The biological variation and fragmentation of Cardiac Myosin- Binding Protein C to diagnose acute and chronic myocardial injury

Alaour, Bashir

Awarding institution:
King's College London

The biological variation and fragmentation of Cardiac Myosin-Binding Protein C to diagnose acute and chronic myocardial injury

Dr Bashir Alaour

MD MRCP (UK) PG Cert Clin Ed FHEA

Submitted for the Degree of Doctor of Philosophy

School of Cardiovascular Medicine
King's College London
British Heart Foundation Centre of Research Excellence
The Rayne Institute
St Thomas' Hospital
London

Supervisors
Professor Michael Marber
Professor Simon Redwood

Dedication

There are too many people I need to thank than I could possibly list, each of whom has played a unique role in my academic journey and personal life. And when I try to put pen to paper my gratitude towards them, I fear being let down by words, but never by thoughts.

To my late mother and father, you were not destined to see me walk the path you had scarified so much for, but your presence has always been overwhelmingly felt in every step I took. You taught me well that candles can put out the storms, roses can water the thirsty rain and that dedication and passion is all it takes to pursue dreams and goals. I hope all my achievements, including this academic work, will make you proud and will live up to your sacrifices.

To my sisters Abir and Souad: through all adversities, we walked our path in life together, hand-in-hand, and I learnt from you that survival might be a destiny, but thrival can only be a choice. I am so proud of you, always inspired by you and ever grateful for your support.

My friend, and my rock Salim Elyas, I could not have asked for a better friend in this life, and I am ever so grateful for our friendship and for your endless support.

To late uncle Salim Kassam and uncle Ammar Al-Khatib: I had been looking forward to handing you a copy of this work and watching you read these words. Thank you for supporting me through the most difficult times, and for inspiring me.

Acknowledgments

I very much remember my first visit to the Rayne Institute for my first meeting with Professor Mike Marber. At the time I was simply hoping for a good project and a good supervisor, but never expected that the man sitting opposite me would soon become a great friend, let alone the best supervisor anyone could wish for.

Prof, I feel privileged to have had the opportunity to spend all those hours by your side. You have taught me almost everything I know about academia and science, but also a lot of what I know about having a positive attitude, about inspiring and supporting others, leading by best example, about generosity beyond limits, being a good company and being an all-round amazing human being. It fills me with great pride to see my name associated with yours in my academic and professional journey. “Thank you” falls too short of my gratitude to you.

To my secondary supervisor Professor Simon Redwood: I am very grateful for your time, support and help. I hope our professional and academic paths continue to cross.

To Mr Tony Cavalheiro, for the extensive and generous admin support throughout. Thank you so much Tony.

Academia also granted me a family of esteemed colleagues who I now call friends: Professor Divaka Perera, Dr Thomas Kaier, Dr Jasmine Quraishi, Dr Ozan Demir, Dr Haseeb Rahman, Dr David Sanchez Tatay, Dr Yu Jin Chung, Dr Olena Rudyk, Professor James Clark, Dr Matt Rayan, Dr Aish Sinha, Dr Holly Morgan, Dr Emma Burnhope, Mr Howard Ellis, Dr Rekha Bassi, Dr Helen Heath, Dr Rhys Anderson and Dr Mohamed Aboudounya. Thank you all for the support and for the many memories to cherish forever.

I am forever in debt to our esteemed international collaborators. This research would not have been completed without their contribution and collaboration:

Dr Kristin Aakre. Department of Clinical Science, University of Bergen, Bergen, Norway.

Dr Torbjørn Omland. Institute of Clinical Medicine, University of Oslo, Oslo, Norway.

Dr Steven Meex. Cardiovascular Research Institute Maastricht (CARIM), Maastricht University Medical Center (MUMC), the Netherlands.

Professor Christian Mueller, University Hospital Basel, Switzerland.

Professor Ekkehard Weber. Institute for Physiological Chemistry, Martin Luther University, Halle-Wittenberg, Germany.

Dr Kasper Iverson. Department of Emergency Medicine, Herlev and Gentofte Hospital, Copenhagen, Denmark

Dr Rasmus Bo Hasselbalch. Department of Emergency Medicine, Herlev and Gentofte Hospital, Copenhagen, Denmark

Dr Asthildur Arnadottir. Department of Emergency Medicine, Herlev and Gentofte Hospital, Copenhagen, Denmark

Associate Professor Brian Weil, University at Buffalo, New York, USA

Professor John Canty, University at Buffalo, New York, USA

Professor Sakthival Sadayappan. University of Cincinnati, USA.

I am grateful for my clinical supervisors in Wessex Deanery, who supported me before and during my out-of-programme period, in particular: Professor Nick Curzen, Dr Andrew Bishop, Dr Iain Simpson, Dr Benoy Shah, Dr Michael Mahmoudi, Dr James Wilkinson, and Dr John Paisey.

And last, but certainly not least, I am so grateful to the British Heart Foundation, the charity and its generous doners, for funding this research work, and for always aspiring to improve the lives of our patients, and also to all the participants who very kindly took part in the studies.

Academic output directly related to this thesis (first authorship only)

Publications

Direct Comparison of The Circadian Rhythm of Cardiac Myosin-Binding Protein C (cMyC) and Cardiac Troponin

Bashir Alaour , Thomas E Kaier , Rasmus Hasselbalch , William van Doorn , Steven Meex , Michael Marber.

The Journal of Applied Laboratory Medicine 2023. In press (accepted March 2023)

Temporal Release of Cardiac Myosin-Binding Protein C Compared to Cardiac Troponin After Brief Coronary Artery Balloon Occlusion in Humans

Bashir Alaour, Rasmus Bo Hasselbalch, Asthildur Arnadottir, Thomas E Kaier, Sune Ammentrop Haahr-Pederson, Henning Bundgaard, Michael Marber, Kasper Karmark Iversen.

European Heart Journal 2023, Revision stage.

Phosphorylation and Fragmentation Patterns of Cardiac Myosin-Binding Protein C for the Differentiation Between Different Types of Myocardial Injury.

Bashir Alaour , Yu Jin Chung , Thomas Edward Kaier , Jasmine India Helen Quraishi , Helen Heath , Zilan Demir , Sakthivel Sadayappan , Simon R. Redwood , Brian Raymond Weil , John M. Canty , and Michael S. Marber

J Am Coll Cardiol. 2022 Mar, 79

Biological variation of cardiac myosin-binding protein C in healthy individuals

B. Alaour, T. Omland, J. Torsvik, T. E. Kaier, M. S. Sylte, H. Strand, et al.

Clin Chem Lab Med 2022 Vol. 60 Issue 4 Pages 576-583

Cardiac Troponin - diagnostic problems and impact on cardiovascular disease

B. Alaour, F. Liew and T. E. Kaier.

Ann Med 2018 Vol. 50 Issue 8 Pages 655-665

Awards

Young Investigator Award finalist, British Society of Interventional Cardiology, 2022

Presentations

The circadian rhythm of cardiac myosin-binding protein C and cardiac troponin

ESC congress 2020

Phosphorylation and fragmentation pattern of cMyC for the differentiation between different types of myocardial injury

ACC 2020

ABSTRACT

Background

Cardiac myosin-binding protein C (cMyC) is a novel biomarker of myocardial injury, with a promising role in the triage and risk stratification of patients presenting with acute and chronic cardiac disease. To assess the suitability of cMyC for the diagnosis, monitoring and risk stratification of acute and chronic myocardial injury, we investigate (i) long- and short-term biological variation of cMyC (ii) the diagnostic performance of cMyC for suspected acute myocardial infarction in patients with renal dysfunction (iii) and the temporal release of cMyC after a brief induced ischaemia.

In an attempt to overcome a major shortfall of cardiac troponin, which is low specificity towards acute myocardial infarction and the inability to differentiate between different types of myocardial injury, we study the phosphorylation and fragmentation pattern of cMyC in different types of myocardial injury in-heart and in-circulation, which includes the creation of immunoassays that would allow the quantification of intact and fragmented cMyC in the circulation from animal models and human participants with different types of myocardial injury.

Methods

For the long- and short-term biological variation studies, blood was sampled weekly and hourly from 30 and 24 healthy participants, respectively, for the determination of the biological variation, reference change value, and index of individuality.

Retrospective analysis of a prospective cohort was performed to determine the diagnostic performance of cMyC compared to cardiac troponin cTn within the European Society of

Cardiology ESC 0/1 h triage algorithm for the diagnosis of acute myocardial infarction, stratified by renal function. The outcome measures were safety defined by sensitivity for NSTEMI in the rule-out group; accuracy as defined by the specificity for NSTEMI in the rule-in group; NPV; PPV; and overall triage efficacy defined by the proportion of patients outside the observe zone.

For the determination of the temporal release of cMyC after induced ischaemia, compared to that of cTn, biomarkers concentrations were measured at baseline and in serial blood samples from 34 consented patients who were randomly assigned to 0, 30, 60, or 90 seconds of intracoronary balloon occlusion of the left anterior descending artery. Temporal release and kinetics of the biomarkers were studied and compared.

To study the phosphorylation and fragmentation pattern of cMyC in different types of myocardial injury, propofol-anesthetized swine were subjected to one of two myocardial injury protocols, model (a): 1- hour phenylephrine PE infusion for stretch-induced left ventricular (LV) stunning and cMyC release (n=5), and model (b): 1-hour Left Anterior Descending LAD occlusion for myocardial infarction MI (n=3).

Myocardial tissue samples from euthanized animals at 1- and 24-h post myocardial injury were immunoblotted using high affinity anti-N-terminal cMyC antibodies and anti-phosphoserine 282 antibodies.

To straddle the cleavage site of cMyC and create a selective full-length FL (intact) circulating cMyC assay, high affinity anti-N-terminal and anti-C-terminal cMyC monoclonal antibodies were paired to create a sensitive electrochemiluminescence sandwich ELISA assay to complement our in-house total (all species) cMyC assay. The

sensitivity of the latter was optimised to enable in-house quantification of low cMyC concentrations.

Porcine serum samples from the PE model (n=3) and MI model (n=3) above, and human serum from patients with Type 1 acute myocardial infarction AMI (ST-segment elevation myocardial infarction, n=8) and Type-2 AMI (tachyarrhythmia-induced myocardial injury, n=5), were tested using both the selective FL cMyC assay and the total (all species) cMyC assay to determine the ratio of FL (intact) cMyC to total (all-species) cMyC.

Results

The weekly biological variation, reference change value RCV and index of individuality II with 95% confidence interval (CI) were: analytical variation CV_A (%) 19.5 (17.8 – 21.6), intra-individual biological variation CV_I (%) 17.8 (14.8 – 21.0), inter-individual biological variation CV_G (%) 66.9 (50.4 – 109.9), RCV (%) 106.7 (96.6 – 120.1)/ -51.6 (-54.6 – -49.1) and II 0.42. There was a trend for women to have lower CV_G. The calculated RCVs were comparable between genders.

The hourly biological variation, reference change value RCV and index of individuality II with 95% confidence interval (CI) were: CV_A (%) 11.1 (10.1-12.2), CV_I (%) 13.4 (11.5-14.2), CV_G (%) 72.7 (61.6-84.7), RCV (%) 61.5 (53.0-68.4)/ -38.1 (-50.6 – -29.3) and II 0.23

All long- and short-term biological variation parameters for cMyC were comparable to the respective parameters derived for cTn.

On population-mean cosinor analysis of biomarker concentrations in the hourly biological variation study, cMyC and cardiac troponin T cTnT exhibited significant circadian

rhythm (p = 0.015 and <0.001, respectively), with 5-hours acrophase difference between cMyC and cTnT (cMyC ahead of cTnT). The impact of this physiological phenomenon on the performance of the biomarkers within unadjusted diagnostic algorithms is yet to be determined.

The sensitivity of rule-out as an assessment of safety of the triage algorithm in patients with renal dysfunction was preserved using all three biomarkers, compared to patients without renal dysfunction, safety: 98.5 % [95% CI, 95.4-100] vs 96.8 % [95% CI, 94.1-99.5], respectively, p=0.776 for cMyC; 96.9 % [95 % CI, 92.7-100] vs 97.4 % [95% CI, 95.0-99.9], respectively, p=1, for hs-TnI; and 100 % [95% CI [100-100] vs 100% [95% CI, 100-100, respectively, p=1 for hs-TnT.

Accuracy of rule-in as quantified by specificity for NSTEMI of the triage algorithm in patients with renal dysfunction was lower in patients with- compared to patients without-renal dysfunction with all three biomarkers. Accuracy: 81.5 % [95% CI, 75.2-87.8] versus 94.5 % [95% CI, 93.0-95.9], respectively, p <0.001 for cMyC; 84.2 % [95% CI,78.3-90.1] vs 93.5 % [95% CI, 99.1-95.1], respectively, p <0.001, for hs-TnI; and 86.3 % [95% CI, 80.7-91.8] vs 96.7 % [95% CI, 95.5-97.8], respectively, p <0.001 for hs-TnT.

Overall efficacy of the algorithm using all biomarkers was reduced, with significantly more patients allocated to the observe zone in patients with renal dysfunction compared to patients without. Overall efficacy: 57.7% [95%CI, 52.1-65.4] vs 83.0 % [95%CI, 80.7-85.2], respectively; p <0.001, for cMyC; 52.1% [95%CI, 45.3-58.8] vs 73.4% [95%CI, 70.8-76.8], respectively, p <0.001 for cTnI; and 49.7% [95%CI,43.0-56.5] vs 79.2% [95%CI, 76.9-81.6], respectively, p <0.001 for cTnT.

Adjusting the rule-in thresholds for cMyC, could not optimise accuracy without further compromising the overall efficacy.

In patients with renal dysfunction, safety, accuracy, overall efficacy and discrimination power as quantified by AUC of ROC of the 0/1 h triage algorithm for all three biomarkers were comparable.

After 90 seconds of induced ischaemia, there was a significant increase at 90 minutes in the concentrations of cMyC, cTnT and cTnI, compared to baseline ($p < 0.05$). After normalizing biomarker concentrations to baseline values to allow inter-biomarker comparison, there was no significant difference between the changes of cMyC, cTnT and cTnI concentrations relative to baseline at all time points. However, there was a significantly more rapid decline in the hourly change of concentration for cMyC compared to cTnI ($p=0.0312$) and cTnT ($p=0.048$) between 3 and 4 hours after ischemia.

In the study of the phosphorylation and fragmentation pattern of cMyC in different types of myocardial injury, immunoblotting showed reduced phosphorylation and increased fragmentation of cMyC in porcine myocardium subjected to ischaemic, compared to LV stretch induced, injury. There was a tendency in the latter for an increase in cMyC phosphorylation above the baseline (control) 24 hours after PE infusion.

Immunoassay of cMyC in the porcine serum samples at 1-h post injury showed an increase in circulating FL (intact) cMyC in parallel with total (all species) cMyC in the PE model, whereas no detectable FL (intact) cMyC was observed in the MI model (only fragmented cMyC was present in the circulation).

Immunoassay of cMyC in the human serum showed significantly higher ratio of FL (intact) to total (all species) cMyC in the tachyarrhythmia-induced myocardial injury subtype of Type 2 AMI compared to Type 1-AMI.

Conclusion

cMyC exhibits acceptable weekly RCV, low hourly RCV, and low weekly and hourly II, suggesting that it could be suitable for diagnosis, monitoring and risk stratification of chronic and acute myocardial injury if measured serially.

In patients with renal dysfunction, the performance of cMyC-guided ESC 0/1-h triage algorithm for suspected NSTEMI is comparable to that of cardiac troponins, however, while the safety of the algorithm is high, the accuracy and overall efficacy is reduced.

Induced ischemic can causes significant and comparable increase in the concentrations of cMyC and cTns, however, a faster decline in cMyC concentrations compared to cTn is observed between 3 and 4 hours after injury.

Phosphorylation and fragmentation patterns of cMyC are dependent on the type of myocardial injury and might aid in the differentiation between different types of myocardial injury.

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1 Chapter 1. Introduction

1.1 Shortfalls in troponin-guided triage of patients with suspected myocardial infarction

Cardiac troponins have transformed acute cardiovascular care, including the diagnosis and assessment of prognosis in patients with suspected acute myocardial infarction (AMI). However, there are several shortcomings with current troponin-guided diagnostic and prognostic strategies.

Troponin is, inherently, a slow-rising and late-peaking protein, [2,3] which impairs its ability to assess and triage patients early after the onset of suspected myocardial injury and introduces a blank pre-triage zone, where early presenters have to wait This has spurred a flurry of commercial investments in assays of ever-increasing analytical sensitivity aiming to rule-out/rule-in AMI sooner and sooner after the suspected index event, and multiple platforms and generations of high-sensitivity troponin (hs-cTn) assays have been introduced.[4] Paradoxically, the journey has not ended here, in fact, it has only just begun.

By definition, high-sensitivity troponin assays must be able to detect troponin in at least 50% of healthy individuals. [5,6] In reality, they could yield quantifiable results in a vast majority of healthy individuals, [7] and nearly all individuals with co-morbidities or risk factors that are commonly associated with ischaemic heart disease, in the absence of clinical evidence of acute myocardial injury.[8] This super-enhanced sensitivity has come at a cost: specificity. It has become widely accepted that troponin is an organ-specific, but no longer condition-specific, biomarker. This liability is particularly apparent in its classic clinical application: identification of acute myocardial infarction (AMI) secondary to atherosclerotic plaque rupture.

Additionally, the concept of the 99th percentile that was traditionally used to dichotomise troponin-guided triage into simple rule-out/rule-in has come under increased scrutiny:

- The 99th percentile as a reference interval for the diagnosis of myocardial infarction is strongly dependent on the reference population and the assay used.[9]
- High-sensitivity troponin assays have allowed the study of biological variation of troponins in healthy subjects, such studies showed that within-subject biological variation is much smaller than between-subject variability, [10,11] indicating significant individuality and low applicability of population-based reference interval, instead, subject-based reference change would be more useful.
- By accepting the concept of biological variation of troponins, results can shift from below the reference interval to above it without clinical significance, which could translate into a false positive result. [12]
- The enhanced analytical sensitivity and the utilisation of high-sensitivity assays allows low concentrations to be detected with acceptable coefficients of variation, therefore, it has possible to detect clinically meaningful change even at low concentrations.[9,13]

Therefore, it seems reasonable to propose new metrics in the form of reference change values rather than the 99th percentile-based reference concentration [12] in an attempt to maximise the benefit of enhancing sensitivity, and minimise the liability of reduced specificity. Subsequently, international guideline societies endorsed the use of assay-specific diagnostic cut-off thresholds, that rely on changing patterns of concentrations, called a delta change value. These thresholds are derived from large, adjudicated cohorts

and incorporated within diagnostic algorithms to produce a three-tier triage and risk stratification system: rule-out, observe, and rule-in. [14,15] The derivation of thresholds considers a pre-defined, clinically desired balance between sensitivity and specificity.

In addition to safety and accuracy, measured by sensitivity of rule-out and specificity of rule-in, respectively, the three-tier triage system adds a third parameter to the diagnostic performance of the biomarker: efficacy, a parameter that is inversely related to the proportion of patients left in the "observe" zone.

The proposed triage algorithms improved diagnostic performance of hs-cTn [16-24] , however, several gaps and deficiencies have become apparent:

- Diagnostics thresholds were derived from cohorts with low proportions of early presenters, [14,15,25] therefore, guidelines adopting them do not advocate reliable and safe rule-out using a single sample before 3 hours from the onset of chest pain. [15]
- Accurate reporting of low change values used for rule-out (< 3 ng/L for hs-cTnT and < 2 ng/L for hs-cTnI) requires: 1) high analytical precision which could be hard to achieve in those who present with low levels such as early presenters,[25-27] 2) low biological variation, which is inherently not the case with cardiac troponins, with a reported within-subject coefficient of variation (CV_I) up to 24% for hs-cTnI,[28] and up to 21% for hs-cTnT [26] on hourly repeat of measurements in healthy volunteers.
- Real-life data suggests rule-out sensitivity of ~ 97% only, [29] which might be suboptimal for emergency physicians.
- Rapid triage requires a very short analytical turnaround, ideally suited to a point-of-care test (POCT) with devices that meet analytical goals, a journey

that has proved tortuous for cTn, [30,31] although promising advances have been achieved recently.[32]

- The proportion of patients left in the observe zone even after the second blood draw varied between 15 and 70%[8,33,34], of which, 5 to 22% had AMI. [8]
- Low to sub-optimal specificity [25,35,36] and positive predictive value PPV for acute myocardial infarction. [14,29] It has been proposed that the "rule-in" arm should be renamed the "rule-in myocardial injury arm", acknowledging the ambiguity inherent in this diagnostic group and the futility of the quest for high specificity in rule-in for acute myocardial infarction. [37]

In parallel, the definition of MI itself had to be refined using a biochemical and clinical approach, a task that was undertaken by international guideline societies including the European Society of Cardiology ESC, American College of Cardiology ACC, American Heart Association AHA, and the World Heart Federation WHF. To minimise the confusion and conflicting consensuses, Global MI Task Force, produced the Universal Definition of Myocardial Infarction UDMI; fundamentally, a major shift from the old epidemiological-based definition of *myocardial infarction* to a more granular, aetiology-based understanding and classification of *myocardial injury*.[38]

UDMI, in its most recent iteration the (4th UDMI) [39], defines myocardial injury, as elevated cTn above the 99th percentile upper reference limit URL. Injury is then classified into acute, in the presence of a new dynamic rise/or fall of cTn values with at least one value above the 99th percentile, or chronic, if cTn is persistently elevated with relatively stable values. The definition then differentiates between two sub-entities of acute myocardial injury, namely ischaemic and non-ischaemic, based on symptoms, clinical context, and electrocardiographic and imaging (invasive or non-invasive) criteria. Acute

ischaemic myocardial injury is then sieved into different entities (Type 1, Type 2, Type 3, Type 4a, Type 4b, Type 4c, and Type 5), figure 1-1.

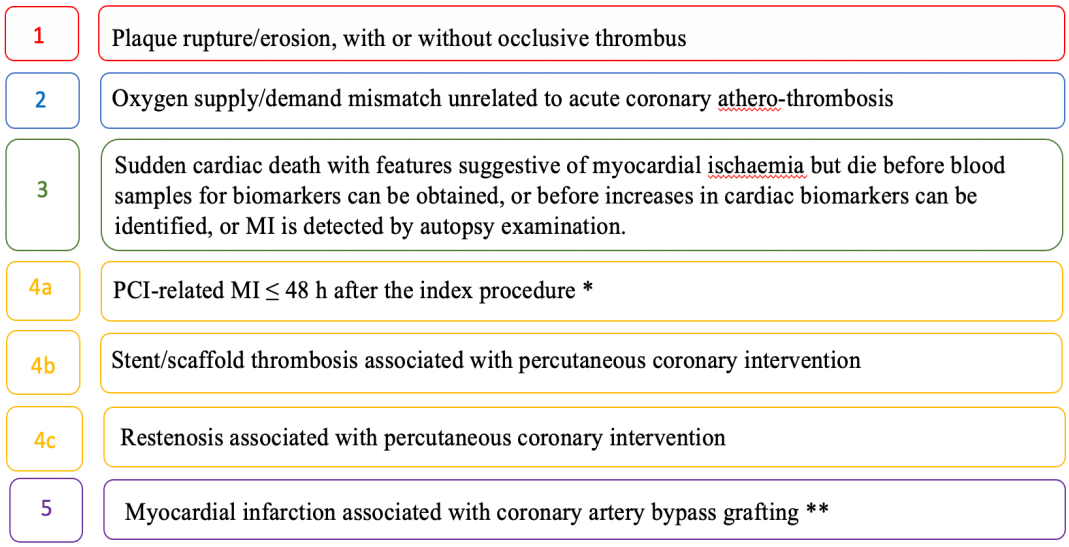

Figure 1.1 Proposed subclassification of acute myocardial injury as per the 4th Universal Definition of Myocardial Infarction

* Arbitrarily defined as elevation of cTn values > 5 times the 99th percentile URL in patients with normal baseline values. In patients with elevated preprocedure cTn in whom the cTn level are stable (≤20% variation) or falling, the postprocedure cTn must rise by >20%. However, the absolute postprocedural value must still be at least 5 times the 99th percentile URL

** Arbitrarily defined as elevation of cTn values >10 times the 99th percentile URL in patients with normal baseline cTn values. In patients with elevated preprocedure cTn in whom cTn levels are stable (≤20% variation) or falling, the postprocedure cTn must rise by >20%. However, the absolute postprocedural value must still be >10 times the 99th percentile URL

This aetiology-based approach proposed by the UDMI is novel, logical, and clinically justifiable. However, in reality, defining the aetiology and refining the diagnosis is less straightforward; the most classic example being the differentiation between Type 1 and Type 2 acute myocardial infarction AMI, [40] as both types share the same biomarker, clinical, ECG and non-invasive imaging criteria, with the only definite difference being the presence of acute coronary athero-thrombosis in Type 1. [39] The presence of which, is not available to the frontline adjudicator, and may not even be certain after invasive coronary angiography.

cTn measurements alone, even including changes in concentration over time, are not sufficient to aid the differentiation between subtypes of acute myocardial infarction. [41]

Concepts like Type 2 AMI and non-ischaemic acute myocardial injury as defined by the UDMI remain hypothetical [42] This in part explains the vast disparities in the reported incidence of Type 2 AMI which can vary between 2% and 37% in hospitalised patients, [43,44] and between 5% and 71% in patients attending the Emergency Department.[41,45]

The gap between pathophysiological basis and clinical translation also extends to other concept AMI and myocardial injury including peri-intervention AMI (Type 4a and 5) as defined by the UDMI. [46] This has been problematic enough for studies to adopt their own protocol specific definitions and interpretation of the MI, including the use of different biomarker and biomarker-defined thresholds. [46-50]

Overall, for physicians, the 4th UDMI is itself a "translation" that requires "translation" [51]. This is because the 4th UDMI is grounded in pathophysiological concepts that our clinical investigations are often unable to confirm or refute. Unfortunately, troponin, that were adopted by the UDMI to aid the diagnosis and classification of myocardial infarction, didn't help much with the "translation".

In summary, cardiac troponins have transformed acute cardiovascular care, but clear deficiencies in troponin-guided triage of suspected acute myocardial infarction remain, including 1) slow release, 2) ambiguous safety of triage in early presenters and 3) low specificity for Type 1 AMI. The ideal biomarker of myocardial injury would possess the same cardiac selectivity as cardiac troponin, but with a faster release profile, enabling safe triage of very early presenters and in pre-hospital setting a higher specificity for

classic myocardial infarction (Type 1 AMI), allowing timely and accurate triage and delivery of evidence-based therapies.

1.2 Cardiac myosin-binding protein C (cMyC)™

Cardiac myosin-binding protein C (cMyC) is a cardiac-restricted thick myofilament structural and regulatory protein that was identified in the cardiac effluent.[52] A pre-commercial quantitative assay was developed by King's College London using high-affinity mouse monoclonal antibodies to cardiac-specific epitopes within the N-terminal domain.[53] This assay can detect minute quantities of myocardial necrosis, equivalent to 0.07 mg of the intact human heart and only few thousandths of the myocardial mass needs to die in order to exceed the 99th percentile. [54] Using the same assay, cMyC was shown to have a 10-fold greater abundance than cTnI or cTnT .[54] Additionally, the cMyC concentration rises more quickly in the systemic circulation than hs-cTnT after iatrogenic AMI (transcoronary ablation of septal hypertrophy) or spontaneous type 1 AMI.[52,55,56]

Additionally, the clearance of cMyC is much faster than cTnT with a decay half-time of 5.5+/-0.8hrs vs 22+/- 5hrs, $p<0.0001$.[52]

The favourable physiological release and kinetic profile of cMyC translates into diagnostic advantages. An analysis confined to the initial presentation blood sample in a large prospective cohort showed that cMyC, compared to hs-cTnT or hs-cTnI, more accurately classified patients into rule-out or rule-in categories: Net Reclassification Improvement NRI +0.149 versus hs-cTnT, +0.235 versus hs-cTnI; ($P < 0.001$ for both), all at equivalent sensitivity for rule-in, and specificity for rule-out. A greater improvement was seen in early presenters (chest pain <3 h): NRI +0.256 versus hs-cTnT and +0.308 versus hs-cTnI; ($P < 0.001$ for both), with superior diagnostic accuracy of cMyC over cTnT (area under the curve AUC, 0.915 versus 0.892: $p = 0.022$).[57]

Further, in patients with chest pain undergoing in-ambulance blood draws (median time of 70 minutes after symptom onset), cMyC improved diagnostic discrimination of AMI over hs-cTnT. (AUC, 0.839 versus 0.813; P = 0.005).[58]

Diagnostic performance of cMyC within a state-of-art algorithm that incorporates change in concentration over 1-hour as well as presentation level of cMyC has also been examined and compared against that of hs-cTnI.

cMyC guided rule-in/rule-out pathway based on the ESC 0/1h-algorithm for triage of patients with suspected myocardial infarction was developed by Kaier et al. [1]

Diagnostic cMyC thresholds based on the best performing cut-off thresholds were derived and validated in the APACE cohort.

