFR, hs-cTnT and NT-proBNP on the first visit to ensure the participants are healthy.

Simple linear regression will be used to identify trends that could indicate that a non-steady-state situation is present. Analytical outliers will be defined according to Burnett.[186] Outliers in mean values will be defined according to Reed's criteria.[187] If the residuals of the data do not conform to a Gaussian distribution, the data will be transformed into natural logarithms. As suggested by Fraser and Harris[188], the variance homogeneity in the analytical and within-person variances will be tested using Cochrane's and Bartlett's tests. Participants exhibiting non-homogeneity will be identified by plotting the cumulated fractions of the ranked individual variation results (consisting of both CV_I and CV_A) on a Rankin scale as a function of the

within-person variation of cMyC, and participants are excluded until homogeneity of variance is achieved.[189]

8.2.4 cMyC and cardiovascular disease monitoring

Use of the cMyC assay on a large-scale, high-throughput laboratory platform would facilitate testing of large cohorts, to validate our published findings in the application of cMyC as a marker in the diagnosis of AMI – in particular, the cut-off values require external validation to avoid overfitting due to the derivation in a single cohort. With regards to cardiovascular risk-prediction, measuring cMyC in the biomarker sub-study of an outcome trial such as ODYSSEY[190,191] would be highly attractive. ODYSSEY evaluates Alirocumab – an antibody to PCSK9, started 1 to 12 months after an acute coronary syndrome – in a placebo-controlled study involving about 18,000 patients. Patients with inadequate LDL control despite optimized conventional lipid treatment were eligible for recruitment. The event-driven follow-up (up to 5 years) finished in December 2017. The primary endpoint of the study is the composite of CHD death, any non-fatal MI, fatal and non-fatal ischaemic stroke, and unstable angina requiring hospitalisation.

The greater abundance of cMyC allowed the detection of quantifiable levels in a much larger proportion of stable patients than either hs-cTnT or hs-cTnI[84]. The correlation between cMyC and cTnI and cTnT enables ratio-metric comparisons between biomarkers.[84]

In contrast to cTn, cMyC may not just be a bystander biomarker of cardiac injury but may lie on the causal pathway leading to myocardial disease. It is a key regulator of cardiac contractility and release may depend on adrenergic drive. At baseline, cMyC is highly phosphorylated, a condition required for normal cardiac function. However, the level of cMyC phosphorylation is significantly decreased during heart failure, indicating that the level of cMyC phosphorylation

is directly linked to signalling and cardiac function. cMyC is phosphorylated by a variety of protein kinases at critical serine residues within the M domain. When phosphorylated, these residues more effectively guard the calpain cleavage site within the M domain. Cleavage at this site releases a 40-kDa N-terminal fragment, the dominant fragment observed in serum of patients with acute myocardial infarction. This fragment may act as a 'poison peptide' causing cardiac dysfunction.[84] Thus, the circulating cMyC concentrations in stable patients may provide an indirect readout of 'myocardial health/attrition'. It would be very attractive to examine the use of cMyC as a biomarker of risk that may be modifiable with an intervention. This requires a large, well characterised population with rigorous prospective follow-up taking contemporary background therapies. The ODYSSEY biomarker sub-study would be ideally suited for this study.

8.2.5 Point-of-Care Testing for cMyC and final

It seems attractive to further evaluate the performance of the cMyC assay with the current monoclonal antibody pair on a Point-of-Care Testing, near-patient platform. Given relative abundance of cMyC in comparison to cTnT and cTnI[84,122], the former might overcome the biggest hurdle for handheld devices in the cardiovascular space – analytical sensitivity. Two characteristics of cMyC could enable migration to POCT: (i) the protein is relatively more abundant – both in the sarcomere and in the circulation following myocardial injury; (ii) the proposed threshold for safe rule-out of AMI (10 ng/L) is about 25-fold the LoD of the current laboratory assay – to match, hs-cTn assays would have to achieve the same LoD on the POCT platform as on the laboratory analyser. As shown before, we contracted a POCT device manufacturer for an initial foray into migration – this achieved an LoD of 10 ng/L (see Diagnostic proportions of hs-cTnT and cMyC, Figure 46). The use of cMyC in a POCT-setup

is compelling from a number of perspectives: a device that can achieve a LoD of 10 ng/L in a near-patient setting would be sufficiently sensitive to enable immediate & safe rule-out of 7-20% more patients than with (laboratory) hs-cTn assays. In healthcare modelling performed previously, on the basis of the study presented in Chapter 3, this could translate into 'savings' of 1,000 bed days per year if a cMyC POCT device was used at St Thomas' Hospital, through admission-avoidance and shorter time-to-discharge.[192] Economically, this could be attractive to the healthcare organisation as well – a cost-effectiveness analysis comparing hs-cTn and cMyC has been performed and published before.[192]

8.3. Summary

In conclusion, cMyC is a cardiac-restricted protein which enters the systemic circulation after myocardial injury. The work described in this thesis shows that it performs favourably in the diagnosis of AMI against hs-cTn. The relative abundance of cMyC, and its rapid rise after myocardial injury, make it particularly well-suited to a point-of-care diagnostic platform.

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