An ESC 0/1 h algorithm using cMyC safely ruled-out a greater proportion of patients than that using hs-cTnI and reduced the number of patients allocated to the stratification/triage grey zone (proportion of patients allocated to rule-out/rule-in: 67.1% vs 56.5%, for cMyC vs hs-cTnI, respectively, $p < 0.05$).[1,59]

The advantages summarised above are predominantly through the greater abundance of cMyC enhancing sensitivity. Additionally, the greater abundance of cMyC with a lower limit of quantification an order of magnitude below the rule out threshold could theoretically facilitate the migration of the cMyC assay onto a handheld device. [58]

In summary, cMyC is a novel biomarker of myocardial injury, with a promising role in the triage and risk stratification of patients presenting with acute cardiac disease.

The research work presented in this thesis aims to further examine the characteristics of cMyC in the following settings:

1. The biological variation of cMyC in healthy volunteers
2. The diagnostic performance of cMyC in patients with renal dysfunction
3. The kinetic profile of cMyC after induced ischaemia
4. The fragmentation pattern of cMyC in different types of myocardial injury.

1.3 Hypotheses

1.3.1 *Hypothesis 1*

Measuring the short- and long-term biological variability of cMyC will allow us to determine the minimal change in concentration on serial testing that indicates acute or chronic myocardial damage.

1.3.2 *Hypothesis 2*

In patients with renal dysfunction the diagnostic performance of a cMyC-based 0/1 h triage algorithm for myocardial infarction is comparable to that of cardiac troponins (cTnI and cTnT)

1.3.3 *Hypothesis 3*

Brief induced ischaemia in humans causes temporal cMyC release that is comparable to that of cardiac troponin

1.3.4 *Hypothesis 4*

Calpain mediated cleavage of cMyC is dependent on its phosphorylation status and can therefore aid the differentiation between Type 1 AMI and other types of myocardial injury

2 Chapter 2. The biological variability of cardiac myosin-binding protein C

Hypothesis

Measuring the short- and long-term biological variability of cMyC will allow us to determine the minimal change in concentration on serial testing that indicates acute or chronic myocardial damage.

2.1 Introduction

2.1.1 Biological variability of an analyte

All biological analytes change with time.[60] This is not limited to pathological states where temporal change and rates of change have diagnostic and prognostic applications, but also occurs in health.

Three types of biological variation of laboratory analytes in the healthy state are described:

1. Age related variation, or variation over the span of life
2. Predictable cyclical variation, which can be diurnal, monthly, or seasonal
3. Random, unpredictable biological variation which applies to most analytes assayed in laboratory medicine. [61,62]

Understanding the biological variation of an analyte has several applications:

1. Calculating the reference change value (RCV) used to assess the significance of changes in serial measurements. Changes that exceed the RCV are deemed clinically significant from a diagnostic or a prognostic point of view. [61]

2. Determining the number and frequency of samples required to establish homeostatic set points of an individual in health and disease. This is usually done with a stated probability. [61,63]
3. Quality control and setting analytical standards (quality goals), by determining precision, bias and the derived allowable total error (TEa) prior to using the test in patients. Total method error TEm must not exceed the total allowable error TEa which is set by regulatory bodies and can vary from one country to another and is also calculated from published biological variation data. [61,64,65]

2.1.2 Reference change value (RCV)

Calculating and interpreting the RCV requires knowledge of three sources of variance:

- Within-subject or intra-individual biological variation (CV_I) is the average random, physiological fluctuation of an analyte around the "inherent" homeostatic set point (normal value of physiological variable) of individuals. In other words, CV_I is the distribution of repeated tests taken from the same subject under the reference condition which could be "health" or "disease". An example of the latter is the natural fluctuation of the levels of biomarkers of myocardial injury in patients with renal failure or heart failure. [61,66]
- Between-subjects or inter-individual biological variation (CV_G) is the variation between the homeostatic set-points of different individuals under the same reference conditions used for determining the CV_I.[61,66,67]
- The analytical variation (CV_A) of the analytical method used to measure the analyte, or the variation of measurements that is attributed entirely to the analyser (also known as the analytical method imprecision or coefficient of variance).

CV_A adds variability to CV_I to make the total intra-individual biological variation

$$CV_I\text{-}T=\sqrt{CV_A{}^2 + CV_I{}^2}$$

The ratio of CV_I-T to CV_G is called the index of individuality (II)

$$II = \sqrt{CV_A{}^2 + CV_I{}^2}\,/CV_G{}^2$$

II is used to assess the appropriateness of applying population-based reference intervals to individuals. Low II indicates high individuality, as a result of more significant CV_G compared to CV_I-T, consequently, population-based reference intervals will be less useful and could increase the fraction of false positive results.[61] Therefore, it would be more useful to measure changes in serial tests. [9,68]

Stratification by sex, age, or risk factors will modify II.[63]

Combining analytical and biological variations CV_I and CV_G is used to calculate the Reference Change Value RCV by using the following formula:

$$RCV = \sqrt{2}\ x\ Z\ x\sqrt{CV_A{}^2 + CV_I{}^2}$$

Z is the number of standard deviations appropriate to the probability, [61,69] and is usually chosen to a 95% probability of significance.

2.1.3 Confounders of biological and analytical variability

Robust, reproducible, and clinically valid determination of RCV requires minimising, ideally eliminating, several potential confounders that are grouped into three classes:

- Pre-analytical

- Analytical
- Post-analytical

Pre-analytical

- *Population:* age, sex, ethnicity and geography are known covariates in clinical pathology, therefore, choosing the right mix that best approximates the known or expected cohort where the RCV will be applied is important.

"Healthy status": In principle, RCV is utilised to determine the difference between "normal" and "pathological" variation in the studied analyte, therefore, it seems reasonable to choose "healthy" individuals as a reference cohort. However, in reality, perfect state of health almost never exists, [70] an alternative would be to select reference individuals with "reasonable" state of health[71]. Further, the state of "health" is variably defined and reported in biological variation studies, using clinical, biochemical or imaging parameters, which adds to the heterogeneity of the results and conclusions. Additionally, the concept of "healthy" might be viewed as too restrictive for deriving clinically relevant reference values that are appropriate to the cohort of interest. RCV derived from healthy populations might vary from RCV calculated from patients with specific conditions, or risk factors i.e. smokers, or patients with renal or heart failure. In theory, an ideal reference population should mirror the patient group, with the exception of the disease in question. Such cohorts could exist within a laboratory database from patients who actually required the test to screen for, rule-in/out the condition of interest, additionally, they would have had their samples collected and processed as would happen in real life.[70]

Pathology can modify the set-point in affected patients and possibly the variation around it.[72] By default, where pathological conditions increase the level of the analyte, similar to the effect of renal failure on the levels of biomarkers of myocardial injury, RCVs tend to be generally higher in healthy individuals due to a higher analytical variability at lower concentrations of the biomarkers. [9]

- *Steady characteristics and health status*

Biological variation studies require steady status of participants.[73] Repeated checks at every visit to ensure stable characteristics including lifestyle, weight, BP etc would minimise the risk of status-drift, unless drift is inherent.

- *Sampling*: timing, especially for analytes that are known to exhibit circadian variation; posture, and fasting and exercise status should be pre-determined and standardised for all participants. Additionally, sample collection technique including blood draw system, collection tubes and sample handling (centrifugation, aliquoting, freezing, storing and transporting) should also be homogenised for all samples.
- *Thaw-freeze cycles*: for analytes that are not intended to be measured immediately after blood draw, it is important to avoid multiple or variable number of thaw-freeze cycles which could compromise the integrity of the analyte or the matrix. Prolonged storage of the analyte could also lead to its decay.

Analytical

Samples should be analysed using the same analyser, ideally in one batch if stored before analysis, by the same technician, and with minimal number of plates to minimise differences in standard curves.

Analytical specifications of the analyser used must be stated clearly.

Post-analytical (statistical)

Ensuring steady-state:

For the biological variation data to be meaningful, the homeostatic set-point for all individuals is assumed to be static, and therefore all individuals included are assumed to be in steady-state. Simple linear regression can be used to identify trends that could indicate that a non-steady-state situation is present[31,63]

Identification and removal of outliers:

Three groups of outliers should be identified and excluded:

- Outliers between duplicates (analytical): according to Burnett's method [74], an outlier is defined as a result, which lies further than some multiple, *m* (*m* is a constant determined by the sample size) of standard deviations from the mean.
- Outliers between samples within an individual (within-subject)
- Outliers among mean values of subjects (between subjects): where the average concentration of analyte for one subject is clearly different from the others, usually identified by Reed's criterion, which considers the variance between the extreme value and the next highest or lowest value.[75] Subjects are excluded if the variance exceeds one third of the range of all measurements. This test assumes the data are normally distributed.

Normality of distribution:

After removing outliers, the normality of data distribution should be tested since this is a requisite precondition to calculate confidence intervals.

This is done by pooling standardised residuals (differences between values and the mean for all levels; within replicates, within subjects, and between subjects) and assessing the distribution of the pool.[63]

If data does not conform to a Gaussian distribution, values should be transformed into natural logarithms [76], before the assessment of the homogeneity of the analytical and within-subject variances and the calculation of biological and analytical variation.

Homogeneity

Homogeneity of the analytical and within-subject variances is commonly tested using Cochrane's or Bartlett's tests. Subjects exhibiting non-homogeneity can be identified by plotting the cumulated fractions of the ranked individual variation results on a Rankin scale as a function of the within-subject variation, and subjects are excluded until homogeneity of variance is achieved. [77]

Number of excluded subjects/samples should be noted, which is important for assessing the remaining subjects' true representativeness of the overall population. Additionally, excluded subjects could be assessed for presence of common traits that could explain their inherent heterogeneity.[31,63]

It is acknowledged that there is no ideal method for identifying outliers or achieving homogeneity of variances.[78] Additionally, differentiating between true aberrancy and the

extreme values which are part of the normal physiological distribution of results from healthy subjects, is not always straightforward. [78] Consequently, stringent methods for the identification and exclusion of the outliers might produce residuals that are less representative of the population of interest.

2.1.4 Shortfalls of RCV

RCV could allow the definition of the minimal change above which a biomarker's rise/fall could be pathological. However, using RCV to determine the exact pathological magnitude or rise/fall required for diagnosing acute myocardial damage might be ambitious, for several reasons and has inherent flaws, some of which are the same for population-based reference intervals.

RCV is assay dependent, mainly due to differences in the precision of the assays at low concentrations. [10]

RCV is also reference cohort and condition dependent, another reason why studies of biological variation of troponins in healthy volunteers have yielded varying results for RCV.[9] Some might advocate measuring RCV in more "relevant" cohorts than healthy volunteers, i.e. patients presenting to emergency department with non-cardiac chest pain.[79] However, the predictable heterogeneities within and between similar studies are likely to produce confounding results.

Additionally, and of significant relevance, the kinetics of the biomarkers of myocardial injury are time/phase dependent, they appear to rise in the early phase after the event much faster than they fall in the late phase, therefore, the later the presentation after the biomarker has plateaued the smaller the observed change in the concentration of the

biomarker within the index interval, change might also be negligible or hard detect in the very early phase after the event or surrounding peak concentration. [80] Therefore, in an ideal world, different RCVs should be used depending on the phase after the index event. In the real world, this is almost impossible, as there are other factors that are very difficult to adjust for, including the size of the infarct, presence of ongoing ischaemia, unknown degree of coronary occlusion/re-perfusion, and other pre/co-morbidities like renal or cardiac dysfunction. These mean that the phase of the change in biomarker concentration cannot be reliably predicted by time from the event(s) causing realise, even if this can be determined.

2.1.5 Quality control and setting analytical performance standards (quality goals)

Quality control in laboratory medicine requires clearly defined analytical performance standards, to produce limits for tolerable or allowable analytical errors that comprise the following[81]:

- "Imprecision", also referred to as "random error" which assesses the reproducibility of the results obtained from multiple testing of the same sample.
- "Bias", also known as "systematic error" assesses the deviation of the results obtained from one analytical method from those obtained from a "definitive method", which produced the "true" value of the analyte. Bias could be constant, or proportional (varies depending on concentration).

Setting performance goals helps to look at the impact of bias and precision on patients' "misclassification"

Imprecision

The concept of basing analytical quality goals on biology was first proposed by Tonks et al, and Cotlove et al, in the 60s and 70s of last century. [82,83] Four decades later, with an expanding bank of data on biological variation of many analytes, Stockl et al, and Fraser et al [84,85] recommended this approach of using within- and between-subject biological variation to derive desirable performance standards, for diagnostic and monitoring purposes, compared to other approaches such as the "state of the art", "expert groups and expert bodies", "clinical opinions", and analytical goals derived from " specific clinical situation".

Cotlove et al, in 1970 recommended that the analytical coefficient of variation (CV_A) should be less than half the within-subject variation (CV_I)

$$CV_A < 0.5 \times CV_I$$

This was proposed to limit the contribution of CV_A to the total within-subject variability (fraction of test variation caused by analytical variation) to less than 11.8 %, which was considered acceptable.[83]

$$CV_I\text{-}T = \sqrt{CV_A{}^2 + CV_I{}^2}$$

It is important to note that the desirable fraction, known as imprecision, was empirically chosen by Cotlove et al. This was later appraised by Fraser et al, who proposed that one should take into consideration the following factors when determining the desirable imprecision 1) the within-subject biological variability of the analyte and 2) the

achievability of the proposed analytical standards with the available technology and methodology.

Stringent "optimum" analytical standards should be considered for analytes with large within-subject biological variation.

$CV_A < 0.25 \times CV_I$

Whereas, "minimum" standards should be targeted for analytes that exhibit minimal biological variation if the technology or method used is unable to attain the desirable precision.[85]

$CV_A < 0.75 \times CV_I$

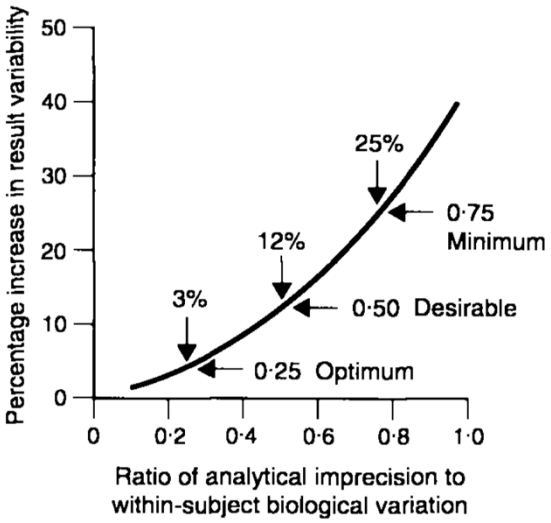

Figure 2.1 The percentage increase in test result variability as a function of the ratio of analytical imprecision to within-subject biological variation. From Fraser at al.[61]

Bias

The concept that the maximum acceptable bias (B_a) should be less than one-quarter of the sum of within- and between-subject biological variation for reference intervals to be used within a geographical area, was first proposed by Gowans et al.[86]

$$B_a < 0.25 \times \sqrt{CV_I{}^2 + CV_G{}^2}$$

Fraser et al, adopted Gowans proposal, but similar to their approach to imprecision, suggested the addition of two tiers for performance standards: minimum, with a ratio of acceptable bias to biological variation of 0.375

$$B_a < 0.375 \times \sqrt{CV_I{}^2 + CV_G{}^2}$$

And maximum or optimum, with a ratio of 0.125

$$B_a < 0.125 \times \sqrt{CV_I{}^2 + CV_G{}^2}$$

Similar to imprecision, acceptable bias also depends on the biological variation of the analyte and the attainability of the desired performance standards with the technology or methodology used.

Allowable total error TEa

TEa defines the limits for the total allowable variation of the analytical performance that is tolerable. TEa is calculated by combining imprecision and bias using the following formula:

TEa = Z score x (Allowable imprecision) + Allowable bias

Z value corresponds to a defined one-sided confidence probability of inclusion inside the targeted limits and depends on the desired stringency of the test. according to the principle of Gaussian distribution: when a targeted limit is 95% (5% of the results fall outside the targeted limit), the corresponding z value is 1.65, one-sided.

TEa = 1.65 x (Allowable imprecision) + Allowable bias

TEa is also used to define three levels of analytical goals

Using the three-tiered levels of allowable or desired imprecision and bias, TEa is also classed within a three-level model into minimum, desirable and optimum.[73,85,87,88]

Basing analytical performance standards on biology has many advantages, including the simplicity of the model, which is easy to interpret, the increasingly available data on clinical variability, and the reproducibility of within- and between-subject biological variations for many analytes. [84] However, evaluating the effect of analytical performance on clinical outcomes in specific clinical settings is still considered the ultimate method to extract and recommend analytical performance standards, provided, there is robust clinical data to allow such derivation. [70,84,89-91]

2.1.6 Summary and aims of this research project:

Detecting a significant change in consecutive results requires an understanding of analytical and biological variation (BV).

Biological variation studies also help to calculate index of individuality and define analytical quality specifications for routine assays. The latter application of BV studies is

most relevant to new biomarkers such as cMyC where data on clinical outcomes in specific clinical settings is yet to be elucidated.

The purposes of the studies in this chapter are to examine the long- and short-term biological variation of cMyC in healthy individuals to 1) better understand its potential as a marker of acute and chronic myocardial injury, and 2) provide data to inform the analytical quality specification of potential assays for clinical use.

I have conducted three studies to address the hypothesis:

1. Long-term (weekly) biological variation of cardiac myosin-binding protein C in healthy individuals
2. Short-term (hourly) biological variation of cardiac myosin-binding protein C
3. The diurnal variation of cardiac myosin-binding protein C

2.2 Long-term (weekly) biological variation of cardiac myosin-binding protein C in healthy individuals

- -

Published work, please see appendix for full manuscript

Statement of contribution

Bashir Alaour co-designed the study, completed, and submitted the local ethical approval application for London's cohort, recruited all UK participants and led sample collection. He organised sample analysis for all three participating centres. He performed data analysis and wrote the manuscript.

2.2.1 Abstract

Background

Understanding the long-term biological variation of an analyte has several diagnostic and quality control applications. We assess the weekly biological variation of cMyC, to examine its potential in monitoring chronic myocardial injury, and to suggest analytical quality specification for routine use of the test in clinical practice.

Methods

Thirty healthy volunteers were included. Non-fasting samples were obtained once a week for ten consecutive weeks. Samples were tested in duplicate on the Erenna® platform by EMD Millipore Corporation. Outlying measurements and subjects were identified and excluded systematically, and homogeneity of analytical and within-subject variances was

achieved before calculating the biological variability (CV_I and CV_G), reference change values (RCV) and index of individuality (II).

Results

Mean age was 38 (range, 21-64) years, and 16 participants were women (53%).

The biological variation, RCV and II with 95% confidence interval (CI) were : CV_A (%) 19.5 (17.8 – 21.6), CV_I (%) 17.8 (14.8 – 21.0), CV_G (%) 66.9 (50.4 – 109.9), RCV (%) 106.7 (96.6 – 120.1)/ -51.6 (-54.6 – -49.1) and II 0.42 (0.29 – 0.56).

There was a trend for women to have lower CV_G. The calculated RCVs were comparable between genders.

Conclusion

cMyC exhibits acceptable RCV and low II suggesting that it could be suitable for disease monitoring, risk stratification and prognostication if measured serially. Analytical quality specifications based on biological variation are similar to those for cardiac troponin and should be achievable at clinically relevant concentrations.

Keywords

Cardiac myosin-binding protein C, biological variation, Reference Change Value, index of individuality.

2.2.2 Introduction

Understanding the long-term biological variation of an analyte has several applications including suggesting analytical quality specifications for routine assays, determining the number and frequency of sampling required to establish homeostatic set points of an individual, calculating the index of individuality, and determining physiological variations in consecutive results. The latter is useful for prognostication and risk stratification, chronic cardiovascular disease monitoring and detecting or predicting cardiac damage in the context of chronic non-cardiac conditions or long-term exposure to cardiotoxic agents. The EFLM suggest three different models for setting analytical quality specifications [90] of which biological variation seems the most applicable for novel markers, until a larger number of outcome studies or robust state of the art data become available. [92] The purposes of the current study are to examine the biological variation of cMyC in healthy individuals to 1) better understand its potential as a marker of chronic myocardial injury, and 2) provide data as for which analytical quality specification for routine use of the assay may be suggested.

2.2.3 Materials, subjects and methods

Ethics

This study was carried out according to the principles of the Declaration of Helsinki. The protocol was approved by the respective regional ethics committee at each centre: South Central - Berkshire Research Ethics Committee, London, (see appendix), and the Regional Committee for Medical and Health Research Ethics in Bergen (Bergen and Oslo).

Unified informed consent from across centres was obtained from all volunteers.

Volunteers

Thirty healthy volunteers were recruited from London (King's College London and Guys and St Thomas' Hospital), Bergen (Haukeland University Hospital) and Oslo (University of Oslo and Akershus University Hospital), 10 volunteers were recruited from each centre.

The opportunity to participate in the study was advertised locally via posters and circulated "Research Opportunities" emails amongst the staff of each of the participating centres.

Screening, inclusion and exclusion criteria:

Potential participants were screened according to the following criteria:

Inclusion criteria: Healthy individuals of age between 18 and 75.

Exclusion criteria: any evident disease, current pregnancy, use of cardiac medications, previous history of acute or chronic cardiac illness, any chronic non-cardiac illness including cancer in remission during the past 5 years, or any of the following abnormalities on screening blood tests

- eGFR <60 mL/min/1.73m^2
- NT-ProBNP > local reference limit
- Troponin T (hs-cTnT)> 99th percentile value (> 13 ng/L)

Sample collection, processing and analysis

To minimise pre-analytical variability, a unified Standard Operating Protocol (SOP) was used across all centres. Venous blood sampling was performed weekly, on the same weekday +/-1 day, for 10 consecutive weeks from October to December 2018. Non-fasting blood samples were drawn between 08.00 and 10.00 am. Smoking, alcohol intake and exercise were reviewed and documented during each visit.

Participants rested for 15 minutes before blood was collected into 3.5 mL plastic serum-separation Vacutainer SST II Advance gel tubes (Becton Dickinson) using a 21 Gauge winged blood collection set with flexible tube needle (Becton Dickinson). Samples were allowed to clot for 30 minutes at room temperature and then centrifuged at 2200 x *g* for 10 minutes at 20°C. Separated acellular serum (0.9 mL) was then aliquoted into matching cryovial tubes (1.5 ml Mikroröhre PCR-PT, SARSTEDT AG & Co. KG) before being frozen at -80 °C within 2 hours after phlebotomy.

Samples were shipped simultaneously from all centres on dry ice for cMyC measurement.

Sample analysis

All serum samples were tested in duplicate on the Erenna® platform by EMD Millipore Corporation, Hayward California. LoD 0.4 ng/L; LoQ (20% CV_A) of 1.2 ng/L; intra-series precision (CV, 11 +/- 3%) and inter-series precision (CV, 13 +/- 3%) [52]. There were three missing samples.

Statistics

Data were analysed twice by two independent researchers: KMA and BA, using the following platforms: Excel 2016 and SPSS version 26.0 (KMA), and R version 3.6.1 (BA).

Baseline characteristics were described using percentage, means or medians (standard deviation and first quartile-third quartile where applicable).

Student's *t* test and Mann-Whitney U test were used for comparing groups as appropriate.

Shapiro-Wilk test was used to verify the normality of distribution.

Calculation of biological variation parameters

1. *Analytical outliers* were identified as per Burnett's method [74]. An outlier was defined as a result, which lies further than some multiple, m (m is a constant determined by the sample size) of standard deviations from the mean.
2. *Stability of subjects*: Subjects that expressed a *non-steady-state* were identified with simple linear regression. The trend was calculated as a percentage of change from the first result. Individual slopes (per participant) of linear regressions were derived. Unstable trends (significantly deviating from 0, $p < 0.01$) were identified and respective subjects were excluded. Then homogeneity of the remaining slopes was tested using linear mixed effect models. ANOVA was used to test whether introducing the slope as a random effect (allowing the slopes to vary) would improve the fit of the model. High ranked slopes were removed until homogeneity was achieved.
3. *Outliers in mean values of subjects* were defined according to Reed's criterion which rejects extreme values if the difference between them and the next highest (or lowest) exceeds one-third of the range of all values [75].
4. *The distribution of the residual data (means of duplicates)* was tested using Shapiro-Wilk test. As data did not conform to a Gaussian distribution, values were transformed into natural logarithms [76].

5. *Homogeneity of analytical and between-subject variances (ln transformed data)* Analytical (n= residual duplicates) and between-subject (n=residual subjects) variances were calculated and ranked. Homogeneity of variances was tested using Cochran's and Bartlett's methods, outlying values were excluded until homogeneity was achieved [77].
6. Calculations of σ_A, σ_I and σ_G were performed (ln transformed data) using nested ANOVA. The σ was thereafter retransformed into CV_A, CV_I, and CV_G using:

$$CV_{ln} = \sqrt{(\exp \sigma^2 - 1)} \times 100$$

in which σ is the estimated standard deviation for the ln-transformed data and CV_{ln} is the adjoining re-transformed CV.

The RCV values (with 95% confidence intervals) were calculated according to Fokkema et al [76]. This method is applicable for skewed data as it will always return negative RCV data that are interpretable in clinical practice (not exceeding 100%):

$$\text{RCV pos} = \left[\exp\left(1.96 \text{ x } 2^{\frac{1}{2}} \times ({\sigma_A}^2 + {\sigma_I}^2)^{\frac{1}{2}}\right) - 1\right] \times 100$$

$$\text{RCV neg} = \left[\exp\left(-1.96 \text{ x } 2^{\frac{1}{2}} \times ({\sigma_A}^2 + {\sigma_I}^2)^{\frac{1}{2}}\right) - 1\right] \times 100$$

in which σ_A is the analytic standard deviation and σ_I is the within-person standard deviation of the logarithmic data. Due to the CV_I exceeding 12%, we choose to also calculate the RCVs in the total cohort using the non-parametric method, as described by Røraas et al [93]. This method is less precise compared to Fokkema, but fits all measurement distributions.

The index of individuality II was calculated using the retransformed data as follows:

$$II = \sqrt{CV_A{}^2 + CV_I{}^2}\,/CV_G$$

Separate calculations were performed in the total cohort, gender-stratified groups, using the methodology above for excluding the outliers and calculating biological variation, RCV and II.

2.2.4 Results

None of the samples had undetectable cMyC concentrations (below LoD).

Baseline characteristics of participants contributing to total and gender-stratified cohorts are shown in table 2.1.

	Total n=30	Women n=16	Men n=14	p value (women vs men)
Age, mean (range)	38 (21-64)	41 (21-64)	35 (21-44)	0.173
BMI, kg/m2	22.8 (2.6)	22.35 (3)	23.38 (2.2)	0.303
Glucose, mmol/L	5.1 (0.6)	4.9 (0.5)	5.2 (0.6)	0.172
eGFR (CKD-EPIcreat), ml/min/1.73m^2	97.7 (14.7)	95 (13.7)	100.6 (15.9)	0.308
Troponin T, ng/L [a]	3.3 (2)	3.3 (2.2)	3.3 (1.9)	0.917
NT-ProBNP, ng/L	47.9 (31.5)	61 (36.5)	33 (15.7)	0.013
Regular medications (%) [b]	3.3	6.25	0	0.359

Table 2.1 Baseline characteristics.

Data are expressed as medians [1st quartile, 3rd quartile] or means ± standard deviation, for categorical variables are expressed as numbers (percentages). BMI = Body mass index; eGFR = estimated glomerular filtration rate. [a] values below LoD were reported as 50% of the local lower limit of reportable result, 2 ng/L or 1.5 ng/L, respectively. [b] non-cardiac drug.

16 participants were women (53%). Mean age was 38 (range, 21-64), there was no significant age difference between women and men (mean age, 41 and 35 respectively; p = 0.173). Two participants were daily smokers. NT-proBNP concentrations were higher in women compared to men (61 +/- 36.5 vs 33 +/- 15.7 ng/L, respectively; p= 0.013), however, none of the participants had NT-proBNP above the reference interval. Otherwise, both groups had similar baseline characteristics as listed in table 2.1.

The distribution of cMyC concentrations across participants is shown in figure 2.2.

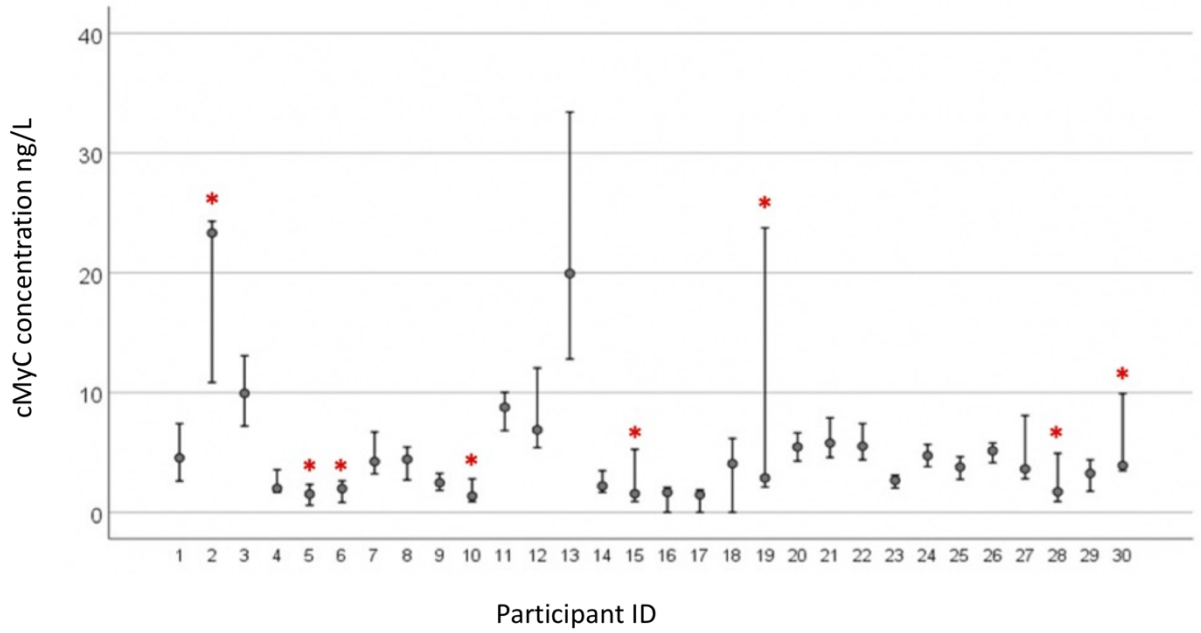

Figure 2.2 Distribution of cMyC concentrations across participants

Concentration (range, median) of cMyC (ng/L) for the 30 participants included in the study.

* Outliers identified and removed from the total cohort

N.B: Different groups of outliers were excluded for the gender-stratified groups.

Total cohort:

216 samples from 22 participants (11 women and 11 men) were included in the calculation of biological variability, after the exclusion of outliers, as described in the method section, figure 2.3 and table 2.2. None of the excluded subjects were smokers.

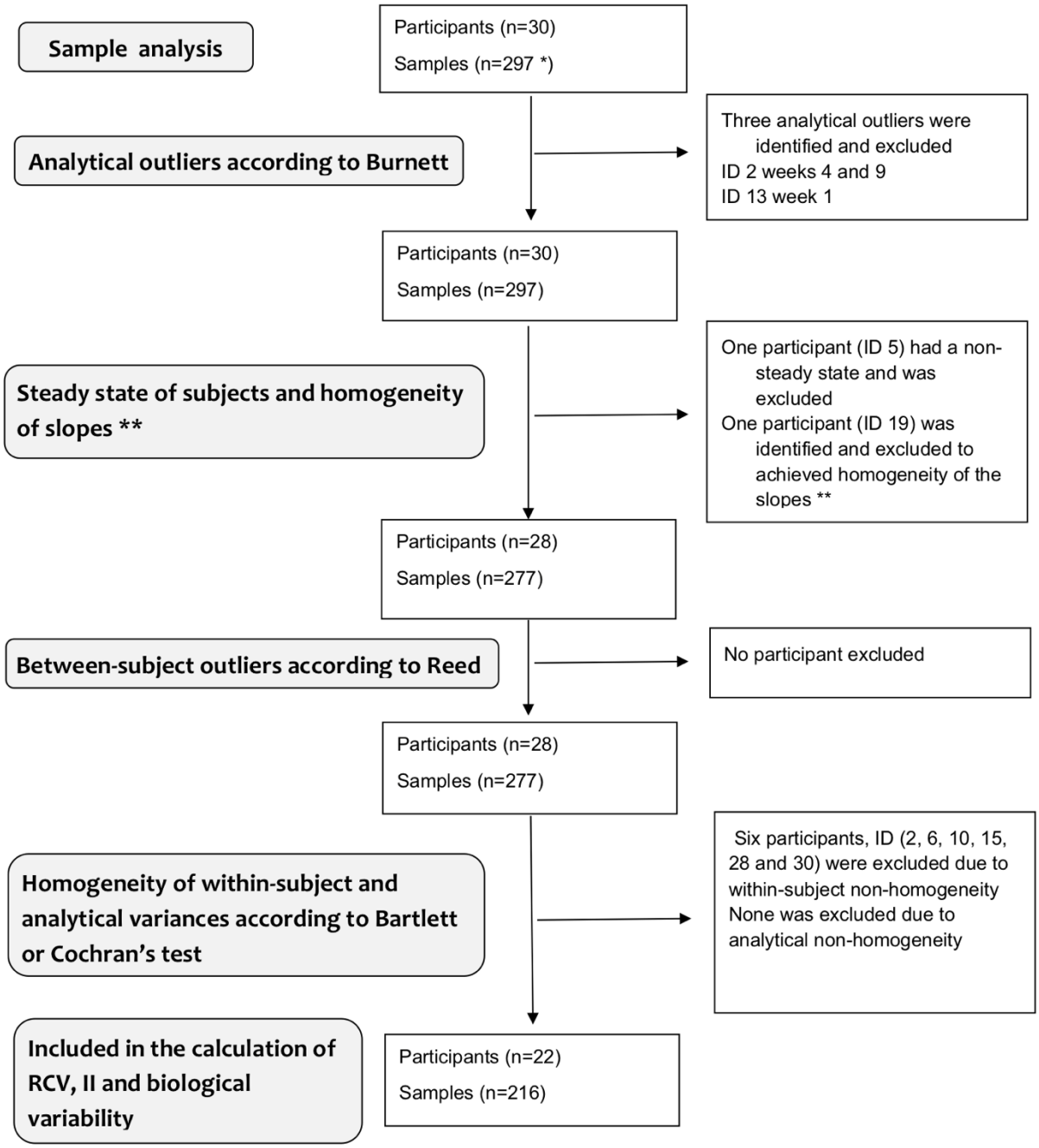

Figure 2.3 Exclusion of outliers from total cohort

* Missing samples; ** slopes of linear regressions of temporal percentage of changes from first readings.

	Total	Women	Men
Analytical outliers	ID 2, week 4 and 9 ID 13, week 1	ID 12, week 2 and 9	ID 2, week 4 and 9
Exclusion due to a significant 10-week trend (p value < 0.01)	ID 5	ID 5	None
Exclusion to achieve homogeneity of slopes (for temporal change)	ID 19	None	ID 19
Exclusion due to Reed's criterion	None	None	None
Exclusion due to within-subject non-homogeneity according to Bartlett or Cochran's test	6 subjects (ID 2, 6, 10, 15, 28, 30) a	3 subjects (ID 6, 15 and 28) a	One subjects (ID 2) a
Exclusion due to analytical non-homogeneity according to Bartlett or Cochran's test	None	None	1 duplicate (ID 17, week 9)

Table 2.2 Exclusion of outliers.

Data are expressed as medians [1st quartile, 3rd quartile] or means ± standard deviation, for categorical variables are expressed as numbers (percentages). BMI = Body mass index; eGFR = estimated glomerular filtration rate. [a] values below LoD were reported as 50% of the local lower limit of reportable result, 2 ng/L or 1.5 ng/L, respectively. [b] non-cardiac drug.

The following results were obtained:

CV_A 19.5 % (17.8 – 21.6 %), CV_I 17.8 % (14.8 – 21.0 %), CV_G 66.9 % (50.4 – 109.9 %), RCV 106.7 % (96.6 – 120.1 %)/ -51.6 % (-54.6 – -49.1 %) and II 0.42 (0.29 – 0.56), (table 2.3).

When RCV was calculated using the non-parametric method, corresponding values were 100.1% and -50.5%, respectively.

	Total	Women	Men
Number of participants	30	16	14
Number of participants [a]	22	12	12
Numbers of samples [a]	216	118	116
cMyC concentration, ng/L, median (Q1-Q3)	4.38 (2.75-5.97)	3.54 (2.47-5.25)	4.58 (3.25-6.58)
CV_A, mean (95% CI), %	19.5 (17.8 – 21.6)	20.2 (17.9 – 23.3)	16.8 (14.9 – 19.4)
CV_I, mean (95% CI), %	17.8 (14.8 – 21.0)	19.7 (15.5 – 24.5)	20.3 (16.6 – 24.6)
CV_B, mean (95% CI), %	66.9 (50.4 – 109.9)	55.7 (37.9 – 110.8)	83.1 (55.6 – 195.9)
Positive RCV, mean (95% CI), %	106.7 (96.6 – 120.1)	117.2 (102.3 – 139.1)	106.2 (92.3 – 126.4)
Negative RCV, mean (95% CI), %	-51.6 (-54.6 – -49.1)	-54.0 (-58.2 – -50.6)	-51.5 (-55.8 – - 48.0)
Index of individuality II	0.42 (0.29 – 0.56)	0.53 (0.30 – 0.78)	0.35 (0.20 – 0.52)

Table 2.3 Analytical and biological variation.

Analytical and biological variation, RCV and II of cMyC. [a] after excluding outliers

Gender- stratified subgroups:

A total of 118 samples from 12 women and 116 samples from 12 men were included in the calculation of gender-specific biological variability. The number of included individuals and samples were different from the total cohort, as the whole procedure of outlier exclusion was repeated in each gender-stratified data set (table 2)

A significant difference in cMyC values between women and men was observed: median (Q1-Q3) 3.54 (2.47 – 5.25) vs 4.58 (3.25 – 6.58) ng/L; respectively; p= 0.007. The CV_I was comparable across both groups, 19.7 % (15.5 – 24.5 %) and 20.3 % (16.6 – 24.6 %) for women vs men, respectively. There was a trend for women to have higher CV_A 20.2 % (17.9 – 23.3 %) vs 16.8 % (14.9 – 19.4 %) and lower CV_G 55.7 % (37.9 – 110.8 %) vs 83.1 % (55.6 – 195.9 %).

Calculated RCVs were comparable in both groups, + 117 %/-54% vs +106 %/-51 % for women vs men, respectively, however, women had higher II at 0.53 (0.30 – 0.78) compared to men 0.35 (0.20 – 0.52) (table 3).

2.2.5 Discussion

The main finding in this study is that the weekly biological variation, RCV of cMyC in healthy individuals, quantified with the Erenna® platform at EMD Millipore Corporation, is moderate and comparable to other cardiac ischemia markers (cardiac troponin). The II is low. No important gender differences were observed. These measures of variation are important to define the minimal magnitude of change in the concentration of cMyC beyond which pathological processes are likely to be present, and to help guide analytical performance criteria for the assay when implemented in the routine laboratory.

Our data demonstrate a within-subject CV_I and between-subject CV_G of 17.8 % (14.8 – 21.0 %), and 66.9 % (50.4 – 109.9 %), respectively. Both fall within the range of respective CV calculated in similar cTn long term biological variability studies (Table 4) [10,26,28,31,94-98]. The derived index of individuality II was also similar to that for cTn. The low II suggesting high individuality. This favours interpreting serial changes of cMyC concentration in the individual patient rather than using population-based reference

intervals, since the later could increase the fraction of falsely interpreted results [61]. Overall, the RCVs were 106.7 % (96.6 – 120.1 %) / -51.6 % (-54.6 – -49.1%), which also lie within the range of RCVs observed for cTn in similar long-term biological variability studies (Table 2.4). The moderate long-term biological variation and RCVs demonstrated in this study suggest that serial measurement of cMyC might have a value in monitoring chronic cardiac disease activity and the vulnerability of the heart to damage secondary to chronic non-cardiac pathology. Of note, the RCV value is dependent on the analytical variation. Laboratories with higher or lower CV_A will produce different RCVs compared to those we report. This could be adjusted for by including the local CV_A in the RCV calculations. The RCV is also reference-cohort and condition-dependent [9]. Cohorts with different types of pathology are likely to modify the haemostatic set-point and the variation around it. As a consequence, some advocate measuring biological variation and RCV in more "relevant" cohorts than healthy volunteers, i.e. measuring long-term RCV in patients with chronic but stable heart failure or renal disease, and short-term RCV in patients presenting to emergency department with non-cardiac chest pain [79]. Such data are of interest and should be reported, preferable together with data from healthy subjects for comparison.

On gender-stratified analysis, slightly higher RCVs were reported in women than in men, driven by higher analytical variability calculated in women, a rather expected result considering that the significantly lower median cMyC concentrations reported in women should return a higher CV_A. Lower levels of cTn in women compared to men have also been reported in healthy individuals in similar studies. Furthermore, a lower CV_G was found in women compared to men, 55.7% vs 83.1%, respectively, resulting in an overall higher II in women, 0.53 vs 0.35. Both IIs remained less than 0.6, suggesting high

individuality in both groups. The overlapping confidence intervals shown for these values indicate that no certain gender difference is evident. The majority of cTn biological variation studies did not report gender-stratified biological variation or RCVs. However, studies are encouraged to do so considering that gender-specific 99th percentile value of biomarkers are increasingly reported.

Until more data from outcome studies investigating the biomarker in different clinical situations become available analytical performance specifications might be based on biological variation. Our data suggest that the CV_A for the cMyC assay at concentrations used for routine diagnosis should be below 9% (half of CV_I) [62], which is very similar to current recommendations for cTn. Our calculated CV_A was 19.5% (17.8 – 21.6%), which is higher than CV_A reported in the majority of long term cTn biological variation studies (Table 3). However, our CV_A % was obtained from duplicates with median cMyC of 4.38 (2.75 – 5.97) ng/L, which is considerably lower than median cMyC found in patient with adjudicated diagnosis of acute coronary syndrome 237 (71 – 876) ng/L in the APACE cohort [57], and only ~ 5% of the 99th percentile (derived from patients without coronary artery disease) [53]. Lower CV_A should be expected at higher (more clinically relevant) concentrations, and we predict lower CV_A with future automated assays of cMyC. Further, a higher ratio of mean cTn to respective assay-specific 99th percentile was reported in similar long-term cTns biological variation studies [94].

Finally, our data indicate that the desirable analytical bias (i.e. calculated as $1/4(\sqrt{CV_I^2 + CV_G^2})$) should be 17% or lower. This is similar to what is commonly seen for lot variations for immunoassays. The allowable total error (precision and bias merged) should be below 28%.

This study has several strengths: 1) it is multi-centre, with unified protocol and standard operating procedure to minimise pre-analytical variability; 2) it included a relatively large number of participants, of which, 53 % were women; 3) "healthy status" was clearly defined, 4) exclusion of outliers was performed systematically and is described in the manuscript; 5) gender-stratified variability, RCV and II were measured. The RCVs were calculated using two different models, ln transformed data according to Fokkema and the more robust but less precise non-parametric method suggested by Røraas, the results were similar. The statistical analysis was performed by two independent researchers using two different software platforms.

The study also has limitations – the participant mean age was lower than in patients with chronic primary or secondary cardiac disease so the reported data may not be valid for cohorts with other characteristics. Samples were analysed 18 months after collection, however, these were continuously stored at -80 °C and thawed once for the analysis.

Author	Year [a]	*n* [b]	Frequency	Period	Age [c]	Assay	RCV (log)	CVA (%)	CVI (%)	CVB (%)	II (%)
cTnI											
Lan et al. [27]	2020	20	weekly	7 weeks	40 (22-70)	hs-TnI Abbott Alinity ci-series	+269.9/-73	14	47.9	25.8	1.69
Ceriotti et al.[86]	2020	89	weekly	10 weeks	20-60	hs-TnI Singulex Clarity	+59.7/-37.4	11.6	16.6	F 40.3 M 65.3	F 0.44 M 0.23
		91				hs-TnI Siemens Atellica	+50.1/-33.4	10.7	13.9	F 36.3 M 36.5	F 0.40 M 0.40
Schindler et al.[87]	2016	10	<= twice a week	3 weeks	50.9 (51-64)	hs-TnI Abbott Architect	+53/-34	4.8	14.5	44	0.3
Aakre et al.[30]	2014	20	weekly	10 weeks	61 (46-68)	hs-TnI Abbott Architect	+77/-44	13.8	15.6	25.9	0.8
Vasile et al.[9]	2011	20	fortnightly	8 weeks	39 (25-56)	hs-TnI Beckman Coulter	+14/-10.6	2.7	2.6	41	0.1
Wu et al.[88]	2009	17	fortnightly	8 weeks	19-58	hs-TnI Singulex	+81/-45	15	14	63	0.39
cTnT											
Meijers et al.[89]	2017	28	monthly	4 months	43 (13)	hs-TnT Roche Modular	+83.4/-27.0	1.5	16	51.2	0.3
Corte et al.[90]	2015	11	weekly	5 weeks	21-50	S-TnT Roche Cobas e411	+35/-26	5.1	5.9	30.4	0.3
Aakre et al.[30]	2014	20	weekly	10 weeks	61 (46-68)	hs-TnT Roche Modular	+42/-30	9.7	8.3	26.8	0.48
Frankenstein et al.[25]	2011	17	weekly	5 weeks	32 (22-59)	hs-TnT E 170	+138/-58	7.8	31	na	na
						hs-TnT Elecsys 2010	+135/-58	9.7	30	na	na
Vasile et al.[91]	2010	20	fortnightly	8 weeks	39 (25-56)	hs-TnT Roche Modular	+315	94	92	94	1.4

Table 2.4 Published long-term biological variation, RCV and II of cTns

Cardiac troponins' long-term biological variation, RCV and II as reported in recent studies. [a] Year published; [b] n=number of subjects; [e] expressed in mean (range) or range only. F, females; M, males.

2.2.6 Conclusion

cMyC exhibits acceptable biological variation, RCV and low II suggesting that it could be suitable for disease monitoring, risk stratification and prognostication if measured serially. Analytical quality specifications based on biological variation data are similar to those for cTn and should be achievable at clinically relevant concentrations. However, testing the RCV in cohorts with chronic cardiac disease and reported/measured outcomes is necessary to testify its ability to monitor disease activity and predict outcomes. However, future use of the biomarker will determine if specification should be based on clinical outcomes or biological variation.

2.3 Hourly biological variation of cardiac myosin binding protein C and cardiac troponins

- -

Statement of contribution:

Recruitment, sample collection and sample analysis for troponin were performed by our collaborators (Professor Steven Meex, et al. Maastricht University Medical Center, the Netherlands) before the start of this research fellowship.

Bashir Alaour organised sample analysis for cMyC, designed and performed all data analyses and statistics outlined in this study.

2.3.1 Abstract

Background:

Cardiac myosin-binding protein C (cMyC) has a promising role in the triage and risk stratification of patients presenting with acute cardiac disease. In this study, we compare the hourly biological variation of cMyC with that of cardiac troponins cTnI and cTnT, to examine the suitability of cMyC for dichotomising chronic from acute myocardial injury by using changes in concentration of overtime.

Methods:

Twenty-six consecutive hourly blood samples were drawn between 08.30 am and 09.30 am (+ 1 day) from 24 individuals without a recent history of acute myocardial infarction, for the measurement of cMyC, cardiac troponin T (Roche hs-cTnT) and I (Abbott hs-cTnI).

Outlying measurements and subjects were identified and excluded systematically, and homogeneity of analytical and within-subject variances was achieved before calculating the biological variability (CV_I and CV_G), reference change values (RCV) and index of individuality (II).

Results:

Mean age was 72yrs (±7). Five participants were women (21%), and 7 (29%) were diabetic (on oral glucose lowering medications).

The biological variation, RCV and II with 95% confidence interval (CI) for cMyC were: CV_A (%) 11.1 (10.1-12.2), CV_I (%) 13.4 (11.5-14.2), CV_G (%) 72.7 (61.6-84.7), RCV (%) 61.5 (53.0-68.4)/ -38.1 (-50.6 – -29.3) and II 0.23.

For hs-cTnT: CV_A (%) 2.4 (2.1-2.8), CV_I (%) 8.8 (6.0-12), CV_G (%) 41.2 (28.7-65.8), RCV (%) 28.71 (21.1-40.7)/ -22.3 (-39.2 – -15.6) and II 0.22.

For hs-cTnI: CV_A (%) 10.1 (9.2-11.4), CV_I (%) 8.9 (7.3-11.5), CV_G (%) 40.1 (30.0-61.9), RCV (%) 45.14 (30.1-61.3)/-31.1 (-41.8 – - 24.7) and II 0.33.

Conclusion:

cMyC exhibits low RCV and low II that are comparable to that of cardiac troponins, suggesting that it could be suitable for diagnosis and risk stratification of acute myocardial injury if measured serially.

2.3.2 Introduction

As discussed earlier, the favourable physiological release and kinetic profile of cMyC translated into diagnostic advantages, enabling timely, and efficient triage of patients with suspected acute myocardial infarction, with both presentation level, and delta (concentration change over one hour) using the state-of-art ESC based 0/1 h triage algorithm.[57,59]

For the latter, best performing diagnostic cMyC thresholds for 0/1 deltas were derived in the APACE cohort, using machine learning, and then tested in the same cohort, in a random 50:50 derivation:validation split.[1]

Understanding hourly biological variation of cMyC in stable individuals will ensure that those derived and tested deltas that indicate likely myocardial infarction are of a larger magnitude than that of the combination of natural physiological and analytic variation. Additionally, beyond acute myocardial infarction, calculation of the RCV will help to define the minimal magnitude of change in the concentration on serial testing, beyond which, acute pathological processes are likely responsible for release. Further, as detailed in 2.1, short-term biological variation studies enable the calculation of index of individuality and set analytical performance standards for routine clinical use of the biomarker in acute settings.[61,81,85,99,100]

Short-term biological and analytical variation of cardiac troponins have been repeatedly studied and presented in literature; however, diverse, and conflicting outputs have been reported. These disagreements are likely the result of differences in the reference populations, definition of health-status, stringency and methods of outlier exclusion, statistical methods and packages used and assays deployed.[10,26,28,79,96,101,102]

In addition to cMyC, studying the biological and analytical variation of current gold-standard biomarkers, cTnI and cTnT, in the same cohort, will provide fair comparison between the biomarkers.

This study aims to determine the hourly biological and analytical variation, RCV and II of cMyC, cTnT and cTnI.

The study was conducted in academic collaboration with the Cardiovascular Research Institute Maastricht (CARIM), Maastricht University Medical Center (MUMC), the Netherlands who kindly provided the samples for this study and the results of troponin measurements.

2.3.3 Methods

Ethics

This study was carried out according to the principles of the Declaration of Helsinki and Approved by the local Institutional Review Board and Ethics Committee. Written informed consent was obtained from all participants.

Volunteers, inclusion and exclusion criteria

Study participants are described by Klinkenberg et al.[103]

24 volunteers were included in the study. The exclusion criteria were active cardiac disease (angina pectoris, cardiomyopathy, and myocarditis), uncontrolled hypertension, anemia (Hb < 10.5 g/dL), renal dysfunction (eGFR < 59 mL/min/1.73m^2), and a history of AMI or stroke within 12 months before inclusion.

Participants were asked to refrain from strenuous exercise for 2 days and to fast overnight before the test period.

Sample collection and processing

From 8:30 AM till 9:30 AM the next day, participants were restricted to the laboratory environment and 8 mL of blood was drawn hourly in serum separating and EDTA tubes via an antecubital venous catheter. Extension lines were used between 11:30 PM and 07:00 AM to minimize sleep disturbance. Meals were consumed at 8:30 AM, 12:30 PM, and 5:30 PM. Blood samples were centrifuged at room temperature, then aliquoted into matched cryovial tubes before being frozen at -80 °C.

Sample analysis

All samples were tested in duplicate.

cMyC was tested on Erenna® platform by EMD Millipore Corporation, Hayward California. LoD 0.4 ng/L; LoQ (20% CVa) of 1.2 ng/L; intra-series precision (CV, 11 ± 3%) and inter-series precision (CV, 13 ± 3%) [52].

High sensitivity cTnT was measured with the Cobas 8000 (Roche Diagnostics) , the 99th percentile among healthy subjects is 14 ng/L, with a 10% analytical variation at 13 ng/L.

High sensitivity cTnI was measured with the STAT assay (Architect I 2000 SR, Abbott Diagnostics). According to the manufacturer, the LoD is 1.1–1.9 ng/L, and 99th percentile of this assay is 26.2 ng/L with a corresponding coefficient of variation of <5%

Statistics

Data were analysed using R version 3.6.1.

Baseline characteristics were described using percentage, means or medians (standard deviation and first quartile-third quartile where applicable).

Student's *t* test and Mann-Whitney U test were used for comparing groups as appropriate.

Shapiro-Wilk test was used to verify the normality of distribution.

Calculation of biological variation parameters was performed by following the same methodology detailed in 2.2.3.

Due to the small number of women included in the study (n=5), gender-stratified calculation of biological and analytical variation was not performed.

2.3.4 Results

Baseline characteristics of the 24 participants are shown in table 2.5.

n	24
Male gender	19 (79%)
Age, years	72 ± 7
cMyC, ng/L	9.6 (5.7-14.3)
Hs-cTnT – Roche, ng/L	13.5 (9.8-17.2)
Hs-cTnI – Abbott, ng/L	4.45 (3.7-6.5)
Type 2 Diabetes Mellitus	7 (29%)
BMI, kg/m^2	27 ± 5
Systolic blood pressure, mm Hg	140 ± 15
Diastolic blood pressure, mm Hg	68 ± 8
Fasting glucose, mmol/L	5.9 (5.2-6.7)
HbA1c, %	5.7 (5.3-6.5)
Total cholesterol, mmol/L	3.7 (4.8-5.4)
HDL cholesterol, mmol/L	1.3 (1.6-1.9)
LDL cholesterol, mmol/L	1.7 (2.5-3.0)
Triglycerides, mmol/L	1.2 (0.8-1.8)
NT-proBNP, pmol/L	10.9 (4.9-38.8)
eGFR, mL/min/1.73m^2	75 (61-92)
Oral blood glucose-lowering medication	7 (29%)
Lipid-lowering medication	12 (50%)
Anti-hypertensive medication	10 (42%)

Table 2.5 Baseline characteristics.

Data are expressed as medians [1st quartile, 3rd quartile] or means ± standard deviation, for categorical variables are expressed as numbers (percentages). BMI = Body mass index; eGFR = estimated glomerular filtration rate.

Mean age was 72yrs (±7). Five participants were women (21%), and 7 (29%) were diabetic (on oral glucose lowering medications)

None of the samples had undetectable cMyC, hs-TnT or hs-TnI concentrations (below LoD).

Distribution of measurements across participants for all biomarkers is shown in figure 2.4.

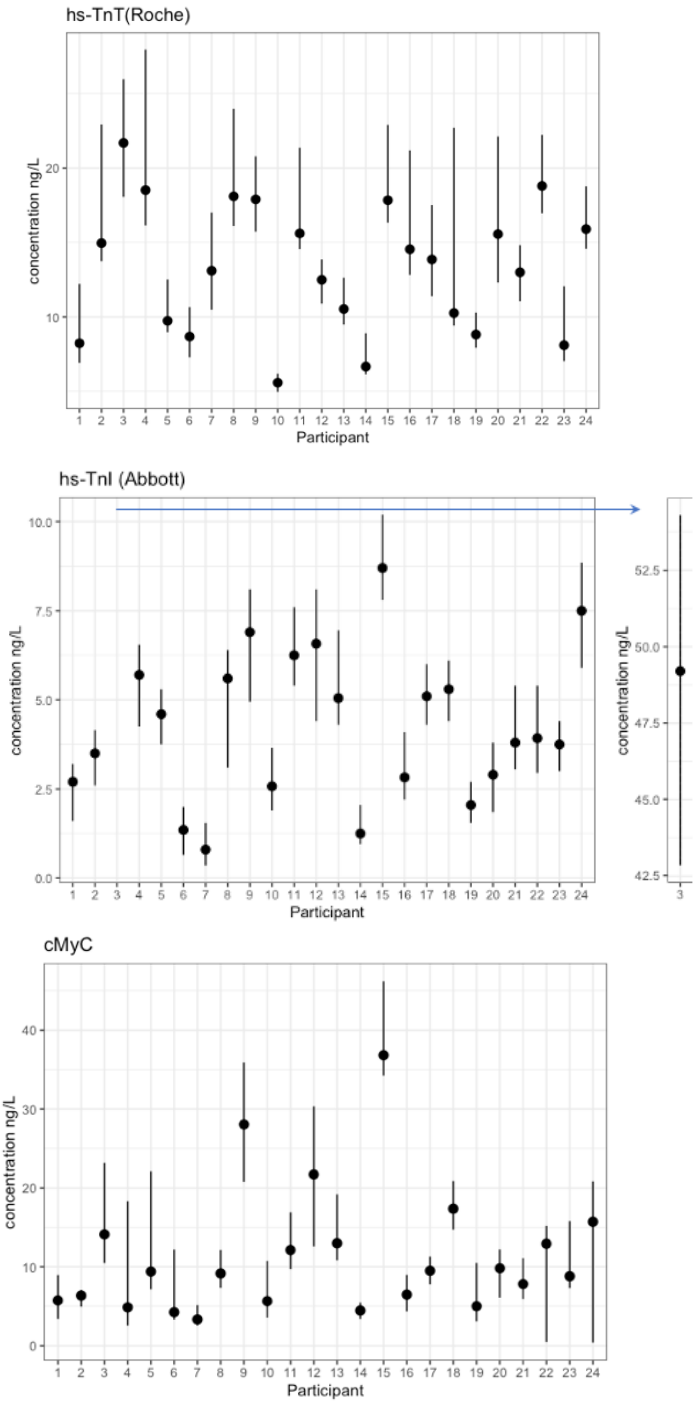

Figure 2.4 Distribution of concentration (median, range) of cMyC, cTnT and cTnI (ng/L)

Assessment of steady-state and diurnal rhythmicity

On population-mean cosinor analysis, significant circadian rhythm was detected for cMyC and hs-cTnT (p = 0.015 and <0.001, respectively) as shown in Figure 2.5 and table 2.6

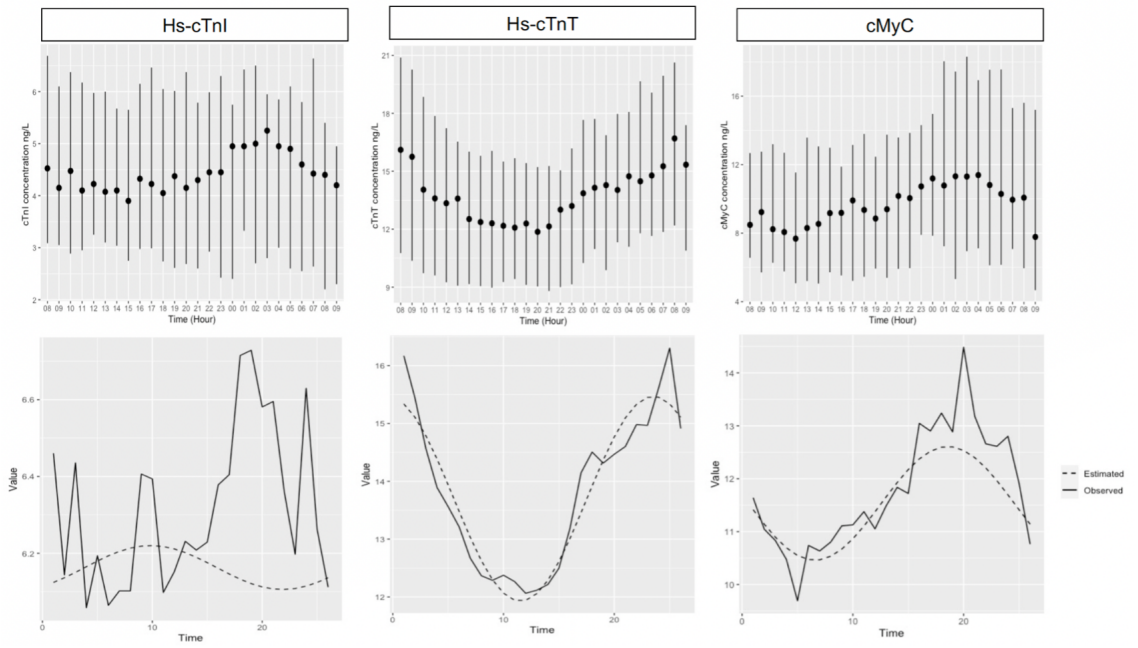

Figure 2.5 **Circadian oscillation of hs-cTnI (Abbott), hs-cTnT (Roche) and cMyC.**

Median concentration (1st, 3rd quartile) (first row) and fitted cosinor model (second row). Dashed- line: cosinor fit estimate, continuous line: actual.

hs-cTnI	6.0-6.7	6.16	0.05	04:01	0.867
Hs-cTnT	12.0-16.3	13.69	1.77	08:01	<0.001
cMyC	9.7-14.5	11.53	1.07	03:03	0.015

Time distributed measurements of cMyC oscillated around a mesor of 11.5 ng/L (95% CI 8.1-14 ng/L), spanning an amplitude of 1.1 ng/L (95% CI 0.4-1.8).

hs-cTnT values oscillated around a mesor of 13.7ng/L (95% CI 11.7-15.6 ng/L) with an amplitude of 1.8 ng/L (95% CI 1.3-2.2 ng/L).

The circadian rhythm of cMyC is characterised by gradually increasing concentrations from early afternoon until early morning (acrophase 03:03 am, 95% CI 01:54-04:26 am) compared to hs-cTnT concentrations which exhibits a later rise in concentration from early evening with a later peak (acrophase, 08:01 am, 95%CI 07:10-08:51 am), p = 0.028 for acrophase difference.

There was 5-hours acrophase difference between cMyC and cTnT (cMyC ahead of cTnT). There was no significant difference between the amplitudes of cMyC and cTnT after normalising to the respective 08.30 value (amplitude ng/L, 0.12, 95% CI 0.07-0.15 vs 0.11, 95% CI 0.08-0.12, for normalised cMyC vs hs-cTnT, respectively; p = 0.67).

The absolute hour-to-hour within-subject variation due to diurnal rhythm of ± 0.09 ng/L for cMyc and ± 0.15 ng/L for cTnT, in participants with significant diurnal rhythm. Since these concentrations are negligible, diurnal variation has been ignored in the calculation of hourly biological variation and RCV.

Table 2.6 Population-mean fitted cosinor sine regression model parameters for hs-TnT, hs-TnT and cMyC

hs-cTnI exhibited random diurnal oscillation (p = 0.867)

None of the remaining participants exhibited non-steady state.

	cMyC	hs-TnT (Roche)	hs-TnI (Abbott)
Analytical outliers identified as per Burnett's (samples)	4	7	8
Outliers in mean values of subject due to Reed's criterion (subjects)	0	0	3
Exclusion due to within-subject non-homogeneity according to Bartlett or Cochran's test (subjects)	7	9	5
Exclusion due to analytical non-homogeneity according to Bartlett or Cochran's test (samples)	1	1	5

Table 2.7 Excluding outlying samples and subjects

Numbers of excluded outlying samples and subjects are presented in table 2.7.

Analytical and biological variation, RCV and II of cMyC, hs-TnT and hs-TnI are shown in table 2.8.

	cMyC	hs-TnT (Roche)	hs-TnI (Abbott)
Number of participants	17	15	18
Number of samples	585 a	584	579
CVA mean (95% CI), %	11.1 (10.1-12.2)	2.4 (2.1-2.8)	10.1 (9.2-11.4)
CVG mean (95% CI), %	72.7 (61.6-84.7)	41.2 (28.7-65.8)	40.1 (30.0-61.9)
CVI mean (95% CI), %	13.4 (11.5-14.2)	8.8 (6.0-12)	8.9 (7.3-11.5)
Positive RCV, mean (95% CI), %	61.5 (53.0-68.4)	28.71 (21.1-40.7)	45.14 (30.1-61.3)
Negative RCV, mean (95% CI), %	-38.1 (-50.6 – -29.3)	-22.3 (-39.2 – -15.6)	-31.1 (-41.8 – - 24.7)
Index of individuality II	0.23	0.22	0.33

Table 2.8 Analytical and biological variation, RCV and II of cMyC, hs-TnT and hs-TnI

CV_A was comparable for cMyC and hs-TnI, 11.1 % (10.1-12.2 %) and 10.1 % (9.2-11.4 %) respectively, but a lower CV_A was calculated for hs-TnT, 2.4 % (2.1-2.8 %).

No significant inter-biomarker differences were found for either CV_I or CV_G. CV_I 13.4 % (11.5-14.2 %), 28.71 % (21.1-46.7 %), 8.9 % (7.3-11.5 %), and CV_G 72.7 % (61.6-84.7 %), 72.7 % (61.6-84.7 %), 40.1 % (30.0-61.9 %), for cMyC, hs-TnT and hs-TnI, respectively.

Calculated RCVs were 61.5 % (53.0-68.4 %) / -38.1 % (-50.6 – -29.3 %), 28.71 % (21.1-40.7 %) / -22.3 % (-39.2 – -15.6 %) and 45.14 % (30.1- 61.3 %) / -31.1 % (-41.8 – - 24.7 %), for cMyC, hs-TnT and hs-TnI, respectively.

II was comparable for all three biomarkers: 0.23, 0.22 and 0.33, for cMyC, hs-TnT and hs-TnI, respectively.

2.3.5 Discussion

This is the first study of the hour-to-hour analytical and biological variation, RCV and II of the novel biomarker cMyC in healthy individuals, quantified with the Erenna® platform by EMD Millipore Corporation. We also present a head-to-head comparison of biological and analytical variation of cMyC and two hs-cTns (hs-TnT and hs-TnI), calculated in the same cohort.

We have demonstrated a comparable RCV for cMyC and hs-TnI: 61.5 % (53.0-68.4 %) / -38.1 % (-50.6 – -29.3 %), vs 45.14 % (30.1- 61.3 %) / -31.1 % (-41.8 – - 24.7 %), both of which were higher than the RCV calculated for hs-TnT: 28.71 % (21.1-40.7 %) / -22.3 % (-39.2 – -15.6 %), driven by the lower analytical variability CV_A of the latter: 2.4 % (2.1-2.8 %) for hs-TnT vs 11.1 % (10.1-12.2 %) and 10.1 % (9.2-11.4 %) for cMyC and hs-TnI, respectively.

Within- and between-subject coefficient of variations, CV_I and CV_G were comparable between all three biomarkers. CV_I, 13.4 % (11.5-14.2 %), 28.71 % (21.1-46.7 %), 8.9 % (7.3-11.5 %), and CVG, 72.7 % (61.6-84.7 %), 72.7 % (61.6-84.7 %), 40.1 % (30.0-61.9 %), for cMyC, hs-TnT and hs-TnI, respectively.

The derived IIs were low and similar across all three biomarkers (< 0.6), suggesting high individuality, and favouring interpreting serial changes of cMyC concentration in the individual patient rather than using population-based reference intervals.

All reported variation parameters of hs-TnI and hs-TnT in our study appear to fall within the ranges of respective parameters described in similar short-term biological variation studies of hs-TnT and hs-TnI, table 2.9 [26,28,96,101,102,104-106]. However, those ranges are broad, reflecting the heterogeneity of the studies in respect to defining "healthy-status", number and age of participants, assay used, exclusion of outliers and the software used for statistics [9].

In our cohort, ratio of mean biomarker concentration to respective 99th percentile for myocardial injury was: 12 %, 95% and 16 % for cMyC, hs-TnT and hs-TnI, respectively. This might well explain the low CV_A calculated for hs-TnT. The original study was designed to examine the biological variation and diurnal variation of cTnT levels near the 99th percentile, which explains the inclusion of participants who fulfil this condition [101,103].

We have also demonstrated that significant circadian rhythm exists for cMyC and hs-cTnT, with 5-hours phase difference between the two biomarkers (cMyC ahead of hs-cTnT).

This phase difference is consistent with the previously described disparity in the release of cMyC and cTnT after iatrogenic myocardial injury [52].

The difference in the time of day for peak concentrations of cMyC and cTnT could either relate to clearance or release. Renal clearance may differ by molecular weight (23.5, 33.5 and 140 kDa, for the non-fragmented species of cTnI, cTnT, and cMyC, respectively). However, renal clearances of two different sized proteins: Albumin (66 kDa) and Macroglobulin (11 kDa) are reported to peak simultaneously around 2 pm [107]. In contrast, we have previously shown that the concentration in the blood of cMyC rises more rapidly and peaks many hours before cTnT after timed iatrogenic myocardial necrosis to treat hypertrophic obstructive cardiomyopathy. Suggesting the concentration of both markers likely reflect the underlying diurnal rhythm of myocardial release and thereby myocardial injury.

Epidemiological studies have shown a circadian rhythm for cardiovascular events including myocardial infarction, angina, arrhythmias, decompensated heart failure and sudden cardiac death, with a peak in the incidence of events in the morning hours (6 am to noon) [108,109].

Additionally, myocardial infarct size measured by CK and imaging and the resulting degree of left ventricular impairment are also influenced by a circadian rhythm with maximal injury following infarctions that begin around midnight [110,111], with a distinctive in-phase circadian pattern for in-hospital mortality [111]. A corresponding increase in 30-day mortality rates in patients who infarcted in the early hours of the morning is also reported [112].

This might reflect a circadian pattern for myocardial vulnerability to ischaemia [111], and in the cases of myocardial infarction at least, this is likely to correspond with a peak in atherosclerotic plaque vulnerability.

It is unknown why cTnI did not exhibit a diurnal rhythm, while both cTnT and cMyC exhibit strong rhythmicity. Unlike cTnI, cMyC has a location within the sarcomere that is distinct from cTnT and it does not from part of the ternary troponin complex that enters the blood. It is also unknown why significant phase differences in the circadian rhythms of cTnT and cMyC exist.

The impact of the strong diurnal rhythmicity of cTnT, and random fluctuation of cTnI didn't appear to impact on the diagnostic accuracy of respective biomarker-assisted triage algorithms, as reported in limited studies[103,109]

The concept of steady-state is violated in analytes with predictable increasing or decreasing trend, or if rhythmic diurnal oscillation is present, where the calculation of an 'overall' within-day RCV is precluded.

To eliminate the trend (either decreasing or increasing), values can be adjusted using the slope or regression equation of mean percentages of change from first value for each time point [31]. The issues become more complicated when trends in both directions exist, such as in the presence of diurnal rhythm. In this case, it has been proposed that the ranges of the systematic change for each time point (as a percentage from the first value) can be calculated [113]. However, for hour-to-hour biological variation, large number of RCVs covering all 24-time points will be calculated.

In our cohort, hour-to-hour within-subject variation due diurnal rhythm was estimated from the amplitude of the fitted population-cosinor model, which predicts the magnitude of change in concentrations due to the circadian oscillation over 12-hours period.

This translated into ± 0.09 ng/L for cMyc and ± 0.15 ng/L for cTnT, both are negligible, and therefore, were not accounted for during the calculation of CV_I.

29% (n=7) of the participants in our study had Type 2 diabetes mellitus, which is similar to the prevalence of Type 2 diabetes in patients presenting to the emergency department with suspected AMI (25%) [114]. Although this precludes the status of "absolute health", we believe that including diabetic patients at around the selected percentage could boost the relevance and applicability of the calculated biological variation components to real life cohorts.

Klinkenberg et al, studied within-day biological variation of hs-TnT in 23 type 2 diabetic patients using the same assay and found comparable results to ours.[101] In our cohort, excluding participants with DM did not result in any significant change to any of the calculated variation parameters for all three biomarkers.

Our data indicate that the desirable analytical bias for cMyC should be < 18.5%, and desirable total error < 29.5%, both calculations are consistent with the desirable analytical specifications calculated in a recent long-term cMyC biological variation study,[100] and should be achievable with clinical assays.

Basing analytical performance standards on biology has many advantages, including the simplicity of the model, which is easy to interpret; the increasingly available data on clinical variability; and the reproducibility of within- and between-subject biological

variations for many analytes [84]. However, evaluating the effect of analytical performance on clinical outcomes in specific clinical settings is still considered the ultimate method to extract and recommend analytical performance standards, provided, there is robust clinical data to allow such derivation [70,84,89-91].

Serial testing using biomarkers of myocardial injury is important in a variety of clinical situations [39,115-119]; however, the magnitude of change required to identify patients at risk varies depending on the assay used, the clinical scenario and the margin of risk accepted by the treating physicians, the latter is also regulated by organisational guidelines.

Of most relevance to this work, is the triage of patients with suspected non-ST-elevated myocardial infarction (NSTEMI) by utilising the change in biomarker concentration, paired with the universal definition of myocardial infarction and the ESC/ACC consensus documents.

In the case of cTn, existing literature describes the attempt to define the magnitude of change in the concentration that best predicts risk and safely and effectively triages patients with suspected NSTEMI.

Whether absolute or relative change should be used, and the respective optimal magnitude in change of concentration to diagnose AMI, has been a subject of debate [9,120-129]. However, for any approach, it would still be essential that the respective change is greater than the analytical variation of the assay used.

Although superiority of absolute change was demonstrated by several studies [122,126,127], this superiority seems to be baseline cTn concentration dependent [80]. and relative change

may outperform the absolute change in patients presenting with intermediate or high cTn values [129].

Additionally, the performance of each approach might be dependent on the serial testing protocol (0-1h, 0-2h, 0-3h, etc) [129].

Whether the conclusions above can be extrapolated to cMyC which has different kinetic profile to cTn, needs to be addressed in clinical studies.

The study has several strengths, it has enabled direct comparison of the biological variation of cMyC and two hs-cTns in the same cohort, it included a relatively large number of participants, "healthy status" was clearly defined, and exclusion of outliers was performed systematically as described in the manuscript.

Our study also has limitations, only 5 participants (21%) were women, and samples were analysed for cMyC 5 years after collection, however, they were continuously stored at -80 °C and thawed only for cMyC analysis.

Author	Year [a]	n [b]	Frequency	Period	Age [c]	Assay	RCV (log-normal)	CVA (%)	CVI (%)	CVG (%)	II (%)
cTnI											
Lan et al.	2020	20	Hourly	4-hours	40 (22-70)	hs-TnI Abbott Alinity ci-series	+111.2/-52.6	14	23.6	92.7	0.38
Zainotto et al.	2020	35	Hourly for 3-hours, 1-sample at 7 hours	7-hours	51 (32-65)	hs-TnI Abbott Architect Plus i2000	+29.49/-22.77	8.39	4.1	17.28	0.54
Van der Linden et al.	2017	18	Hourly	24-hours	76 (68-78)	hs-TnI Abbott Architect Plus i2000	+44/30.6	6.8	10	49.4	0.27
Vasile et al.	2011	20	Hourly	4-hours	39 (25-56)	hs-TnI Beckman Coulter	+45.2/-15.8	3.5	3.5	45.3	
Wu et al.	2009	12	Hourly	4-hours	19-58	hs-TnI Singulex	+46/-32	8.3	9.7	57	0.21
cTnT											
Klinkenberg et al.	2014	23	Hourly	11-hours	63 (7)	Hs-TnT Roche Cobas 6000	+48.6/-32.7 d	2.0 d	14.2 [d]	38.4 d	0.37 [d]
							+42.7/-29.9 e	1.9	12.7 [e]	40.4 e	0.32 [e]
Frankenstein et al.	2011	20	Hourly	4-hours	32 (22-59)	hs-TnT E 170 assay	+62/-39	7.8	15	n/a	n/a
						hs-TnT Elecsys 2010 assay	+90/-47	9.7	21	n/a	n/a
Vasile et al.	2010	20	Hourly	4-hours	39 (25-56)	hs-TnT Roche Modular	84.6 f	53.5	48.2	85.9	0.84

Table 2.9 Published short-term biological variation of cTns.

[a] Year published. [b] n = number of subjects. [c] expressed in mean (range), mean (SD), median (Q1, Q3) or range only, F = females, M = males

2.3.6 Conclusion

cMyC has low and comparable analytical and hourly biological variation, RCV and II to that of two hs-cTns. Analytical quality specifications of cMyC based on biological variation data are similar to those for hs-cTn and should be achievable at clinically relevant concentrations. Significant circadian rhythm exists for cMyC and hs-cTnT, with 5-hours phase difference between the two biomarkers (cMyC ahead of hs-cTnT). The cause of this rhythmic variation is unknown

Studies are required to establish the performance of biology-derived relative change of cMyC concentration in the triage and risk-stratification of patients with suspected AMI.

3 Chapter 3. The diagnostic performance of cMyC-guided 0/1hour triage algorithm for myocardial infarction in patients with renal dysfunction

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Hypothesis

In patients with renal dysfunction the diagnostic performance of a cMyC-guided 0/1 h triage algorithm for myocardial infarction is comparable to that of cardiac troponins (cTnI and cTnT)

Statement of contribution:

Recruitment, sample collection, sample analysis and adjudication were performed by our collaborators (Professor Christian Mueller, et al) before the start of this research fellowship.

Bashir Alaour designed and performed all data analyses outlined in this study.

3.1 Abstract

Background

Guidelines endorse the use of state-the-art triage algorithms that incorporate assay specific reference change values (delta) for rapid rule-out/in of acute myocardial infarction.

Ischaemic heart disease and chronic myocardial injury are highly prevalent in patients with renal dysfunction. The safety of troponin-guided 0/1 h triage algorithm for the diagnosis of acute myocardial infarction in patients with renal dysfunction is preserved, however, the accuracy and overall efficacy are compromised. The diagnostic performance of cMyC-guided 0/1 h triage algorithms in patients with renal dysfunction is unknown and will be examined in this chapter.

Methods

In a retrospective analysis of a prospective multicentre cohort of unselected patients presenting with suspected non-ST elevation myocardial infarction NSTEMI, the diagnostic performance of cMyC guided 0/1 h triage algorithm was assessed and compared to the performance of troponin (hs-cTnt and hs-cTnI)-guided 0/1 h triage algorithm in patients with renal dysfunction.

Safety was quantified as sensitivity of rule-out, accuracy as the specificity of rule-in, and efficacy as the proportion of patients assigned to either rule-out or rule-in zone.

Results

1317 patients were included in the analyses, of which 211 patients had renal dysfunction as defined by eGFR < 60 mL/min/1.73 m^2.

Patients with renal dysfunction were significantly older (76 ± 10 vs 59 ± 15, p < 0.001) and had a higher prevalence of risk factors for ischaemic heart compared to patients with normal renal function.

Compared to patients with normal renal function, safety of the ESC 0/1 h triage algorithm in patients with renal dysfunction was high and preserved, however, accuracy and overall efficacy of the algorithm were compromised for all three biomarkers.

In patients with renal dysfunction, safety, accuracy, overall efficacy and discrimination power as quantified by AUC of ROC of the 0/1 h triage algorithm were comparable between all biomarkers.

Adjusting the rule-in thresholds, could not optimise accuracy without further compromising overall efficacy.

Conclusion

In patients with renal dysfunction, the performance of cMyC-guided ESC 0/1-h triage algorithm is comparable to cardiac troponins, however, while the safety of the algorithm is high, the accuracy and overall efficacy is reduced.

3.2 Introduction:

Accurate interpretation of elevated cTn concentrations in the presence of renal disease is an everyday clinical challenge. There are several factors that confound the recognition of AMI in this group, including the increased likelihood of atypical presentations, [130,131] and

the increased prevalence of chronic elevations of troponin concentrations above the 99th percentile (chronic myocardial injury).[132-134]

The exact pathophysiology underlying chronic stable cTn elevation, and whether this is due to reduced renal elimination or increased cardiac release caused by coexistent coronary artery disease and/or accumulating toxins, remains unclear.[135-137]

Additionally, it is well recognised that patients with renal dysfunction have an increased risk of cardiovascular events compared to patients with normal renal function, adding to the anxiety of troponin interpretation.[138-141]

In order to enhance the triage efficacy and accuracy, the concept of a 99th percentile or of a reference interval as a single parameter for diagnosing myocardial infarction is now widely replaced by state-of-the-art triage algorithms incorporating bespoke, assay-specific cut-off concentrations and reference change values or deltas.[142]

The safety and efficacy of contemporary troponin-assisted triage pathways has been assessed in recent studies, including pathways with integrated concentration change (delta).

Of most relevance, is the recent work by Twerenbold et al who assessed the performance of the European Society of Cardiology ESC 0/1 h triage algorithm for the diagnosis of NSTEMI in patients with renal dysfunction. This study assessed; safety by sensitivity of rule-out; accuracy by specificity of rule-in and overall efficacy by the proportions of patients allocated to rule-in, rule-out or indeterminate/grey-zone groups.

While the safety of cTn-based 0/1 algorithms was preserved, specificity suffered (88.7 vs 96.5, $p < 0.001$, in patients with- compared to without- renal dysfunction, respectively).

Overall efficacy in renal dysfunction was also compromised, due to significantly higher proportion of patients allocated to the grey zone.[138]

The diagnostic performance of cMyC-based triage algorithms in patients with renal dysfunction, has not been tested and will be addressed in this chapter.

The diagnostic thresholds of cMyC in patients with suspected myocardial infarction were derived and validated in the Advantageous Predictors of Acute Coronary Syndromes Evaluation (APACE) prospective cohort in collaboration with the University of Basel. The diagnostic thresholds were validated in all patients irrespective of their renal function.[57,143] Only patients with end-stage renal failure were excluded in the original APACE study design.

Our aim in this chapter was to test the derived/validated diagnostic thresholds for cMyC in patients with and without renal dysfunction to determine if cMyC, cTnI and cTnT are similarly confounded.

3.3 Methods

Study population

APACE (Advantageous Predictors of Acute Coronary Syndrome Evaluation) is an ongoing prospective international multi-centre diagnostic observational study with 12 centres in 5 European countries aiming to advance the early diagnosis of AMI (ClinicalTrials.gov.NCT00470587). https://clinicaltrials.gov/ct2/show/NCT00470587

APACE recruited all adult patients presenting to the ED with suspected AMI within 12 hours of relevant symptom onset. Only patients with terminal kidney failure on chronic

dialysis were excluded. Serial blood samples were drawn (0, 1, 2, 3 and 6h) for detection of biomarkers of myocardial injury.

cMyC was measured in 7000 sera from approximately 2000 patients presenting with suspected NSTEMI of whom 340 had an adjudicated AMI.

In this analysis, only patients with recorded eGFR who had both 0h and 1h concentrations of cMyC, cTnT and cTnI were included, to ensure fair comparison between the performance of all three biomarkers in the same cohort with the same events.

Renal dysfunction was defined as an eGFR of <60 mL/min/1.73 m^2

Adjudication

Patients were adjudicated by the APACE investigators. Adjudication of the final diagnosis based on the universal definition of myocardial infarction [38,142] was performed by two independent cardiologists at the co-ordinating centre (University Hospital Basel), incorporating all available clinical and research data. The adjudication biomarker in the APACE study was hs-cTnT (see below for assay characteristics).

Assays characteristics

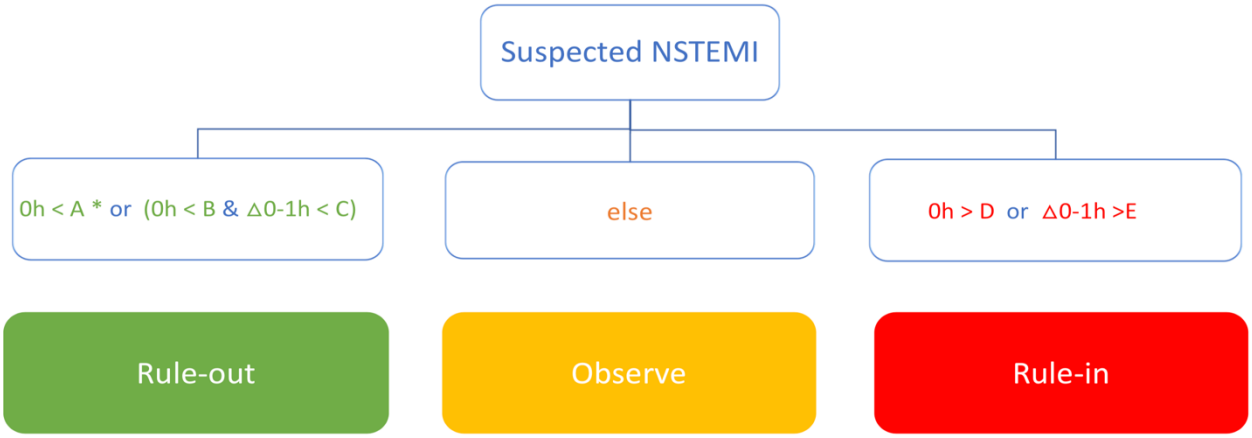
cMyC assay (EMD Millipore on the Erenna® platform) has a LoD of 0.4 ng/L and LoQ (20% CVa) of 1.2 ng/L

hs-cTnT assay (Elecsys 2010 high-sensitivity troponin T, Roche Diagnostics) has a 99th percentile concentration of 14 ng/L, CV 10% at 13 ng/L, and limit of detection of 5 ng/L.

hs-cTnI assay (ARCHITECT High Sensitive STAT Troponin I, Abbott Laboratories) has a 99th percentile concentration of 26.2 ng/L with a corresponding coefficient of variation of <5% and a limit of detection of 1.9 ng/L.

ESC 0/1 h triage algorithm

The ESC 0/1 h triage pathway with assay specific diagnostic cut-off thresholds of the relevant biomarkers is displayed in figure 3.1.



	A	B	C	D	E
cMyC (EMD Millipore)	10	26	8	136	15
hs-cTnT (Roche)	5	12	3	52	5
hs-cTnI (Abbott)	2	5	2	52	6

* chest pain onset > 3h before presentation to A&E. All units in ng/l

Figure 3.1 European Society of Cardiology 0/1-hour triage algorithm for myocardial infarction assay specific cut-off thresholds displayed in table. Concentrations for cMyC were derived from the APACE cohort by Kaier & Twerenbold et al [1]

cMyC cut-off thresholds were previously derived from the APACE cohort using machine learning by selecting the best performing diagnostic cut-off combinations. [1,143]

with assay specific cut-off thresholds displayed in table. Concentrations for cMyC were derived from the APACE cohort by Kaier & Twerenbold et al [1].

Main outcome measures

The outcome measures are safety defined by sensitivity for NSTEMI in the rule-out group; accuracy as defined by the specificity for NSTEMI in the rule-in group; NPV; PPV; and overall triage efficacy defined by the proportion of patients outside the observe zone.

Statistical analysis

All data are expressed as medians (1st quartile, 3rd quartile) for continuous variables and as numbers and percentages for categorical variables. Continuous variables were compared with the Mann-Whitney U test, and categorical variables using the chi-square test or Fisher exact test as appropriate for unpaired samples, or the McNemar test for paired samples.

Classification models' performances were quantified by the area under the receiver-operating characteristics curve (AUC) for each biomarker using 100,000 stratified boot-strap replicates to calculate confidence intervals (CIs).

Cross tables derived from the application of the ESC assay-specific cut-off criteria for rule-out or rule-in were used to calculate diagnostic performance parameters and their 95% confidence intervals.

Sensitivity, specificity, NPV, PPV, and efficacy were compared using a chi-square or Fisher exact test for unpaired samples and the McNemar test for paired samples.

To visualise the temporal change in the concentrations of the biomarkers in patients with acute myocardial infarction, stratified by renal function, time variables from pain onset to blood sampling were created, polynomial regression graphs were generated with increasing orders, orders were compared with ANOVA for the selection of the best

predictive line. Interaction graphs were generated between time from onset of pain and eGFR with biomarker concentration as outcome.

All hypothesis testing was 2-tailed, and P values of <0.05 were considered to indicate statistical significance.

All statistical analyses were performed with the use of R statistical software version 3.6.1

3.4 Results

3.4.1 Baseline characteristics

1317 patients were included in the analyses, of which 1106 had normal renal function as defined by eGFR > 60 mL/min/1.73 m^2 and 211 had renal dysfunction.

Patients with renal dysfunction were significantly older (76 ± 10 vs 59 ± 15, p < 0.001).

The prevalence of risk factors for ischaemic heart disease including diabetes, hypertension, hypercholesterolaemia and smoking history was significantly higher in patients with renal dysfunction. Similarly, the prevalence of a previous history of cardiovascular or cerebrovascular disease was significantly higher in patients with renal dysfunction (p <0.001, for history of each of myocardial infarction MI, coronary artery bypass grafting CABG, percutaneous coronary intervention PCI, cerebrovascular accident CVA, and peripheral vascular disease PVD, individually).

Significantly higher rates of acute coronary syndrome (NSTEMI and unstable angina) and lower rates of non-cardiac and cardiac non-coronary chest pain occurred in patients with- compared to without- renal dysfunction (p < 0.001, for all diagnoses). Table 1.

Significantly higher concentrations of presentation (0h) cMyC and cTnT but not cTnI were present in patients with renal dysfunction compared to patients with normal renal function ($p < 0.001$, < 0.001 and 0.1, respectively). At 1h post presentation, significantly higher concentrations of all biomarkers were seen in patients with-compared to without-renal dysfunction ($p<0.01$)

Baseline characteristics classified by renal function are shown in table 3.1.

	Normal renal function n=1106	Renal dysfunction n=211	*p* value
Age	59.0 (15.2)	76.57 (10)	<0.001
Male gender	786 (71.1)	115 (54.5)	<0.001
Cardiac risk factors			
BMI	27 (4.7)	27.4 (4.7)	0.2
Diabetes	196 (17)	58 (26.4)	<0.001
Hypertension	639 (57.8)	190 (90.0)	<0.001
Hypercholestrolaemia	540 (48.8)	140 (66.4)	<0.001
Smoking history	707 (63.9)	108 (51.2)	<0.001
Cardiac history			
Previous MI	230 (20.8)	86 (40.8)	<0.001
Coronary artery disease	356 (32.2)	125 (59.2)	<0.001
Previous CABG	91 (8.2)	41 (19.4)	<0.001
Previous PCI	252 (22.8)	72 (34.1)	<0.001
Previous CVA	52 (4.7)	24 (11.4)	<0.001
PVD	51 (4.6)	26 (12.3)	<0.001
Vital signs			
Pulse	79 (19)	81 (24)	0.2
Systolic BP	145.4 (24.5)	139.82 (26.7)	0.0
Diastolic BP	82.9 (14.6)	75.45 (16.6)	<0.001
eGFR			
Admission eGFR	92.3 (20.6)	45.5 (11.1)	<0.001
Adjudicated diagnosis			
NSTEMI	159 (14.4)	65 (30.8)	<0.001
Type 1 AMI	139 (12.6)	47 (22.3)	<0.001
Type 2 AMI	20 (1.8)	18 (8.5)	<0.001
Unstable angina	110 (9.9)	28 (13.3)	<0.001
Non-cardiac chest pain	650 (58.8)	66 (31.3)	<0.001
Cardiac non-coronary chest p	143 (12.9)	45 (21.3)	<0.001
Biomarkers			
cMyC 0h	93.5 (307.6)	242.0 (479.5)	<0.001
cTnT 0h	22.6 (59.1)	54.0 (89.8)	<0.001
cTnI 0h	95.3 (605.8)	175.4 (760.0)	0.1
cMyC 1h	117.6 (372.3)	287.6 (595.5)	<0.001
cTnT 1h	26.4 (69.9)	58.3 (98.5)	<0.001
cTnI 1h	123.5 (729.7)	198.9 (782.2)	0.0
cMyC 0-1h delta	24.1 (148.9)	45.6 (205.01)	0.1
cTnT 0-1h delta	4.6 (18.2)	6.01 (16.8)	0.3
cTnI 0-1h delta	32.2 (175.1)	29.30 (85.4)	0.8

Table 3.1 Baseline characteristics stratified by renal function

Renan dysfunction: (eGFR of <60 mL/min/1.73 m^2). Figures presented as mean (SD) or number (percentage), as appropriate. Biomarker concentration expressed as ng/mL. BMI, body mass index; MI, myocardial infarction; CABG, coronary artery bypass surgery; CVA, cerebrovascular accident; PVD, peripheral vascular disease; eGFR, estimated glomerular filtration rate.

3.4.2 Performance of the ESC 0/1-h triage algorithm utilising cardiac troponins

	Abbott hs-TnI			Roche hs-TnT		
	Normal renal function (n=1106)	**Renal dysfunction (n=211)**	***P value***	**Normal renal function (n=1106)**	**Renal dysfunction (n=211)**	***P value***
Prevalence of NSTEMI	14	31	<0.001	14	31	<0.001
Sensitivity of rule-out	97.4 (95.0-99.9)	96.9 (92.7-100)	1	100(100-100)	100 (100-100)	1
NPV of rule-out	99.3 (98.7-99.9)	94.8 (87.9-1.0)	0.046	100 (100-100)	100 (100-100)	1
Specificity of rule-in	93.5 (99.1-95.1)	84.2 (78.3-90.1)	<0.001	96.7 (95.5-97.8)	86.3 (80.7-91.8)	<0.001
PPV of rule-in	68.0 (61.4-74.6)	67.6 (56.7-78.4)	1	80.5 (74.3-86.6)	70.5 (59.7-81.4)	0.2397
Proportion ruled out	56.1 (53.2-59.0)	18.5 (13.2-23.7)	<0.001	64.9 (62.1-67.3)	17.5 (12.4-22.6)	<0.001
Based on 0-hour sample only	9.8 (8.0-11.6)	1.4 (0.1-3.0)	<0.001	15.6 (13.5-17.7)	1.9 (0.05-3.7)	<0.001
Based on 0/1-hour concentration change	52.2 (49.3-55.2)	10.9 (6.6-15.1)	<0.001	64.9 (62.1-67.7)	17.5 (12.4-22.6)	<0.001
Proportion ruled-in	17.2 (15.0-19.4)	33.6 (27.2-40.0)	<0.001	14.4 (12.3-16.4)	32.3 (25.8-38.5)	<0.001
Based on 0-hour sample only	11.8 (9.9-13.7)	25.1 (19.2-13.9)	<0.001	8.2 (6.6-9.8)	26.5 (20.5-32.4)	<0.001
Based on 0/1-hour concentration change	15.6 (13.5-17.7)	29.8 (23.6-36.0)	<0.001	12.3 (10.4-14.3)	22.7 (17.0-28.4)	<0.001
Overall efficacy	73.4 (70.8-76.8)	52.1 (45.3-58.8)	<0.001	79.2 (76.9-81.6)	49.7 (43.0-56.5)	<0.001
Prevalence of NSTEMI in the observe group	8.5 (7-10.1)	14.8 (11.3-16.8)	0.1835	13.5 (11.5-15.8)	16 (13.1-19.4)	0.843

Table 3.2 Performance of the 0/1-h ESC algorithm using hs-TnI and hs-TnT.

Performance measures presented as percentages and 95% confidence intervals.

The sensitivity of rule-out as an assessment of safety of the triage algorithm in patients with renal dysfunction was preserved using both troponin assays, compared to patients without renal dysfunction, (96.9 % [95 % CI, 92.7-100] vs 97.4 % [95% CI, 95.0-99.9], respectively; *p*=1, for hs-TnI and 100 % [95% CI [100-100] vs 100% [95% CI, 100-100, respectively; *p*=1 for hs-TnT).

The specificity of rule-in was significantly compromised in the renal dysfunction group, (84.2 % [95% CI,78.3-90.1] vs 93.5 % [95% CI, 99.1-95.1], p <0.001 with hs-TnI; and 86.3 % [95% CI, 80.7-91.8] vs 96.7 % [95% CI, 95.5-97.8], p <0.001 with hs-TnT), table 3.2.

NPV with hs-cTnI guided algorithm was lower in patients with renal dysfunction compared to patients without (94.8 % [95 % CI, 87.9-1.0] vs 99.3 % [95% CI, 98.7-99.9], respectively; p=0.046). Whereas NPV was similar between groups with hs-cTnT-guided triage, (100 % [95% CI [100-100] in both groups; p=1)

PPV with both troponins were comparable across patient groups.

With both troponins, efficacy of rule-out was significantly reduced in patients with renal dysfunction, regardless of whether rule-out was based on presentation or 1-h sample.

Whereas efficacy of rule-in with both troponins was significantly higher in patients with renal dysfunction, and the difference was consistent with both the presentation sample and 1-h change.

Overall efficacy of the algorithm using either troponin was reduced, with significantly more patients allocated to the observe zone in patients with renal dysfunction compared to patients without. (52.1% [95%CI, 45.3-58.8] vs 73.4% [95%CI, 70.8-76.8],

respectively; p <0.001 for cTnI; and 49.7% [95%CI,43.0-56.5] vs 79.2% [95%CI, 76.9-81.6], respectively; p <0.001 for cTnT).

Amongst those who were allocated to the observe zone, prevalence of NSTEMI was similar regardless of renal function and triage troponin.

3.4.3 Performance of the ESC 0/1-h triage algorithm utilising cMyC

Diagnostic performance parameters of cMyC in patients with- and without-renal dysfunction are presented in table 3.3.

Safety as represented by sensitivity of rule-out for NSTEMI by the cMyC-guided ESC 0/1-h triage algorithm was high in patients with renal dysfunction and similar to that in patients with normal renal function (98.5 % [95% CI, 95.4-100] vs 96.8 % [95% CI, 94.1-99.5], respectively; p=0.776).

NPV was also similar in patients with renal dysfunction (98% [95% CI, 94.1-100]) compared to patients with normal renal function (99.3 % [95% CI, 98.7-99.9], respectively; p=0.304)

Accuracy of rule-in as quantified by specificity for NSTEMI was lower in patients with- compared to patients without-renal dysfunction (81.5 % [95% CI, 75.2-87.8] versus 94.5 % [95% CI, 93.0-95.9], P<0.001). However, PPV was also similar in both groups. (63.5 % [95% CI, 52.5-74.4] vs 70.06 % [95% CI, 63.9-77.3]; p=0.593).

	cMyC		
	Normal renal function (n=1106)	**Renal dysfunction (n=211)**	***P value***
Prevalence of NSTEMI	14	31	<0.001
Sensitivity of rule-out	96.8 (94.1-99.5)	98.5 (95.4-100)	0.776
NPV of rule-out	99.3 (98.7-99.9)	98 (94.1-100)	0.304
Specificity of rule-in	94.5 (93.0-95.9)	81.5 (75.2-87.8)	<0.001
PPV of rule-in	70.06 (63.9-77.3)	63.5 (52.5-74.4)	0.593
Proportion ruled out	67.0 (64.2-69.7)	23.7 (17.9-29.4)	<0.001
Based on 0-hour sample only	38.8 (35.9-41.8)	4.2 (1.5-6.9)	<0.001
Based on 0/1-hour concentration change	66.4 (63.6-69.2)	23.7 (17.9-29.4)	<0.001
Proportion ruled-in	16 (13.8-18.1)	35.07 (28.6-41.5)	< 0.001
Based on 0-hour sample only	10.01 (8.3-11.9)	23.69 (20.5-32.4)	<0.001
Based on 0/1-hour concentration change	12.68 (10.6-14.6)	23.69 (17.9-29.5)	<0.001
Overall efficacy	83.0 (80.7-85.2)	58.7 (52.1-65.4)	<0.001
Prevalence of NSTEMI in the observe group	15.43 (13.8-17.6)	19.5 (17.3-21.9)	0.72

Table 3.3 Performance of the 0/1-h ESC algorithm using cMyC.

Performance measures presented as percentages and 95% confidence intervals.

With the cMyC-guided triage algorithm, the proportion of patients who were ruled-out was significantly reduced in patients with renal dysfunction (23.7 % [95% CI, 17.9-29.4] vs 67.0 % [95% CI, 64.2-69.7]; p=<0.001) regardless of whether rule-out was based on presentation (0h) or concentration change (delta) over 1h.

Whereas proportion of patients that were ruled-in with both was significantly higher in patients with renal dysfunction, (35.07 % [95% CI, 28.6-41.5] vs 16% [95% CI, 13.8-18.1]; p=<0.001), the difference again was consistent in both presentation sample and 0/1-h delta triage ($p < 0.001$)

However, overall efficacy of cMyC-guided algorithm was significantly reduced in patients with renal dysfunction, with significantly more patients allocated to the observe zone compared to patients with normal renal function (overall efficacy, 57.7 % [95%CI, 52.1-65.4] vs 83.0 % [95%CI, 80.7-85.2], respectively; p <0.001)

3.4.4 Pairwise comparison between the performance of all three biomarkers in patients with renal dysfunction

Pairwise comparison between performance parameters of the three biomarkers in patients with renal dysfunction is shown in table 3.4.

Although cMyC ruled out a significantly higher proportion of patients with renal dysfunction compared to cTnI and cTnT: (23.7 % [95% CI, 17.9-29.4] versus 18.5 % [95% CI, 13.2-23.7], for cMyC vs cTnI respectively; p=0.035) and (23.7 % [95% CI, 17.9-29.4] versus 17.5 % [95% CI, 12.4-22.6], for cMyC vs cTnT respectively; p= 0.045), overall efficacy remained comparable between all three biomarkers.

	cMyC	Abbott hs-TnI	Roche hs-TnT	p value (cMyC vs TnI)	p value (cMyC vs TnT)
Sensitivity of rule-out	98.5 (95.4-100)	96.9 (92.7-1.0)	100 (100-100)	0.991	0.476
NPV of rule-out	98 (94.1-100)	94.8 (87.9-1.0)	100 (100-100)	0.993	0.579
Specificity of rule-in	81.5 (75.2-87.8)	84.2 (78.3-90.1)	86.3 (80.7-91.8)	0.339	0.641
PPV to rule-in	63.5 (52.5-74.4)	67.6 (56.7-78.4)	70.5 (59.7-81.4)	0.473	0.143
Proportion ruled out	23.7 (17.9-29.4)	18.5 (13.2-23.7)	17.5 (12.4-22.6)	0.035	0.042
Based on 0-hour sample only	4.2 (1.5-6.9)	1.4 (0.1-3.0)	1.9 (0.05-3.7)	0.032	0.045
Based on 0/1-hour delta	23.7 (17.9-29.4)	10.9 (6.6-15.1)	17.5 (12.4-22.6)	0.004	0.148
Proportion ruled-in	35.07 (28.6-41.5)	33.6 (27.2-40.0)	32.3 (25.8-38.5)	0.987	0.984
Based on 0-hour sample only	26.5 (20.5-32.4)	25.1 (19.2-13.9)	26.5 (20.5-32.4)	0.872	0.999
Based on 0/1-hour delta	22.07 (17.0-28.4)	29.8 (23.6-36.0)	22.7 (17.0-28.4)	0.136	0.726
Overall efficacy	58.7 (52.1-65.4)	52.1 (45.3-58.8)	49.7 (43.0-56.5)	0.117	0.219
Prevalence of NSTEMI in the observe group	19.5 (17.3-21.9)	14.8 (13.0-16.3)	16 (13.1-19.4)	0.436	0.581

Table 3.4 . pairwise comparison between performance measures of all biomarkers in patients with renal dysfunction.

Performance measures presented as percentages and 95% confidence intervals.

There was no statistically significant difference in any diagnostic performance parameter between the three biomarkers in patients with renal dysfunction. Prevalence of NSTEMI among patients triaged to the observe groups by all three biomarkers was similar.

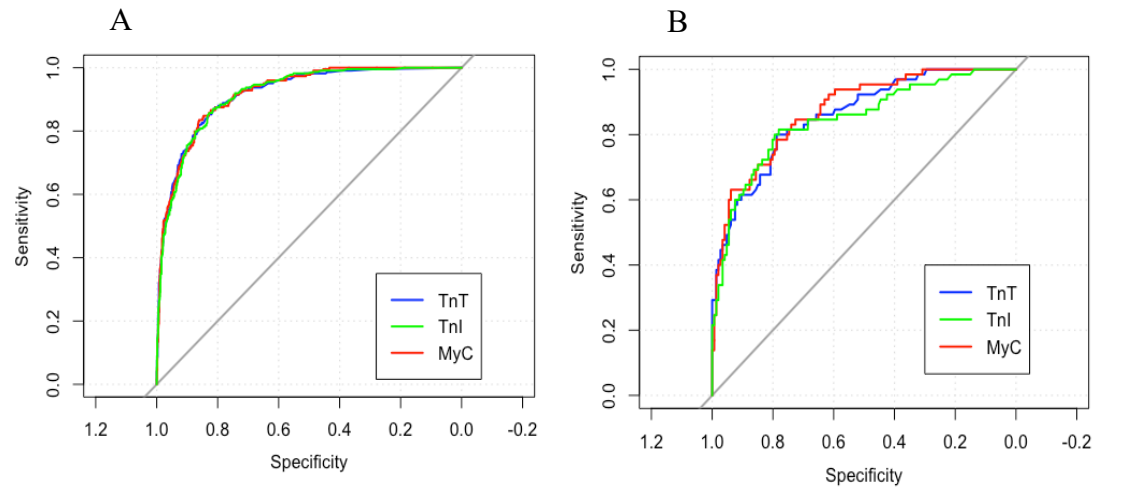
3.4.5 Discrimination power of the biomarkers in patients with renal dysfunction and with normal renal function

Receiver operating characteristic (ROC) curves for individual biomarkers utilising the respective biomarker guided 0/1h triage algorithm are shown in figure 3.2.

In figure 3.2 (A), discrimination power of the biomarkers for acute myocardial infarction was compared in patients with normal renal function. AUC for cMyC was 0.919 [95% CI, 0.901-0.937], compared to the AUC for hs- cTnT, (0.917 [95% CI [0.89-0.935], p = 0.744) and hs-cTnI (AUC, 0.916 [95% CI [0.898-0.9343]; p = 0.620).

Figure 3.2 (B) shows ROC curves for all three biomarkers in patients with renal dysfunction.

The discrimination power of cMyC for acute myocardial infarction as quantified by the AUC was 0.873 [95% CI, 0.820-0.919], compared to the AUC for hs- cTnT, AUC, 0.858 [95% CI, 0.802-0.909], p = 0.413 for direct comparison; and hs-cTnI, AUC, 0.8457 [95% CI, 0.783-0.901], p = 0.115 for direct comparison.



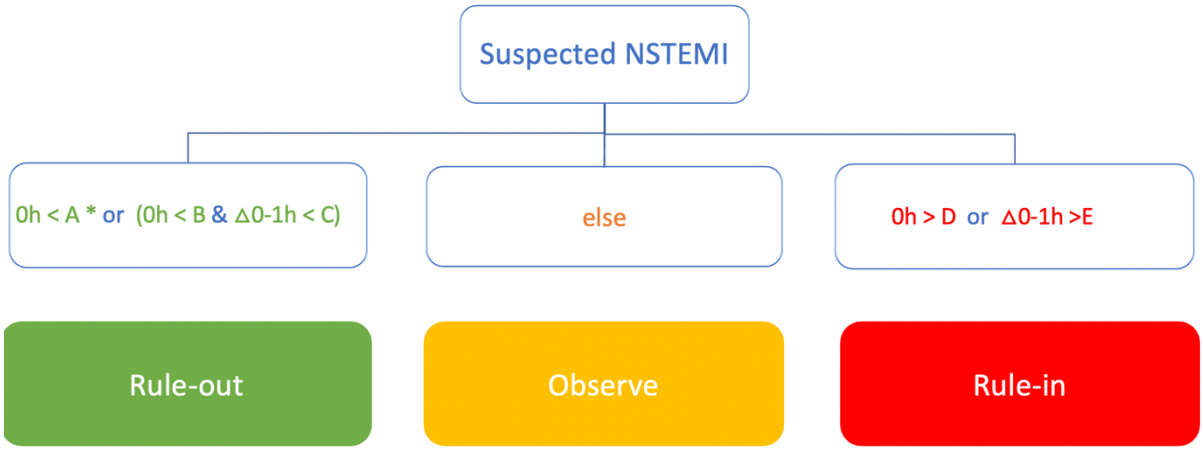
	cMyC AUC (95% CI)	hs-cTnT AUC (95% CI)	hs-cTnI AUC (95% CI)	*p* (cMyC vc hs-cTnT)	*p* (cMyC vc hs-cTnI)
Normal renal function	0.919 (0.901-0.937)	0.917 (0.898-0.935)	0.916 (0.898-0.934)	0.744	0.62
Renal dysfunction	0.873 (0.820-0.919)	0.858 (0.802-0.909)	0.845 (0.783-0.901)	0.413	0.115

Figure 3.2 Diagnostic performance of cMyC, hs-cTnT and hs-cTnI for the diagnosis of acute myocardial infarction based on whole ESC 0/1-h triage algorithm.

The discrimination power of all biomarkers for acute myocardial infarction as quantified by the AUC in patients with normal renal function (A) and patients with renal dysfunction (B). ROC curves describe the performance of cMyC (red line), hs-cTnT (blue line) and hs-cTnI (green). (CI: 10000 stratified bootstrap replicates). Table: depicted AUC for each biomarker in patients with-and without-renal dysfunction with pairwise direct comparison between cMyC and hs-cTnT/hs-cTnI.

3.4.6 Adjusting cMyC diagnostic thresholds for the optimisation of cMyC performance in patients with renal dysfunction

To optimise the performance of cMyC-guided 0/1-h algorithm in patients with renal dysfunction, I utilized machine learning iterations spanning experimental thresholds above and below each cut-off thresholds in the ESC 0/1-h algorithm. Figure 3.3.



		A	B	C	D	E
	Current cMyC thresholds	10	26	8	136	15
Experimental threshold range	Lower limit	5	16	5	120	10
	Upper limit	15	36	14	150	25

Figure 3.3 Current and ranges of experimental diagnostic thresholds combined for optimising the diagnostic performance of cMyC in patients with renal dysfunction.

A total of 1,038,345 threshold combinations were produced. Summary of the ranges of resulting diagnostic performance parameters are shown in table 3.5. To maintain safety, threshold combinations with a NPV of rule-out > 98 % were selected.

The impact of adjusting rule-in thresholds on the rule-in diagnostic parameters (specificity of rule-in and PPV) and overall efficacy is illustrated in figure 3.4.

	With current thresholds	With experimental thresholds	
		Minimal	Maximum
Sensitivity of rule-out %	98.5	95.4	100
NPV of rule-out %	98	94.4	100
Specificity of rule-in %	81.5	75.3	88
PPV of rule-in %	63.5	57.1	69.7
Overall efficacy %	57.7	43.6	71.6
Prevalence of NSTEMI in the observe group %	19.5	15.6	25

Table 3.5 cMyC 0/1h triage algorithm's diagnostic performance parameters with current and experimental thresholds.

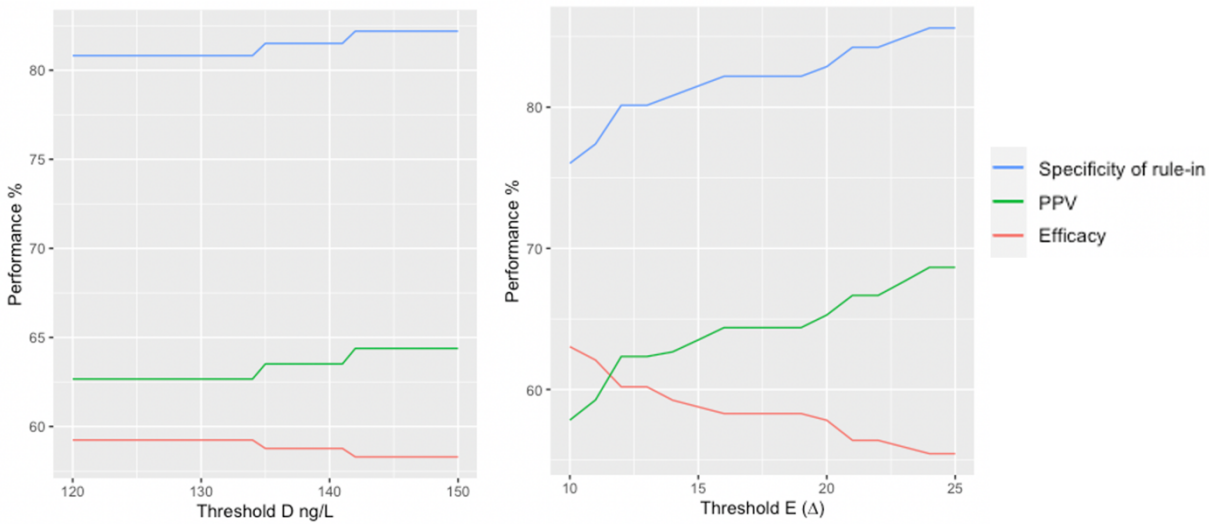


Figure 3.4 Impact of rule-in threshold adjustment in cMyC-guided 0/1 h triage algorithm on the specificity of rule-in, PPV and overall efficacy. (Thresholds D and E are explained in figure 3.3)

The specificity of rule-in and PPV are optimised by increasing thresholds D and E, at the cost of a reduction in the overall efficacy.

3.4.7 The kinetic profile of cMyC and cardiac troponins (cTnT and cTnI) in patients with- and without -renal dysfunction

To further understand the effect of renal dysfunction on the concentration/dynamics of the biomarkers, polynomial regression models were designed to produce representative interaction graphs to demonstrate the temporal change of the biomarker concentration in patients with-and without-myocardial infarction MI, stratified by 4 specific eGFR values, 30, 60, 90 and 120 mL/min/1.73 m^2. For this analysis, all patients in the APACE cohort were included.

There was an inverse relationship between eGFR and the baseline concentration in patients with-and without-MI. However, in patients with MI, the pattern is reversed 3-6 hours from onset of pain, into higher biomarker concentrations with improved eGFR, suggesting a more dynamic change in the biomarker concentration with higher eGFR values.

No dynamic changes in the biomarker concentration were observed in patients without MI across all eGFR values, figure 3.5.

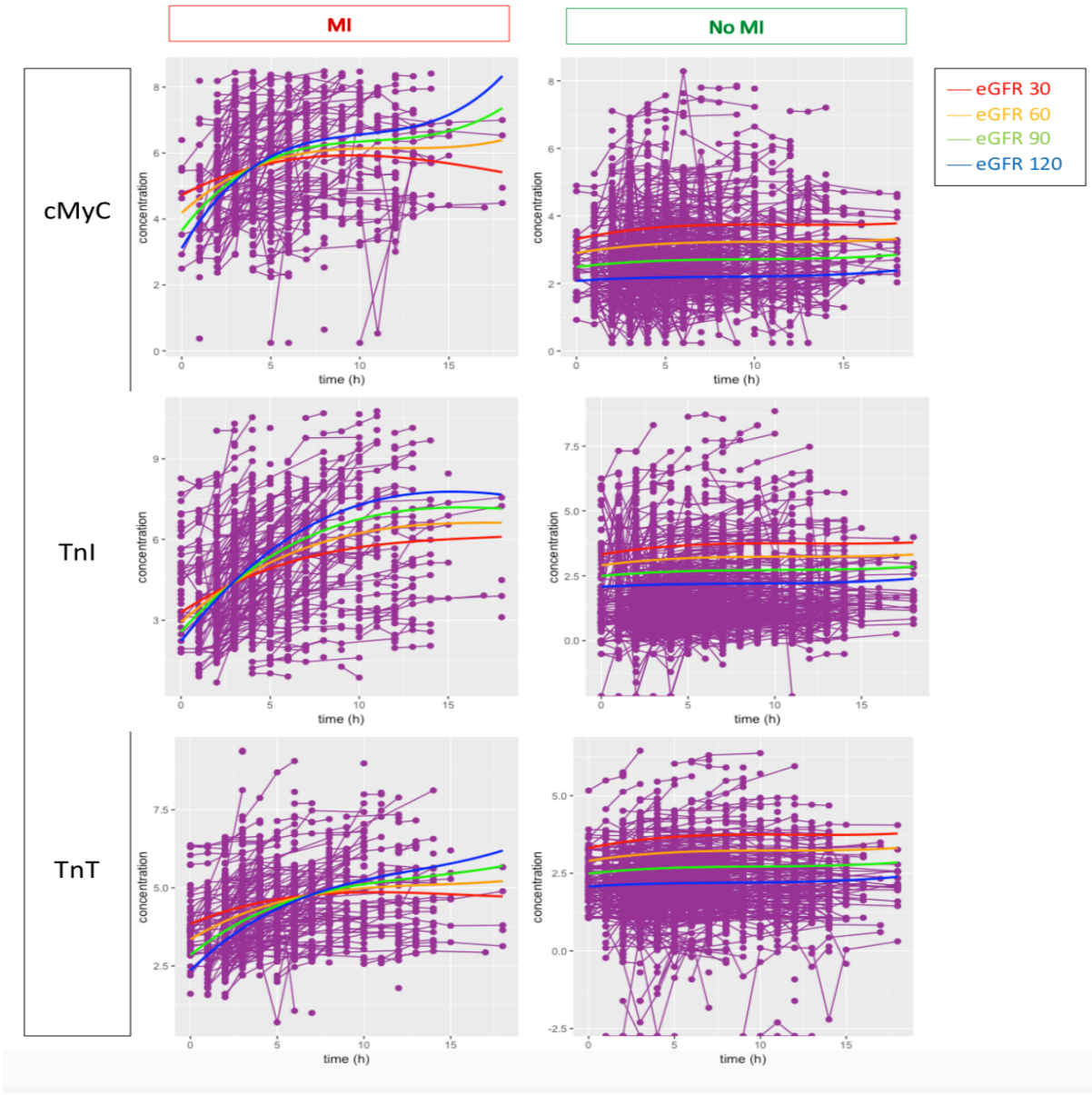


Figure 3.5 temporal changes in cMyC, hs-cTnI and hs-cTnT concentration stratified by eGFR

Polynomial regression models demonstrating the temporal change in cMyC, hs-cTnI and hs-cTnT concentration on serial samples stratified by 4 eGFR values 30, 60, 90 and 120 mL/min/1.73 m^2 in patients with- and without final adjudicated diagnosis of MI. Time (h) measured from onset of pain. Joined dots represent samples obtained from the same subject. Concentrations are natural logged. Polynomial regression graphs were generated with increasing orders, orders were compared with ANOVA for the selection of the best predictive model

3.5 Discussion:

This is the first study assessing the diagnostic performance of the cMyC-guided ESC 0/1-h triage algorithm in patients with renal dysfunction. The study was conducted on the same unselected cohort (APACE) used for deriving and validating the diagnostic cut-off threshold for cMyC for the triage of patients with suspected myocardial infarction [1]. The study showed several important findings:

In patients with renal dysfunction as defined by eGFR < 60 mL/min/1.73 m2, the safety of the triage algorithm as estimated by sensitivity of rule-out was high and preserved for cMyC as well as for hs-cTnI and hs-cTnT (98.5%, 96.9% and 100%, respectively); however, all three biomarkers ruled-out fewer patients in this group compared to patients with normal renal function. The reduction in the efficacy of rule-out was of sufficient magnitude to significantly compromise the overall efficacy of the algorithms by allocating significantly higher proportions of patients to the observe zone in the group with renal dysfunction compared to the group with normal renal function, for all three biomarkers.

On the other hand, cMyC and cTns ruled in a higher proportion of patients with renal dysfunction compared to patients without, but this was at the cost of accuracy as assessed by specificity of rule-in, which was significantly compromised in the former group, but without parallel reduction in the PPV, owing to the higher prevalence of NSTEMI in renal dysfunction group (30% vs 14%, $p < 0.001$).

While the NPV remained high with cMyC- and hs-cTnT-guided algorithms regardless of renal function, hs-cTnI achieved lower NPV in patients with reduced- compared to

normal- renal function (NPV, 94.8% [95% CI, 87.9-1.0] vs 99.3% [95% CI, 98.7-99.9], respectively; p=0.046).

In patients with renal dysfunction, compared to hs-cTns, cMyC ruled out significantly more patients, however the overall efficacy as well as all other performance measures remained comparable across all three biomarkers.

Although AUC for cMyC was higher than AUCs for hs-cTnI and hs-cTnT, the difference did not reach statistical significance.

The results of this study are comparable with the findings of Twerenbold et al, which was the first study examining the performance of the ESC 0/1-h triage algorithm using hs-cTnT and hs-cTnI in the APACE cohort. [138]

With machine learning iteration spanning experimental thresholds above and below each cut-off thresholds, it was possible to increase specificity of rule-in without unacceptable reduction in the sensitivity of rule-out, however, the overall efficacy suffered even further compromise. This is expected when the diagnostic thresholds for rule-in are increased leading to widely spaced rule-out and rule-in thresholds thereby increasing the number of patients left in the observe zone.

Previous attempts to adjust the 99th percentile reference interval of troponin in patients with renal dysfunction yielded reasonable results,[144] however, similar challenges to the ones we describe have been reported when attempting to adjust the thresholds within the troponin-guided 0/1-h triage algorithm.[138]

To further explain the deficiencies in the ESC 0/1-h triage algorithm when applied to patients with renal dysfunction, the dynamics of the biomarkers in this group of patients were examined.

The higher baseline concentrations of the biomarkers in renal dysfunction could explain the lower proportion of patients ruled-out and the higher proportion allocated to the observe and rule-in zones. The inverse relationship between eGFR and presentation concentration of the biomarker is present up to 3-6 hours after onset of pain, the time period during which most patients present to the hospital and have their first blood draw.

On the other hand, the reduced temporal dynamism and magnitude of change in the group with renal dysfunction, possibly the result of reduced renal clearance, would be expected to lead to an underperformance of deltas in rule-in and overperformance of deltas in rule-out. However, the deltas incorporated into the algorithms for all-comers are perhaps still too small for the dynamic variation seen in this group.

Biological variation data in patients with and without chronic kidney disease showed similar intra-individual variability regardless of renal disease, but the analytical variation was significantly different, owing to the concentration dependency of analytical variation, thereby leading to lower Reference Change Values in patients with chronic renal disease.[102] However, proposing smaller deltas for patients with renal dysfunction is likely to further reduce the efficacy of rule-out and the accuracy of rule-in with the algorithm.

Our study has some limitations; foremost, this was a retrospective analysis of data derived from an hs-cTnT adjudicated cohort, which would, by default, affect the competitiveness of cMyC and hs-cTnI. In addition, some events might have been missed or misdiagnosed during the adjudication process and these decisions may have been biased by unblinded

measures of renal function. Furthermore, in this analysis, only patients with recorded eGFR who had both 0h and 1h concentrations of cMyC, cTnT and cTnI were included. This was done to ensure fair comparison between the performance of all three biomarkers in the same cohort with same events; however, it is recognised that some patients who have been triaged with presentation sample alone might not have had repeated testing, and therefore were excluded from the analysis. Finally, it is not determined whether the reduced eGFR at presentation was acute or chronic, the acuity of renal dysfunction might also have some impact on the serum concentrations of the biomarkers that is different to the effect of the severity of renal disease.

3.6 Conclusion

In patients with renal dysfunction, the performance of cMyC-guided ESC 0/1-h triage algorithm is comparable to cardiac troponins, however, while the safety of the algorithm is high, the accuracy and overall efficacy is reduced.

4 Chapter 4. Temporal Release of Cardiac Myosin-Binding Protein C Compared to Cardiac Troponin After Brief Coronary Artery Balloon Occlusion in Humans

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Hypothesis

Brief induced ischaemia in humans causes temporal cMyC release that is comparable to that of cardiac troponin

Statement of contribution:

Recruitment, sample collection and sample analysis for troponin were performed by our collaborators (Professor Kasper Iversen, et al. Department of Cardiology, Herlev-Gentofte Hospital, Copenhagen, Denmark) before the start of this research fellowship.

Bashir Alaour organised sample analysis for cMyC, and designed and performed all data analyses outlined in this study.

4.1 Introduction

Our collaborators at Herlev-Gentofte Hospital, Copenhagen, Denmark demonstrated that as little as 30 seconds of induced myocardial ischemia in humans was sufficient to cause acute myocardial injury, as assessed by an increase in cardiac troponin cTn[145].

cMyC resides outside the troponin complex and has a substantially larger molecular weight than either cTnT or cTnI. In this chapter I examine whether the release of cMyC after brief induced ischemia is similar to that of cTn.

4.2 Methods

Ethics

The study was approved by the local Research Ethics Committee in Denmark (H-16027749) in accordance with Danish law and was registered at https://www.clinicaltrials.gov (Unique identifier: NCT03203057).

Participants

A detailed description of methods has been published previously [145]. In brief, 34 consented patients who underwent invasive coronary angiography on clinical grounds but were found to have normal coronary arteries, were randomly assigned to 1 of 4 groups and underwent 0, 30, 60, or 90 seconds of intracoronary balloon occlusion of the left anterior descending artery between the first and second diagonal branch. An appropriately sized balloon (3.0–4.0 mm) was inflated to no more than 4 atm. Balloon occlusion was documented by contrast angiography. Baseline and serial serum samples drawn at intervals up to 240 min after occlusion, were frozen at -80°C until analysis.

Sample analysis

cMyC was measured using an established high sensitivity assay on the Erenna platform by Millipore Sigma [3]. LoD 0.4 ng/L; LoQ (20% CVA) of 1.2 ng/L; intra-series precision (CV, 11 +/- 3%) and inter-series precision (CV, 13 +/- 3%), 99th percentile is 87 ng/L.

The hs-cTnT was measured on the Elecsys 2010 (Roche Diagnostics). The limit of blank (LoB) was 3 ng/L, and the limit of detection was 5 ng/L. The 99th percentile of a healthy

reference population was 9 ng/L for female patients and 17 ng/L for male patients, with a coefficient of variation of 10% at 13 ng/L.

The hs-cTnI was measured on the Siemens Centaur platform. The LoB and limit of detection were 1 ng/L and 2 ng/L, respectively. The 99th percentile was 37 ng/L for female patients and 57ng/L for male patients [145].

Statistics

Continuous variables are presented as medians and interquartile ranges. Categorical variables are presented as numbers and percentages. Figures comparing concentrations at different time points were presented with log10–transformed y axes. Continuous variables across groups were compared using the Mann-Whitney U test. For comparisons of categorical variables, $\chi 2$ test was used.

4.3 Results

Baseline characteristics of participants are shown in table 4.1.

Characteristic	0-s ischemia (n=9)	30-s ischemia (n=8)	60-s ischemia (n=9)	90-s ischemia (n=8)
Age, y, median (IQR)	62 (53–66)	61 (45–66)	55 (47–68)	60 (56–68)
Male sex, n (%)	4 (44)	3 (38)	5 (55)	3 (38)
Hyperlipidaemia, n (%)	2 (22)	4 (50)	4 (44)	4 (50)
Diabetes, n (%)	1 (11)	0 (0)	0 (0)	0 (0)
Baseline high-sensitivity cardiac troponin T, ng/L, median (IQR)	5 (4–7)	5 (3–8)	7 (4–9)	6 (5–8)

Table 4.1 Baseline characteristics

Baseline high-sensitivity cardiac troponin I (Siemens), ng/L, median (IQR)	4 (3–9)	4 (2–7)	3 (3–7)	4 (2–9)
Baseline cMyC, ng/L, median (IQR)	5 (3–9)	10 (2–28)	8 (3–22)	3 (2–5)

Whiskers plots of the biomarker concentrations at different timepoints for each group are shown in Figure 4.1, panels A-D.

In the 30 seconds and 60 seconds groups, only cTnI concentration increased significantly compared to baseline (at 180 minutes). In the 90 seconds group, there was a significant increase at 90 minutes in the concentrations of all three biomarkers compared to baseline ($p < 0.05$).

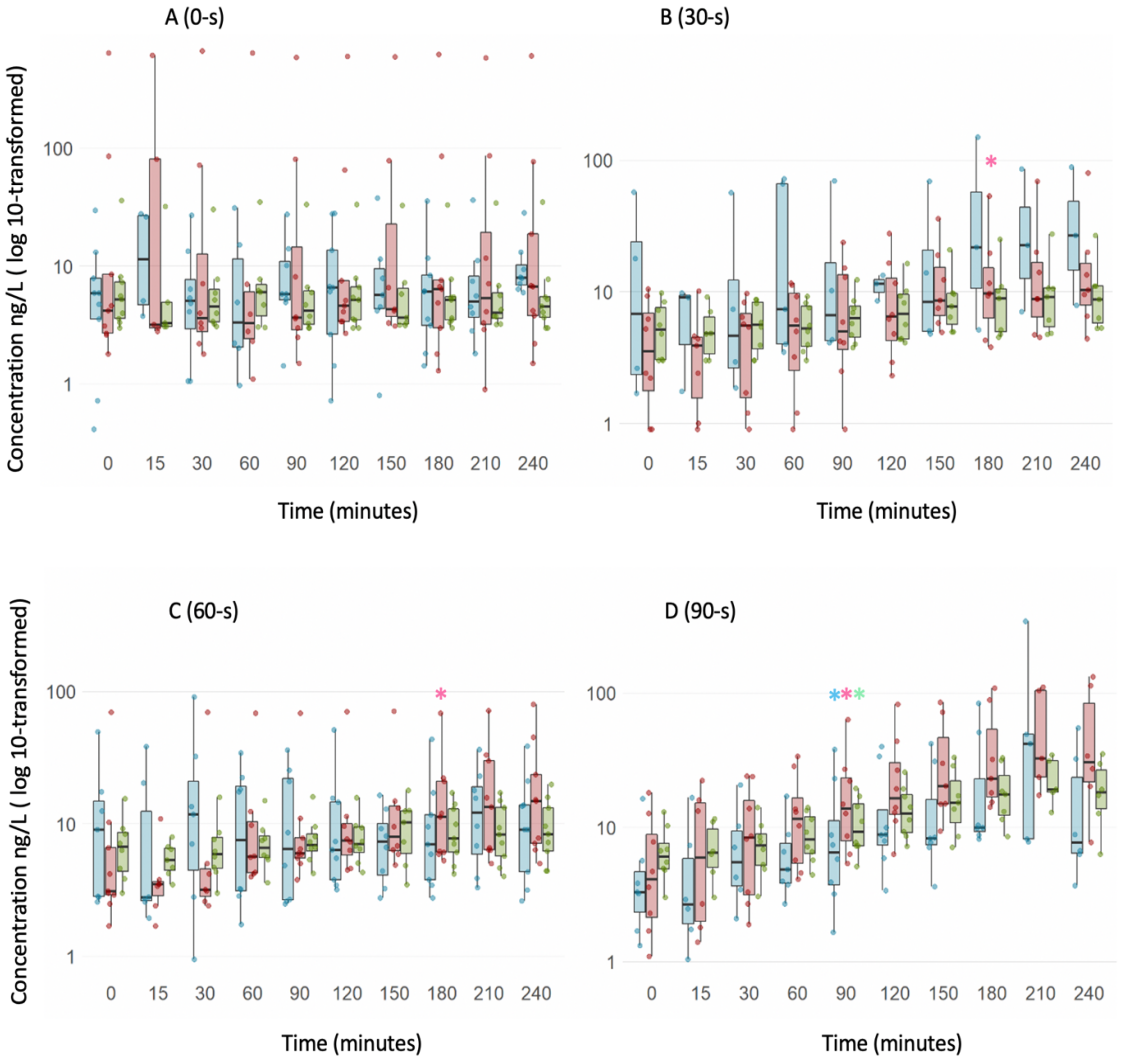

Figure 4.1 Biomarker concentrations against time after myocardial ischemia induced by intracoronary balloon occlusion

Panel A – Control (0-s) group, Panel B – 30-s ischemia group, Panel C – 60-s ischemia group, Panel D – 90-s ischemia group. Boxes depict median, 1st and 3rd quartiles, lower and upper whiskers and outliers. (Blue: cMyC; pink: Siemens hs-cTnI; green: Roche hs-cTnT). * p <0.05 for concentration difference compared to baseline.

After normalizing biomarker concentrations to baseline values to allow inter-biomarker comparison in the 90-s ischaemia group, there was no significant difference between the changes of cMyC and cTnI concentrations relative to baseline at all time points. But there was a tendency for a lower concentration change relative to baseline for cTnT and cMyC compared to cTnI, although inconsistent across time points. Figure 4.2.

Concentrations of cMyC, cTnI and cTnI were above the upper reference limit for the respective biomarker in one participant (25%) in the 30-seconds group (counting only the 4-participants who had all three biomarkers measured).

cMyC, cTnI and cTnT values exceeded the upper reference limit in 1 (11%), 2 (22%) and 2 (22%) participants, respectively, in the 60-seconds group; and in 1 (12.5%), 2 (25%) and 6 (75%) participants, respectively, in the 90-seconds group.

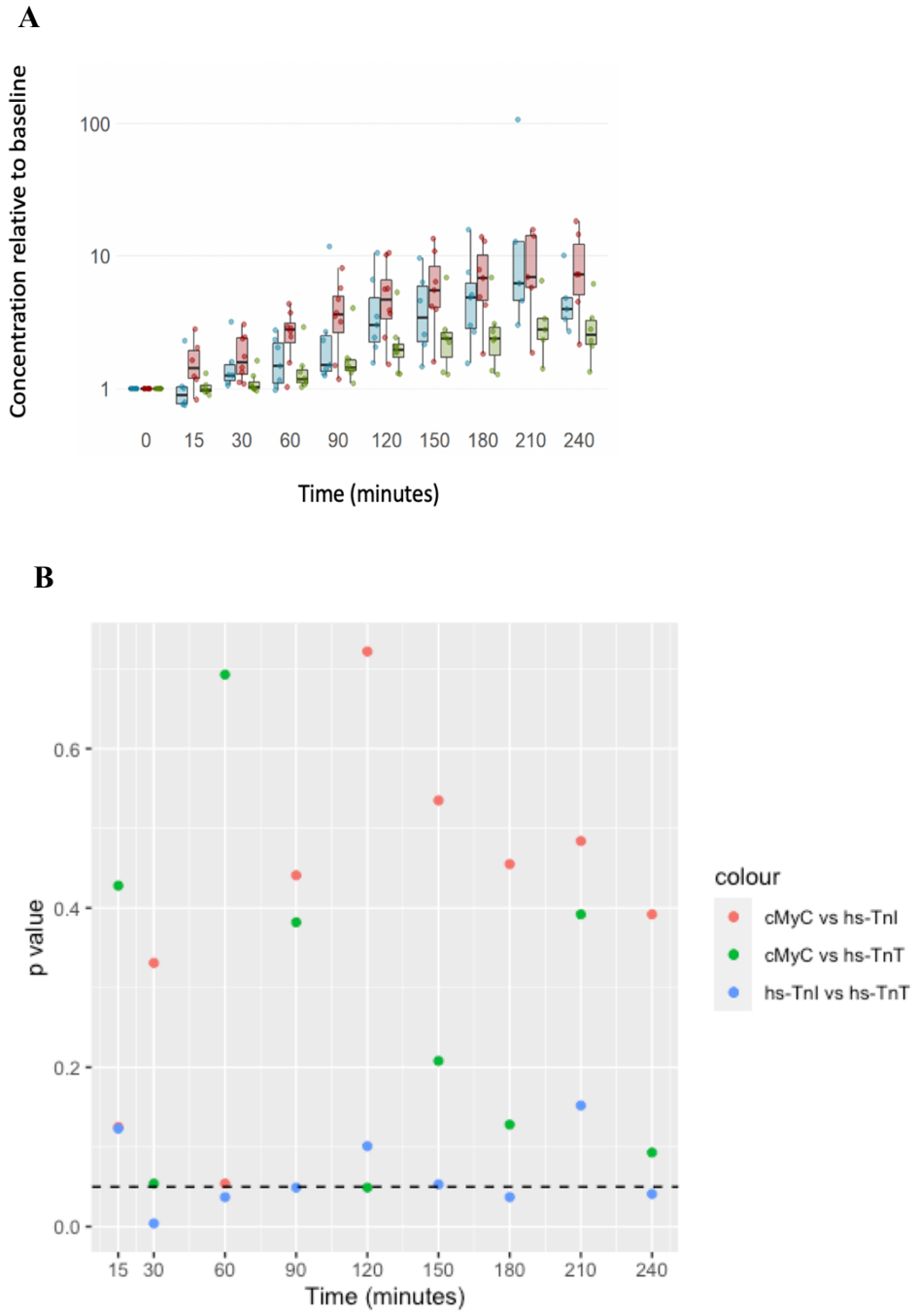

Figure 4.2. Biomarker concentrations (log 10-transformed) against time after myocardial ischemia induced by intracoronary balloon occlusion.

Panel A biomarkers concentrations relative to baseline against time after myocardial ischemia induced by intracoronary balloon occlusion for 90-s ischemia group. Boxes depict median, 1st and 3rd quartiles, lower and upper whiskers and outliers. (Blue: cMyC; pink: Siemens hs-cTnI; green: Roche hs-cTnT). Panel B, p values for inter-biomarker comparison of concentrations relative to baseline at different time points after 90-s balloon-induced myocardial ischaemia. Dashed line p = 0.05

Hour-to-hour relative concentration changes for each biomarker are shown in figure 4.3. The magnitudes of hour-to-hour change appear to decline from 2 hours after induced ischemia, with a significantly more rapid decline in the hourly change of concentration for cMyC compared to cTnI (p=0.0312) and cTnT (p=0.048) between 3 and 4 hours after ischemia.

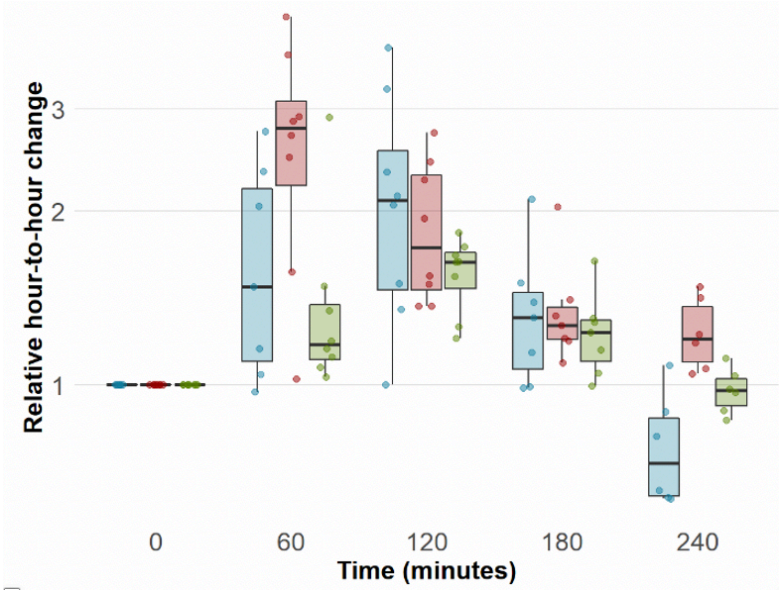

Figure 4.3 Relative hour-to-hour change of biomarker concentrations.

(Blue: cMyC; pink: Siemens hs-cTnI; green: Roche hs-cTnT)

4.4 Discussion

The study shows that 90 seconds of balloon induced myocardial ischaemia in humans causes a significant increase in the concentration of cMyc, cTnT and cTnI, at 90 minutes after injury, with a comparable relative change in biomarker concentration between cMyC and cTnI and a tendency towards a lower relative change for cTnT.

Additionally, between 3 and 4 hours after ischaemia, the magnitude of hour-to-hour change is significantly lower for cMyC compared to cTns, suggesting a more rapid decline in cMyC release.

The results of this human study complement a previous report on troponin release after transient, although more prolonged (10 minutes) inducible ischaemia in a porcine model, without cellular evidence of myocardial necrosis or infarction, but with evidence of caspase-mediated apoptosis. [146] Theories suggesting alternative or adjunctive etiologies such as the release of free cytosolic pools of troponin and increased cell wall permeability, have also been discussed.[147,148]

It is unclear whether the same mechanisms underline the release of cMyC and cardiac troponin in this model of induced ischaemia in humans, as cMyC lies outside the troponin complex and has a substantially larger molecular weight (140.5 vs 23.5 and 33.5 kDa for cMyC, cTnI and cTnT, respectively).

Previous reports showed that cMyC rises faster and peaks earlier than cTnT after iatrogenic or spontaneous acute MI. However, the magnitude of acute myocardial injury was much greater than seen in the current study. A similar pattern is not clearly demonstrated after brief ischemia in this study, however, the magnitude of change in cMyC concentration appeared to decline faster, similar to what is observed after acute MI [52].

Our analysis is limited to 4 hours after injury. It would be useful to observe the kinetic profiles of the biomarkers over a longer period, to determine if time-dependent inter-biomarker differences in the release pattern exist.

Our finding may have implications for clinical practice. Previous reports showed that cMyC rises faster and peaks earlier than hs-cTnT after iatrogenic or spontaneous acute MI, [52] similar differences in the release kinetics were not demonstrated after induced ischemia. Additionally, and interestingly, cMyC concentration exceeded the upper reference limit in a smaller proportion of participants in the 60- and 90-seconds group, compared to cTns, especially cTnT concentrations. However, considering the small number of participants in our study, the relatively short period of follow up of biomarker release, and the differences between participants in our cohort and the typical patients with angina, in whom coronary artery disease might be associated with adaptive changes that could protect the myocardium from significant injury during angina attacks, it is difficult to draw solid conclusions about differences between the biomarkers in the sensitivity and specificity to ischemia, or to extrapolate the results to myocardial infarction, but it is plausible that using multi-biomarkers approach could help differentiate between infarction and ischemic injury.

Overall, our data suggests the need to revisit the diagnostic criteria for unstable angina, and that biomarkers of myocardial injury could play a role in a contemporary refinement of the diagnosis of unstable angina. [148]

In summary, we have shown that after 90 seconds of induced ischemia there is a significant and comparable increase in the concentration of three protein biomarkers of myocardial injury despite marked differences in their protein sizes. This might suggest similar mechanisms of biomarker release, e.g. exocytosis rather than membrane leakage, and indicates that other intracellular myocyte proteins might also be subject to exocytosis. The absence of inter-biomarker differences in the release kinetics in the early phase (up to 3 hours) after induced ischemia might help aid the differentiation between ischemia and myocardial infarction, where clear differences between the kinetic profiles of the biomarkers are recognized. This is of significant relevance to peri-procedure MI but might also be applicable to other types of myocardial injury. We propose that a study with a longer duration of blood sampling would add to the field [149].

4.5 Conclusion

Induced ischemic can causes significant and comparable increase in the concentrations of cMyC and cTns, however, a faster decline in cMyC concentrations compared to cTn is observed between 3 and 4 hours after injury. Studies are needed to establish the mechanism of biomarker release post ischaemia and whether differences in the kinetics of the biomarkers could help differentiate between different types of myocardial injury.

5 Chapter 5. Phosphorylation and fragmentation pattern of cardiac myosin-binding protein C (cMyC) for the differentiation between different types of myocardial injury

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Hypothesis

Calpain mediated cleavage of cMyC is dependent on its phosphorylation status and can therefore aid the differentiation between Type 1 AMI and other types of myocardial injury

Statement of contribution

The porcine animal models were designed by our collaborators (Professor John Canty and Associate Professor Brian Weil, et al. University at Buffalo, USA).

Bashir Alaour performed all laboratory work outlined in this chapter, including all immunoblotting and immunoassay experiments, the creation of the immunoassays for total and FL-cMyC, and all sample analysis. Bashir Alaour led the recruitment for all participants in the study and performed all sample analysis for cMyC.

5.1 Abstract

Background

Cardiac myosin-binding protein C (cMyC) is a novel biomarker of myocardial injury, with apromising role in the triage and risk stratification of patients with acute cardiac disease. Differentiating between different types of myocardial injury is a daily challenge in clinical practice.

We hypothesise that Calpain-mediated cleavage of cMyC is dependent on its phosphorylation status and can aid the differentiation between different types of myocardial injury.

Methods

Propofol-anesthetized swine were subjected to one of two myocardial injury protocols, model (a): 1- hour phenylephrine PE infusion for stretch-induced left ventricular (LV)

stunning and cMyC release (n=5), and model (b): 1-hour Left Anterior Descending LAD occlusion for myocardial infarction MI (n=3).

Myocardial tissue samples from euthanized animals at 1- and 24-h post myocardial injury were immunoblotted using high affinity anti-N-terminal cMyC antibodies and anti-phosphoserine 282 antibodies.

To straddle the cleavage site of cMyC and create a selective fuller-length FL (intact) circulating cMyC assay, high affinity anti-N-terminal and anti-C-terminal cMyC antibodies were paired to create a sensitive electrochemiluminescence sandwich ELISA assay to complement our in-house total (all species) cMyC assay.

Porcine serum samples from the PE model (n=3) and MI model (n=3) above, and human serum from patients with Type 1 AMI (ST-segment elevation myocardial infarction, n=8) and Type-2 AMI (tachyarrhythmia-induced myocardial injury, n=5), were tested using both the new selective FL cMyC assay and the total (all species) assay to determine the ratio between FL (intact) and all-species cMyC.

Results

Immunoblotting showed reduced phosphorylation and increased fragmentation of cMyC in porcine myocardium subjected to ischaemic, compared to LV stretch induced, injury. There was a tendency in the latter for an increase in cMyC phosphorylation above the baseline (control) 24 hours after PE infusion.

Immunoassay of cMyC in the porcine serum samples at 1-h post injury showed an increase in circulating FL (intact) cMyC in parallel with total (all species) cMyC in the

PE model, whereas no detectable FL (intact) cMyC was observed in the MI model (only fragmented cMyC was present in the circulation).

Immunoassay of cMyC in the human serum showed significantly higher ratio of FL (intact) to total (all species) cMyC in the tachyarrhythmia-induced myocardial injury subtype of Type 2 AMI compared to Type 1-AMI.

Conclusion

Phosphorylation and fragmentation patterns of cMyC are dependent on the type of myocardial injury and might aid in the differentiation between different types of myocardial injury.

Large cohorts with carefully adjudicated participants with different types of myocardial injury are required to further test the hypothesis.

5.2 Introduction and literature review

5.2.1 cMyC structure

Cardiac myosin-binding protein C (cMyBP-C or cMyC) is a thick myofilament structural and regulatory protein. It comprises 8 immunoglobulin-like (C0, C1, C2, C3, C4, C5, C8, C10) domains, 3 fibronectin-type III (C6, C7, C9) domains and one phosphorylation motif (M domain).

In comparison to the slow and fast skeletal isoforms, cMyBP-C has distinct insertions and extensions, these include the N-terminal immunoglobulin-like extension (C0 domain), distinct phosphorylation sites in the M domain, and a 28-amino acid residue insertion within the C5 domain.[150]

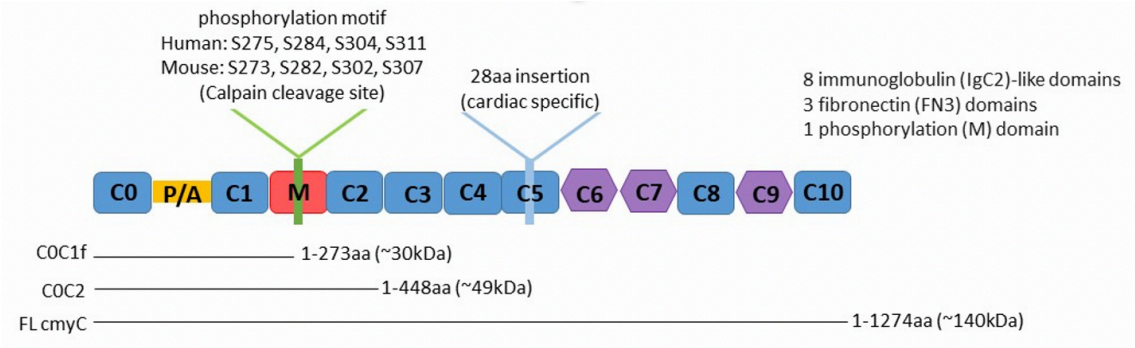


Figure 5.1 cMyC structure. Adapted from Lynch et al 2017

cMyC, like other myofilaments, is subject to a spectrum of post-translation modifications, including acetylation, citrullination, carbonylation, S-nitrosylation and phosphorylation. Phosphorylation occurs at four identified serine residues within the M domain (S273,

S282, S302 and S307 in mouse sequence; S275, S284, S304 and S311 in human sequence) by c-AMP dependant protein kinase A (PKA), PKC, PKD, Ca2+/calmodulin-dependent protein kinase (CaMK), casein kinase 2 (CK2) and p90 ribosomal S6 kinase (RSK).[150-153] Figure 5.1.

Phosphorylation of the serine sites within the M domain occurs selectively or in a hierarchical order,[150,154] and plays a critical role in modulating thick-thin filament interaction and regulating force generation and myocontraction.[154]

In animal models, reduced cMyC phosphorylation was associated with contractile dysfunction and heart failure.[155]

Additionally, and of more relevance to this research, phosphorylation of cMyC appears to protect cMyC against calpain mediated cleavage.

5.2.2 Basal cMyC phosphorylation

It is hard to determine the exact basal level of myofilament (including cMyC) phosphorylation in the hearts of healthy individuals. A pre-existing, probably above-basal level of phosphorylation, is likely to be found in samples derived from "healthy" hearts of brain-dead donors on inotropic support before death;[156] similarly, myofilaments from experimental animal models are likely to be subjected to additional phosphorylation secondary to the sympathetic stimulation accompanying terminal anaesthesia and cardiac excision.[157]

Attempts to experimentally induce "zero-phosphorylation" have been successful and are of great value for studying the functional and physiological effects of different kinases on

the individual phosphorylatable serines.[157] Nonetheless, this complete biochemical dephosphorylation doesn't mimic the "true" basal physiological phosphorylation state.

This uncertainty poses a significant challenge in designing an experimental animal model with a "ground state" of phosphorylation that mimics the real basal situation in healthy hearts of living individuals.

It has been suggested that there is a low level of basal cMyC phosphorylation in healthy hearts, which increases with physiological stress and beta-1 adrenergic stimulation.[151] In contrast, the phosphorylation of cMyC is significantly reduced below baseline with ischaemia-reperfusion injury, low flow ischaemia and myocardial stunning (see below).

5.2.3 cMyC is a substrate for calpain mediated cleavage

In vitro incubation of cMyC myofilament protein with calpain increases cMyC proteolysis and the appearance of a 40 kDa (COC1f) fragment in a calpain concentration-dependent fashion.[157,158]

Calpain-mediated proteolysis of cMyC is Ca^{2+} dependant; it is prevented by the absence of Ca^{2+} and the presence of calpain inhibitors.

In Mice, it is believed that the calpain target site (CTS) of cMyC lies between R-272 and R-280 (272-TSLAGAGRR-280).[158] It contains the S-273 phosphorylation site and precedes the S-282 phosphorylation site by one amino-acid.

Genetic ablation of this calpain-targeted R272-TSLAGAGRR-R280 motif confers cMyC resistance to calpain-mediated cleavage; further supporting evidence of the calpain-targeted site.[158]

The sequence equivalence of calpain target site (CTS) in human cMyC is 274-TSLAGGGRR-282.

The association between cMyC fragmentation and calpain activity in in-vivo experiments is discussed below.

5.2.4 cMyC dephosphorylation and Calpain mediated cleavage of cMyC in infarcted and failing human hearts

Barefield et al studied LV tissue derived from infarcted non-failing human hearts including samples from ischaemic tissue, border zone and remote areas and also samples from non-infarcted hearts as controls. cMyC protein extracted from ischaemic tissue exhibited clear fragmentation compared to border zone and remote tissue, with a concomitant increase in calpain activity and reduction of cMyC phosphorylation at S-273, S-282 and S-302 in the infarct zone compared to border and remote areas.[158]

LV samples derived from explanted failing hearts exhibited a similar pattern of increased site-specific S-273, S-282 and S-302 dephosphorylation with concomitant degradation of cMyC compared to explanted donor hearts.[159] So it is possible that the pattern of cMyC fragmentation maybe similar in different cardiac pathologies.

5.2.5 cMyC dephosphorylation and calpain mediated cMyC proteolysis during ischaemia-reperfusion injury in mouse models

In a mouse heart model, Govindan et al demonstrated significant reduction of cMyC phosphorylation at S-273, S-282 and S-302 and concomitant cMyC degradation in infarcted heart tissue following I-R injury compared to sham operated heart tissue.[159]

Decker et all observed a similar pattern of cMyC degradation and dephosphorylation in mouse heart subjected to prolonged low flow ischaemia caused by partial (50%) ligation of the left circumflex for 5 hours followed by reperfusion for 24 hours. [160]

A significant increase in dephosphorylated cMyC was seen in the territory of the low coronary flow area compared to the remote (normally perfused) area within the same heart or within similar areas in sham-operated hearts. In the remote myocardial territories, a significant increase in poly-phosphorylated cMyC was observed.[160] This is likely to be the result of increased sympathetic drive and consequent beta-1 adrenergic stimulation, activating the relevant kinases (PKA) to phosphorylate cMyC in the remote myocardium. Dephosphorylation of cMyC in the low coronary-flow territory correlated with degradation of cMyC and the appearance of the 40 kDa C0C1f N-terminal fragment. Whereas, in the remote area, where an increase in phosphorylation of cMyC was observed, full length cMyC was conserved. These observations support the idea that cMyC fragmentation patterns may differ according to pathological state of the myocardium at the moment of injury.

Barefield et al induced ischaemic-reperfusion injury by complete ligation of the left anterior descending artery for 60 minutes followed by reperfusion for 24 hours. In addition to cMyC dephosphorylation and fragmentation post I-R injury, calpain activity was also assessed. Degradation of cMyC and calpain activity were significantly increased in the infarct and border zones compared to remote and sham-operated control. Once again, the infarct tissue exhibited significant reduction in cMyC phosphorylation at S-273 and S-282.[158]

Overall, cMyC degradation post I-R injury appears to go hand-in-hand with a reduction in the myofilament phosphorylation and a concomitant increase in Calpain activity.

Although there appears to be an associative relationship between dephosphorylation of cMyC and calpain activity; the direct relationship between the I-R injury-induced dephosphorylation of cMyC and its effect on calpain-mediated degradation is not addressed in these i*n-vivo* studies, but this was studied in-vitro.

5.2.6 The direct relationship between cMyC phosphorylation and calpain mediated cleavage in-vitro

Lambda phosphatase treated (*Zero*-phosphorylated) cMyC myofilaments are susceptible to a potent calpain-mediated proteolysis effect. Prior incubation of the dephosphorylated cMyC preparation with PKA and PKD to achieve tri-phosphorylation of the protein provided a "near complete protection" of cMyC against cleavage with calpain.[157]

This takes us to the next question:

5.2.7 Does cMyC phosphorylation protect the heart against I-R injury?

Sadayappan et al demonstrated that pseudo-phosphorylation of cMyC in a transgenic mouse model does indeed render the heart resistant to I-R injury which translates into a potential cardioprotective effect of cMyC myofilament phosphorylation against I-R injury.[161]

In summary, the literature suggests that I-R injury leads to depressed cMyC phosphorylation which causes an increased susceptibility to calpain-mediated cleavage and the appearance of cMyC fragments.

Phosphorylation of cMyC protects it against calpain induced cleavage and conserves its structure and function. This opens the horizon for potential therapeutic targets; and additionally, of more relevance to my thesis, for major diagnostic benefits by using cMyC fragmentation patterns to distinguish between different types of myocardial injury

I hypothesise that the phosphorylation status of the myofilaments including cMyC varies depending on the injurious conditions that lead to its release into circulation; and considering the protective effect of phosphorylation against calpain-mediated cleavage, the primary structure of the released cMyC may also vary depending on those conditions.

Potentially, this scenario might have major clinical benefits by allowing biomarker-assisted differentiation between different types of myocardial injury including the differentiation between type 1 and type 2 acute myocardial infarction, a dilemma encountered by cardiologists on a daily basis, as discussed further below.

5.2.8 Differentiating between Type 1 and Type 2- AMI- a clinical conundrum

For diagnostic and management purposes, the Fourth Universal Definition of Myocardial Infarction proposed that we classify patients with myocardial infarction based on aetiology. The definition differentiates between Type 1 AMI where myocardial necrosis and ischaemia is due to plaque rupture, ulceration, fissuring, erosion or dissection, and Type 2 AMI where myocardial injury is triggered by conditions that contribute to oxygen supply-demand imbalance e.g. tachy-/brady-arrhythmias, anaemia, respiratory failure, hypotension and hypertension.[162] Differentiating between these types of AMI is crucial since their treatment differs markedly: revascularisation for Type 1 vs. supportive measures directed at the underlying illness for Type 2. Furthermore, subjecting patients with Type 2 AMI to interventions proven to improve outcome in Type 1 AMI (antiplatelet

agents and invasive angiography) wastes resource and is likely to cause harm. For the purposes of my research we will incorporate acute myocardial injury into our definition of Type 2 AMI, since in practice it is difficult to separate these entities.[163,164] In addition, the conditions of spontaneous coronary artery dissection, coronary embolus and coronary spasm, which are classified as Type 2 AMI, will be considered as Type 1. The diagnosis of Type 2 MI has become more prevalent since the introduction of high sensitivity troponin into clinical practice.[165] However, cTn measurements alone, even including changes in concentration over time, are not sufficient to distinguish Type 1 from Type 2 AMI,[41] this is also the case for cMyC.(https://pubmed.ncbi.nlm.nih.gov/33881449/) In clinical practice there is no clear way to determine whether a patient with an elevated cTn is having a Type1 or a Type 2 AMI. The diagnosis of Type 2 AMI in many cases is currently "A concept based on clinical hypothesis and observation without prospective mechanistic evaluation".[42] This in part explains the vast disparities in the reported incidence of Type 2 AMI which can vary between 2% and 37% in hospitalised patients[43,44] and between 5% and 71% in patients attending the Emergency Department![41,45]

There is consistent evidence that patients with Type 2 AMI have poorer clinical outcomes compared to Type 1, with three-fold increased risk of death at 1 (13.6% vs 4.0%, $p<0.0001$) and 12 (23.9% vs 8.6%, $p<0.0001$) months.[166] Accurate identification of Type 2 AMI could help reduce this disparity, by guiding studies to better understanding this heterogeneous condition and how it should be managed.

Additionally, the new ICD-10 (10th Revision of the International Statistical Classification of Disease and Related Health Problems) - effective October 2017- differentiates between

types of MI and has added a new and specific diagnosis code for Type 2 AMI.[167] This is important, as the new codes will guide payers and commissioners in making decisions re payments and reimbursements policies, which places even greater emphasis on having better diagnostic tools for recognition of Type 2 AMI.

Overall, troponin measurements alone, even including changes in cTn concentration over time, are not sufficient to distinguish Type 1 from Type 2 AMI.[41] My premise is that the proportion of cMyC appearing as COC1f will help differentiate between these forms of AMI. In Type 2 AMI the supply:demand imbalance is triggered by increased demand and the PKA/PKC/CAMK signalling pathways are therefore likely activated before cell death; thus the phosphorylation of cMyC should protect it from cleavage. In contrast, in Type 1 AMI plaque rupture is a fast and catastrophic event causing an abrupt reduction in myocardial supply. As noted in the summary above, this is associated with cMyC dephosphorylation to below basal levels, the concurrent activation of calpain and thus the likely dominance of C0C1f over full-length cMyC. Thus, the fraction of cMyC that is intact ([intact]/[total]) will be higher in Type 2, than in Type 1, MI. Admittedly, the scenario depicted does involve some conjecture, however we definitely observe C0C1f predominance in the serum after confirmed Type 1 AMI.[52] As yet there has been no exploration of the cMyC fragmentation pattern in Type 2 AMI, a deficiency I plan to address

The hypothesis will be addressed in the following order

A. In-heart *in-vivo* in animal models: by immunoblotting myocardial tissue samples from euthanized pigs with ischaemic and non-ischaemic myocardial injury, using high affinity anti-N-terminal cMyC antibodies and anti-phosphoserine 282

antibodies, to determine the predominant species of cMyC in the heart and its phosphorylation status.

B. Circulation *in-vivo* (serum) from animals used in (a) by determining the ratio between circulating intact (full length/un-cleaved) and total (all-species) cMyC concentrations in the two models.

C. Circulation *in-vivo* (serum) in human, by determining the ratio between circulating intact (full-length/un-cleaved) and total (all species) cMyC concentrations in the serum of recruited participants with Type 1 AMI (ST-segment elevation myocardial infarction) and Type-2 AMI (tachyarrhythmia-induced myocardial injury).

To allow the testing proposed in (B) and (C) I will create the following:

1. Selective full-length FL (intact) assay for circulating cMyC in both humans and pigs using high affinity anti-N-terminal and anti-C-terminal cMyC antibodies, paired in an electrochemiluminescence sandwich ELISA format assay.
2. Non-selective cMyC assays that allow in-house measurement of total (all species) cMyC concentrations with optimised sensitivity.

5.3 Phosphorylation and fragmentation pattern of cMyC in pig heart subjected to phenylephrine-induced myocardial injury and Left Anterior Descending ligation-induced myocardial infarction

5.3.1 Methods

5.3.1.1 Animal models

Details of the swine closed-chest animal models, designed and conducted by The University at Buffalo collaborators are found in Weil, et al, and Techiryan, et al [168,169].

In summary, propofol-anesthetized swine were subjected to one of two induced myocardial injury protocols:

1. hour phenylephrine PE infusion to induce transient elevation in left ventricular end-diastolic pressure (LVEDP) to approximately 30 mm Hg with left ventricular LV stunning and myocardial injury. Swine were then euthanised 3-h or 24-h post PE infusion, and hearts were excised for histopathological assessment of infarction and cell apoptosis. Endocardial tissue samples were collected from LAD territory. Serial blood sampling from the carotid artery was performed between PE infusion and euthanasia [168].
2. 1-hour Left Anterior Descending (LAD) coronary artery occlusion for myocardial infarction. Swine were then euthanised 3-h or 24-h post ligation, and hearts were excised for histopathological assessment of infarction and cell apoptosis.

Endocardial tissue samples were collected from LAD territory. Serial blood sampling from the carotid artery was performed between reperfusion and euthanasia [169].

Swine were obtained from a USDA certified vendor (Bippert's Farm; USDA license number 21-B-0159) and processed at the University of Buffalo Laboratory Animal Facility (USDA license number 21-R-051).

Blood samples were collected under sterile laboratory conditions, centrifuged, and stored as frozen plasma aliquots (-80 °C) at the University at Buffalo.

Tissue and plasma samples were transferred to London on dry ice with temperature control (-65 C to -80 C). I applied for the samples to be imported to the UK under a Plant & Animal Health Agency licence, authorisation number ITIMP20.1016.

5.3.1.2 Materials

- Lysate buffer: 50mM Tris-HCL PH 7.5
- Protease inhibitor, (Thermo Scientific, #78429, Thermofisher, USA)
- Pierce™ BCA Protein Assay Kit, (Thermo Scientific, # 23225, Thermofisher, USA)
- 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels, 15-well, 15 µl (Bio-Rad, # 4561096, Bio-Rad Laboratories, USA)
- Laemmli sample buffer (Bio-Rad, #1610737, Bio-Rad Laboratories, USA)
- 0.45 uM PVDF membrane (Bio-Rad Laboratories, USA)
- TransBlot Turbo Semi-Dry Transfer System (Bio-Rad Laboratories, USA)

- Washing buffer: PBS-0.05% Tween-20
- Blocking buffer: 1x Tris Buffered Saline (TBS) with 1% Casein (Bio-Rad #1610782, Bio-Rad Laboratories, USA).
- Sheep monoclonal cMyC N-terminal antibodies (3C2, Bioventix, UK)
- Rabbit polyclonal cMyC anti-p282 antibody
- Peroxidase-conjugated AffiniPure Donkey Anti-Sheep IgG (H + L) (Jackson Immuno Research). Code 713-035. Lot: 138813
- Goat Anti-Rabbit IgG H&L (Alexa Fluor® 647) (# ab150079, abcam)
- X-OMAT developer (Kodak, USA)
- iBright Imaging System (Thermofisher, USA

5.3.1.3 Protocols

Tissue lysate preparation

All tissue samples underwent a standard tissue homogenisation protocol as described below:

Heart tissue samples (~50 mg) were aliquoted in 500 ul lysate buffer (50mM Tris-HCL PH 7.5). Protease inhibitor at volume ratio 1:100 (Thermofisher, # 78429) was added to each sample. Samples were sonicated on ice, 10 seconds on/10 seconds off, to a total of 60 seconds on, then vortexed for 10s every 10 min, X 3 (on ice). Aliquots were then centrifuged at 13200 rpm at 4ºC for 20min. Supernatant was carefully collected, and protein concentration was measured with Pierce™ BCA Protein Assay Kit, Thermo Scientific, Cat 23225, following manufacturer's instructions.

https://www.thermofisher.com/documentconnect/documentconnect.html?url=https://assets.thermofisher.com/TFSAssets%2FLSG%2Fmanuals%2FMAN0011430_Pierce_BCA_Protein_Asy_UG.pdf

Desired volumes (50 ul) were aliquoted and stored immediately at -80 C.

SDS-PAGE

Lysate volumes containing 100 ug of protein were eluted in 10 uL of 2X Laemmli sample buffer and diluted in ddH2O to a final volume of 20 uL. Samples were then heated to 65ºC for 1 min. 17 uL of sample was loaded into a 10% SDS-PAGE gel and subject to electrophoresis, 20 min at 90V, then 120V until the dye front reached the edge of the gel. Proteins on the gel were then transferred to a 0.45 uM PVDF membrane (BioRad) using the TransBlot Turbo semi-dry transfer system (BioRad).

Western Blot

The membrane was blocked in 1% casein at room temperature. Primary antibody (Sheep N -terminal mAb 3.45 mg/ml, 1:2000 in blocking buffer, and Rabbit polyclonal anti-p282 antibody 2.68 mg/ml, 1:1000 in blocking buffer) were applied to respective gels and blocked overnight at 4C. The membrane was washed for 10 mins, 3 times, in PBS-0.05% Tween-20 and washed in the respective secondary antibody (Donkey anti-sheep-HRP, 1: 20000, or Goat anti-rabbit-Alexa647, 1:10000) for 1h at room temperature. After washing 3 times in PBS-0.05% Tween-20 for a total of 30 mins, the membrane was exposed on either film using a X-OMAT developer or the iBright Imaging System (ThermoFisher).

Films were scanned and band densities were evaluated using ImageJ (NIH Image) or using the iBright Analysis software.

Coomassie Blue Staining

Following SDS-PAGE electrophoresis, the gels were stained using Coomassie Blue R-250 for 1h at room temperature, then de-stained in 10% acetic acid/50% MeOH solution until the background was clear, changing solutions every 20 minutes. Gels were imaged on the iBright Imaging System.

5.3.2 Results

5.3.2.1 *Tissue lysate preparation*

Total of 19 samples of myocardial tissue were homogenised. Protein concentration was measured with Pierce™ BCA Protein Assay Kit, Thermo Scientific, table 5.1.

Sample ID	Run 1 (ug/ul)	Run 2 (ug/ul)	Average (ug/ul)
16-22	34.6	34.1	34.4
18-55	56.7	59.3	58
08-78	63.1	59.8	61.4
20-25	42.8	42.3	42.5
20-48	58.4	60.7	59.6
16-24	63.1	64.3	35
16-23	42.8	43.6	40
20-05	55.6	51.8	53.7
20-08	50.4	51.7	51
20-23	44.5	45.6	45
08-24	52.8	51.4	52.1
08-70	46.4	47.5	46.9
08-71	33.8	28.6	31.2
16-19	36.6	23.1	29.8
16-20	33.9	31.8	32.9
16-21	41.8	48.7	45.2
20-24	61.2	49.3	55.2
08-72	43.6	39.1	41.3
16-71	51.1	49.4	50.2

Table 5.1. Tissue lysate protein concentration measured with Pierce™ BCA Protein Assay Kit.

5.3.2.2 *Fragmentation and phosphorylation of cMyC according to type of myocardial injury*

Total of 19 samples were immunoblotted

Control: 5 samples

PE 3h: 5 samples

PE 24h: 5 samples

MI 3h: 3 samples

MI 24h: 1 sample

Samples were clustered in the following order: Control, PE 3h, PE, 24h, MI 3h +/- MI 24h (only one MI 24h sample available for analysis). Table 5.2.

Gel 1		
Well	**Group**	**Sample**
1	control	16-21
2	PE 3h	20-23
3	PE 24h	20-25
4	MI 3h	16-23
5	control	16-71
6	PE 3h	20-24
7	PE 24h	08-72
8	3h MI	16-22
9	control	16-19
10	PE 3h	08-78
11	PE 24h	08.71
12	MI 3h	16-24
13	MI 24h	18-55
14	control	20-48

Gel 2		
well	**Group**	**Sample**
1	PE 3h	20-05
2	PE 24h	08-24
3	control	16-20
4	PE 3h	20-08
5	PE 24h	08-70

Table 5.2. Sample arrangement within the WB gels

Immunoblotting of myocardial tissue from the MI model showed reduction in intact (FL) cMyC (~135 kDa) and the appearance of cMyC fragments C0C1f (~35 kDa), (columns 4, 8, 12 and 13)

FL-cMyC (~135 kDa) was consistently the predominant species of cMyC in control and PE model myocardial samples (columns 1, 5, 9, 14, 17 and 2, 3, 5, 6, 10, 11, 15, 16, 18, 19, for control and PE models, respectively). Figure 5.2.

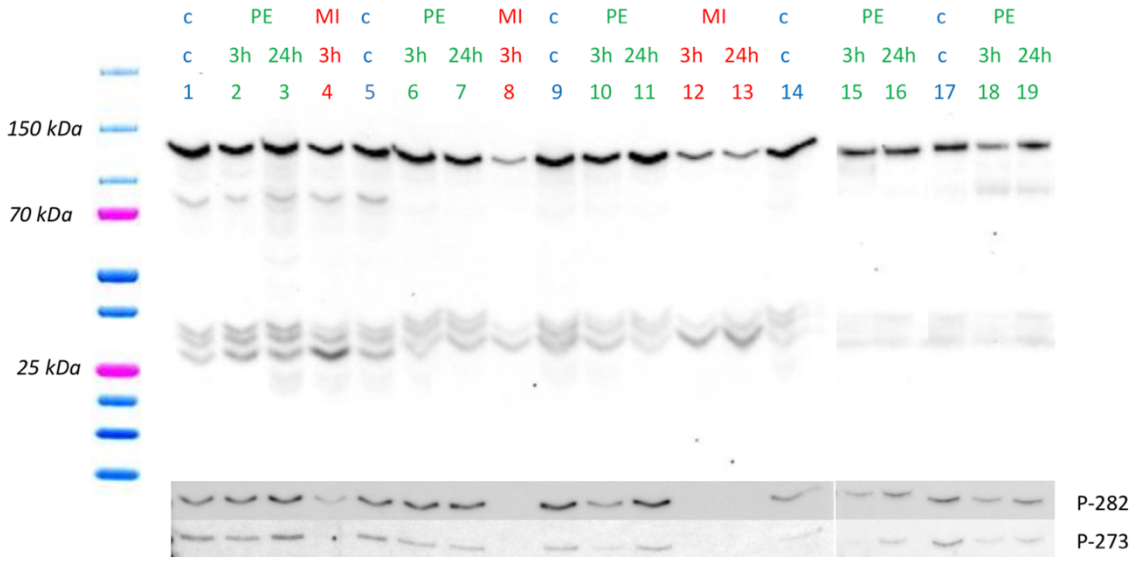


Figure 5.2. Immunoblots of myocardial tissue lysates.

C, control; PE, myocardial tissue from pigs subjected to 1-hour phenylephrine infusion; MI, myocardial tissue from pigs subjected to 1-hour left anterior descending LAD ligation. cMyC was detected with high affinity anti-N-terminal sheep mAb 3C2. Bottom 2 rows: phosphorylated cMyC detected with anti-phosphoserine 282 and 273.

On densitometry analysis, there was a significant decrease in [intact cMyC]/[all species cMyC] ratio in the infarcted myocardium compared to samples from stretch-induced myocardial injury ($p<0.01$), with no significant difference in the ratio between the latter and control.

The increased fragmentation of cMyC in the infarcted myocardium corresponded with a significant reduction in the phosphorylation of serine 282 in the residual intact cMyC, compared to control and PE model ($p < 0.05$, for MI 3h vs control and MI vs PE).

Phosphorylation of cMyC serine 282 was similar between control and PE model ($p=0.99$).

Figure 5.3.

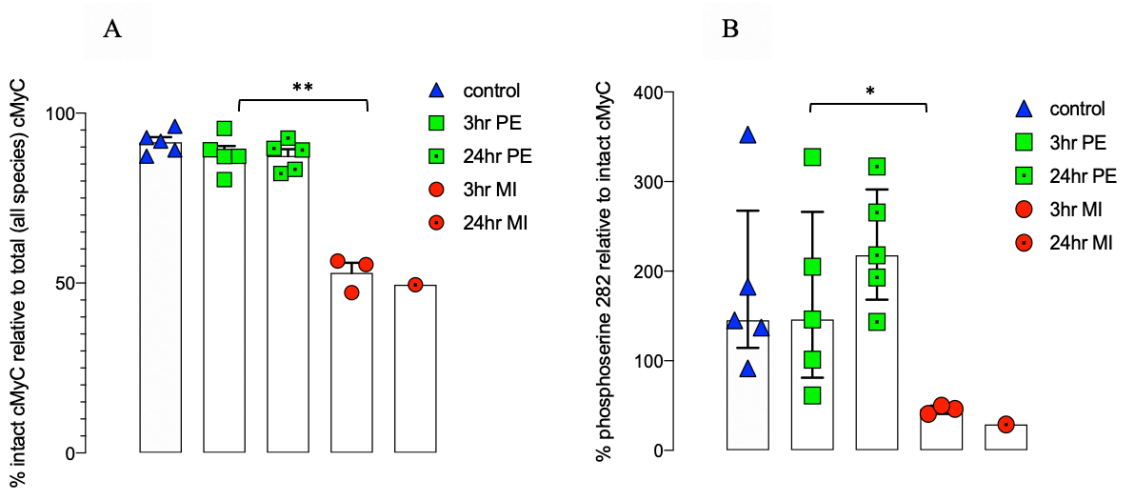


Figure 5.3 Densitometric analysis of cMyC bands.

Control, PE (phenylephrine) and MI (left anterior descending ligation) model groups for the determination of the ratio of intact (FL-cMyC) to total (all species) cMyC (A), and the ratio of phosphorylated cMyC at serine 282 to intact cMyC (B). * $p < 0.05$, ** $p < 0.01$.

Quantification of protein loading in entire lanes was done in ImageJ. Protein load was similar for all samples, within 1% difference.

5.3.3 Discussion

Immunoblotting showed reduced phosphorylation and increased fragmentation of cMyC in ischaemic myocardial injury, compared to LV stretch induced injury with phenylephrine and to control. There was no significant difference in the fragmentation pattern, nor the phosphorylation of intact cMyC between control and LV stretch myocardial injury.

The appearance of C0C1f fragment which borders the calpain target site (CTS) suggests that the cleavage of cMyC is likely to be calpain mediated, with association between the phosphorylation status of cMyC and its susceptibility to cleavage at CTS as shown in previous studies.[159] Additionally, I demonstrated that the phosphorylation status of cMyC is dependent on the type of myocardial injury.

To determine whether the differences in the phosphorylation and fragmentation patterns could be translated into a clinically utilisable diagnostic method that aids the differentiation between different types of myocardial injury, I need to examine the

fragmentation pattern of the circulating species of cMyC and determine the ratio between intact and total (all species) cMyC in the serum of the swine included in the experiments above and in human serum from patients with two likely distinct subtypes of myocardial injury.

To enable this, I need to create the following:

1- A selective full-length FL (intact) circulating cMyC assay for both human and porcine cMyC using high affinity anti-N-terminal and anti-C-terminal cMyC antibodies, paired in an electrochemiluminescence sandwich ELISA format assay.

2- A non-selective cMyC assay that allows in-house measurement of total (all species) cMyC concentrations with optimal sensitivity.

5.4 The creation of immunoassays for FL-cMyC and total (all species) cMyC

5.4.1 Introduction: FL-cMyC vs total (all species) cMyC assay

As discussed above in detail, Cardiac myosin-binding protein C is a thick myofilament structural and regulatory protein. It comprises 8 immunoglobulin-like (C0, C1, C2, C3, C4, C5, C8, C10) domains, 3 fibronectin-type III (C6, C7, C9) domains and one phosphorylation motif (M domain). See full sequence map in figure 5.4.

The identified calpain-targeted site of cMyC lies within the M domain: R272-TSLAGAGRR-R282 (in mice), resulting in the calpain-mediated cleavage of cMyC into the N-terminal fragment C0C1f and the larger C-terminus.

Our current ELISA uses capture and detection monoclonals that bind in near adjacent epitopes between residues 125-160. This assay runs on our in-house MesoScale Discovery analyser with a lower limit of detection of ≈80pg/mL and will reflect total cMyC release since there is no cleavage site between epitopes to decrease detection. The same reagents running on the Erenna analyser at EMD achieve a lower limit of detection of ≈1pg/mL and this was used to determine a 99th centile of ≈90pg/mL.[10] Thus our in-house analyser is able to quantify high-normal and pathogenic concentrations of cMyC and the signal is unchanged by cMyC cleavage by calpain between residues 272-280. However, to enable in-house quantification of total cMyC, the sensitivity of the total

cMyC assay ought to be optimised, which I will attempt by trying new combinations of mouse and newly available sheep N-terminal mAbs.

To create an assay that can selectively measure full length FL-cMyC (intact) I proposed using our current mouse N-terminal mAbs and the newly available sheep N-terminal mAbs (N-terminal anchor) in combination with antibodies with a high affinity to epitopes embedded within C-terminal side of the calpain cleavage site. See figure 4 for target epitopes for the proposed antibodies.

We have supernatants of hybridomas from mice immunised with a synthetic peptide mimetic of the cardiac-specific insert in the C5 domain, These C-terminal anti-C5 mAbs were generated alongside our N-terminal mouse monoclonal antibodies.

The proposed assay will only detect species of cMyC containing both the capture and detection epitopes, which will straddle the calpain cleavage site ([intact cMyC]).

If the C-terminal anti-C5 mAbs and sheep N-terminal mAbs show promise for the development of FL-cMyC assay, we will produce and purify anti-C5 antibodies via a contract research organisation (INVIVO) for further testing and will request purified sheep N-terminal mAbs from Bioventix.

Figure 5.4. Full epitope mapping of human cMyC.
Calpain Target Site, phosphorylation serines and epitope targets for antibodies used for the creation of assays are shown.

Reference www.blast.ncbi.nlm.nih.gov. NCBI Reference Sequence: NP_000247.2

CTS: Calpain Target Site; S275, S284, S304 and S311: phosphorylation serines.

In summary, the creation of the FL-cMyC assay will be performed using the following method

1. Antigen recognition by the new antibodies (mouse C-terminal anti-C5 MAbs and Sheep N-terminal mAbs)
2. Sandwich ELISA for the detection of full-length cMyC (FL-cMyC)
    i) Mouse C-terminal anti-C5 mAbs paired with Sheep N-terminal mAbs
    ii) Mouse C-terminal anti-C5 mAbs paired with mouse N-terminal mAbs 1A4 and 3H8
    iii) ATLAS rabbit C-terminal polyclonal antibodies paired with mouse N-terminal mAbs 1A4 and 3H8

For the creation of total (all species) cMyC assay, two N-terminal antibodies will be paired (sheep N-terminal mAbs/ mouse N-terminal mAbs)

We have recombinant human cMyC to use in the development of the human cMyC assays, but there were no similar reagents in-house or commercially available for swine cMyC at the time of this research work. To overcome this shortfall, I will perform and conduct the following:

1. Cross-species (human-pig) antibody target-epitope peptide sequence alignment. If significant similarity (~ > 70%) in sequence is present across species, this would suggest that the antibodies generated against human cMyC are likely to recognise porcine cMyC. Although affinity might be different.

2. I will make pig heart lysates as a matrix containing cMyC for antigen recognition by the antibodies tested with immunoblotting.
3. Creating human cMyC assays using available recombinant human cMyC antigens.

5.4.2 Cross-species (human-pig) antibody target-epitope peptide sequence alignment

5.4.2.1 Methods

Using Basic Local Alignment Search Tool (US National Centre of Biotechnology Information), the following antibody target-epitopes in human and pig were compared (see full cMyC epitope mapping, figure 5.4)

N-terminal antibodies

- Mouse N-terminal mAbs 1A4 and 3H8
- Sheep N-terminal mAbs

C-terminal antibodies

- Mouse C-terminal anti-C5 antibodies mAbs
- Rabbit C-terminal polyclonal antibodies (ATLAS, Lot A115415, Atlas Antibodies, Sweden)

5.4.2.2 Results

Peptide sequence alignment of anti-cMyC antibody-target epitopes in human and pig are shown in table 5.3. Adequate (> = 70%) alignment was found for all antibody-target epitopes was found.

The exact peptide sequences targeted by the sheep monoclonal N-terminal antibodies is not released by the manufacturers (Bioventix), however, target epitopes overlap with our mouse monoclonal N-terminal antibodies as mapped below (figure 5.5). 3C2 and 5G2 sheep mAbs share target epitope with 1A4 and 3H8 mouse mAbs, respectively, and will be tested accordingly.

Antibody	Sequence	Identities	Positives	Gaps
1A4	[AAELGESAPSPK]	80%	80%	0%
3H8	[APDDPIGLFVM]	90%	91%	1%
Anti-C5	[AITQGNKAPARPAPDAPEDTG DSDEWVFDK]	70%	70%	6%
ATLAS	[TGDSDEWVFDKKLLCETEGR VRVETTKDRSIFTVEGAEKED EGVYTVTVKNPVGEDQVNLT VKVIDVPDA]	97%	97%	0%

Table 5.3 Peptide sequence alignment of anti-cMyC antibody-target epitopes in human and pigs.

Source: US National Centre of Biotechnology Information

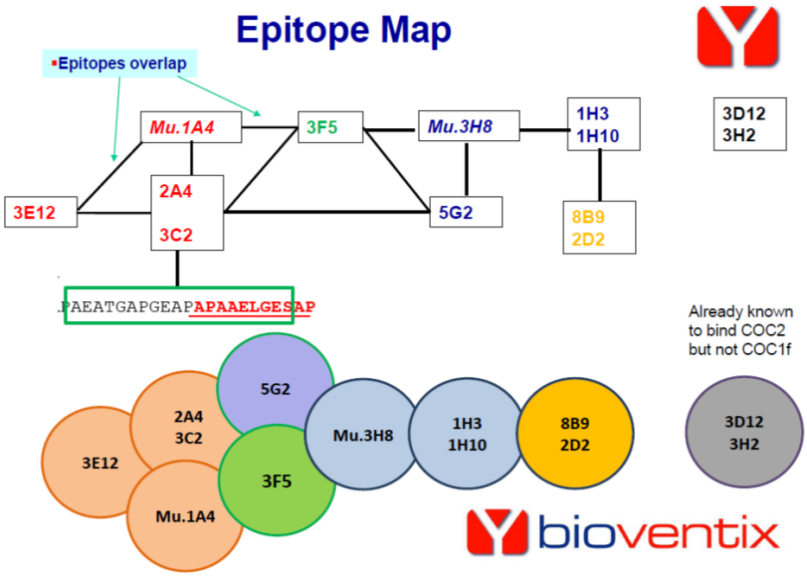


Figure 5.5 Overlap map of target epitopes of mouse monoclonal N-terminal antibodies 1A4 and 3H8 and sheep monoclonal N-terminal antibodies (all other antibodies). Produced by Bioventix.

5.4.3 Porcine heart lysate for intact and fragmented cMyC

5.4.3.1 *Methods*

Pig heart was obtained from a licenced abattoir within 5 minutes of slaughter and transferred to the laboratory on dry ice.

The heart was defrosted, and suitably sized pieces from healthy looking muscle were resected and incubated at room temperature inside an Eppendorf tube for 5 days.

Tissue lysate was prepared at the following time points of incubation: 0, 12, 24, 36, 48, 60, 72, 96, 120 and 144 hours, using the standard method described previously (5.3.1.4) with one deviation: protease inhibitor was not added.

Lysates were aliquoted and frozen at -80 C immediately after preparation.

At 144 h-time point, all lysates were thawed, and protein concentration in each aliquot was measured using Pierce™ BCA Protein Assay Kit, Thermo Scientific. Protease inhibitor was then added to the aliquots before refreezing.

Lysates from all time points were then Western Blotted following standard WB protocol (5.3.1.3) (no deviation from protocol) using the following antibodies

1. Primary antibody: Sheep N-terminal mAb 3C2 4.5 mg/ml diluted to 1:2000
2. Secondary antibody Donkey-anti-Sheep (see materials) 1:20000

Densitometry (iBright) was used to determine timepoints with predominant FL-cMyC and time points with predominant cMyC fragments.

Centrifugal ultrafiltration (50 kDa) was then used for the separation of N-terminal fragments and to exclude FL-cMyC from lysates with predominant fragments.

1.5 ml pig heart lysate was loaded into Amicon™ ULTRA-4 50 kDa columns (Merck Millipore). Columns were centrifuged @ 7500 G for 10 minutes

Permeate (< 50 kDa protein) and retentate (> 50 kDa proteins) supernatant was collected and aliquoted. Protein concentration in both permeate and retentate was measured with Pierce™ BCA Protein Assay.

5.4.3.2 *Results*

Figure 5.6 shows immunoblotted cMyC in porcine myocardial tissue incubated at room temperature for 0, 12, 24, 36, 48, 60, 72, 96, 120 and 144 hours.

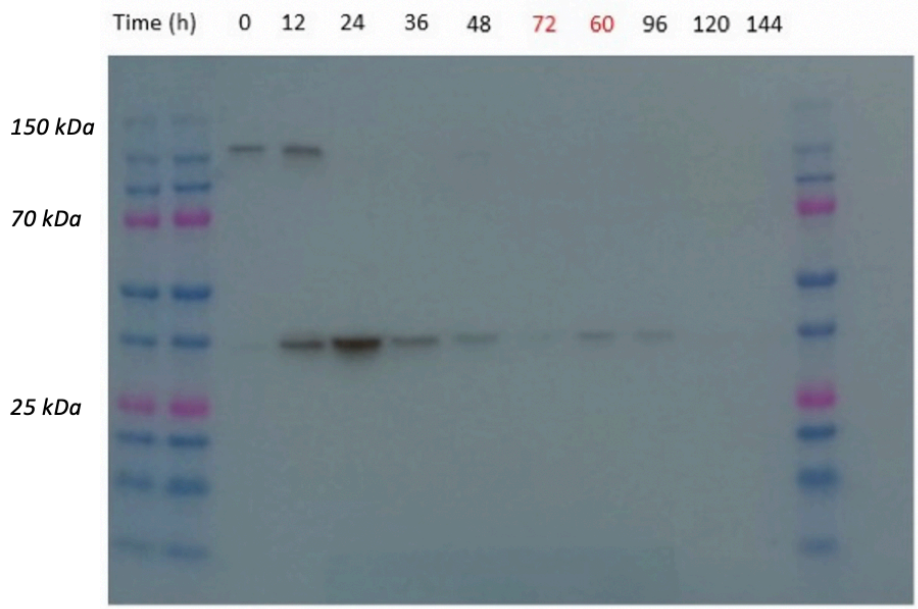


Figure 5.6 Immunoblotted cMyC in porcine myocardial tissue incubated in room temperature. Time points of incubation: 0, 12, 24, 36, 48, 60, 72, 96, 120 and 144 hours

FL-cMyC (~140 kDa) was the predominant species of the protein at baseline (0 h). Cleavage of FL-cMyC and the appearance of a 35 kDa, N-terminal fragment C0C1f, was faintly observed at time 0h and clearly observed as early as 12 hours post incubation at room temperature. N-terminal fragment C0C1f was the predominant species at all subsequent time points up to 96 h, with gradual temporal attenuation of the immunoblotting signal until it almost disappeared at day 6, due to presumed further autolysis.

Lysate from time 0h was selected for FL-cMyC containing matrix and lysate at time 24h for C0C1f.

As faint traces of FL-cMyC were still seen beyond 12 hours, further purification and separation of N-terminal fragments was required for use as a positive control during the

development of the assay and future testing of porcine serum samples. This was achieved with centrifugal ultrafiltration (Amicon™ ULTRA-4 50 kDa columns, Merck Millipore).

Protein concentration in both permeate and retentate was measured with Pierce™ BCA Protein Assay.

- 24 h permeate 0.226 ug/ul
- 24 h retentate 2.087 ug/ul

5.4.3.3 *Discussion*

I demonstrated that cMyC undergoes cleavage at room temperature and can be used to produce what we believe to be the N-terminal fragment C0C1f, based on molecular weight and recognition by N-terminal antibodies on immunoblotting.

It is unclear whether this cleavage of cMyC at room temperature is calpain induced proteolysis or if it is caused by other mechanisms, however, the appearance of C0C1f fragments suggest that the cleavage of cMyC occurred around the calpain target site CTS. Furthermore, freshly excised heart tissue at room temperature is “ischaemic”. Further examination of the mechanism of cMyC autolysis within pig heart at room temperature falls outside the remit of this research project, since our goal was merely to generate fragments of porcine cMyC to control for assay development.

Further gradual degradation of the cMyC protein occurred with prolonged incubation at room temperature until near-complete degradation was observed after day 6. It is unknown whether this additional temporal deterioration is caused by epitope disruption/degradation, leading to attenuated affinity of the primary antibodies used in

the experiment, or due to further fragmentation of cMyC including its N-terminal fraction into sub-fragments at different sites.

The selectivity of the FL-cMyC assay to FL-cMyC needs be tested with N-terminal fragments as positive control.

The retentate should contain cMyC > 50 kDa molecular weight including FL-cMyC (~140 kDa) with some unfiltered < 50 kDa cMyC including C0C1f (~ 35 kDa).

The permeate should contain cleaved < 50 kDa cMyC including C0C1f (~ 35 kDa).

5.4.4 Creating FL-cMyC and total (all species) cMyC assays

5.4.4.1 *Materials and consumables*

Monoclonal antibodies: (see below for generation)

A. N-terminal antibodies
   - Mouse N-terminal mAb 1A4 (clone number AK 2263) and 3H8 (clone number AK2235)-purified.
   - Sheep N-terminal mAbs (Clone numbers: F1783, F1786, F1789, F1793, F1794, F1806, F1809, Bioventix, UK)-supernatant

B. C-terminal antibodies
   - Mouse C-terminal mAbs (clone number: C5C 242)-supernatant.
   - Rabbit C-terminal monoclonal antibodies**:** (see below for generation)

Secondary antibodies

- Peroxidase-conjugated AffiniPure Goat anti-mouse Fc_y fragment specific (Jackson Immuno Research) Code: 115-174. Lot: 139280.
- Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG, light chain specific (Jackson Immuno Research). Code: 115-035. Lot: 139280.
- Peroxidase-conjugated AffiniPure Donkey Anti-Sheep IgG (H + L) (Jackson Immuno Research). Code 713-035. Lot: 138813.

Sulfo-tag kits

- MSD GOLD TM SULFO-TAG NHS-Ester (Lot: R91AO-2. Meso Scale Discovery, USA).

- MSD GOLD TM SULFO-TAG NHS-Ester Conjugation Pack (Lot: R31AA-2. Meso Scale Discovery, USA)

Antigens:

- Recombinant human C0C1f, C0C2 and FL, purified from *E.COLI* (recombinant in NaCl and HEPES pH 7.4)
- Healthy pig heart for preparation of intact and fragmented porcine cMyC

Amicon ™ ULTRA-4 50 kDa column (Merck Millipore)

Pierce™ BCA Protein Assay Kit, Thermo Scientific

96 Well plates

- 96 well plates Costae Assay Plate (Corning, USA-Lot: 26018047)
- MSD 96 wells MULTI-ARRAY plate (MSD, # L16XA-3/L11XA-3, Lot: 21H5AA)

Buffers

- Plating buffers (PBS, TBS, Tris PH 9.6)
- Diluting buffer (MSD diluent-7, Lot: R54BB-3, Meso Scale Discovery, USA)
- Washing buffers (PBS-0.1% Tween-20 or PBS-0.05% Tween-20)
- Blocking buffers with BSA: washing buffer + BSA 1 or 5% (Bovine Serum Albumin Fraction V, Sigma-Aldrich, code: 10735086001)

Plate readers

- BioTek spectrophotometric plate reader. Model EL808IU, USA.

- MSD Electrochemiluminescence MULTI-ARRAY technology plate readers.
    - MSD Sector Imager 2400, Meso Scale Discovery, USA
    - MESO QuickPlex SQ 120 MM Reader, AI1AA-0, Meso Scale Discovery, USA)

5.4.4.2 *Protocols:*

5.4.4.2.1 Direct antigen recognition protocol

1. Dilute antigen to planned concentration in PBS, and ensure enough volume to coat the required number of wells in 96-well plate, 100 ul/well
2. Add antigen 100 ul/well, cover the plate with cling film and store overnight at 4^{o} C
3. Discard the solution from the overnight incubation and blot the plate well on tissue, then wash x 3 with 150 ul washing buffer per well (PBS-0.05% Tween-20). Blot off excess liquid post each wash
4. Add blocking buffer 100 ul/well and incubate the plate at room temperature for 1 hour on a shaker @ 100 rpm
5. Discard the solution and blot the plate well on tissue, then wash x 3 with 150 ul washing buffer per well. Blot off excess liquid post each wash
6. Dilute primary antibody to the planned concentration in blocking buffer, ensure enough volume for the required number of wells.
7. Add primary antibody, 100 ul/well cover the plate with cling film and incubate at room temperature for 1 hour on a shaker @ 100 rpm
8. Repeat step 5
9. Dilute secondary antibodies to the planned concentration in blocking buffer

10. Add secondary antibody, 100 ul/well cover the plate with cling film and incubate at room temperature for 2 hours on a shaker @ 100 rpm
11. Place TMP solution in room temperature 15 minutes before end of incubation period
12. Repeat step 5
13. Add TMP, 100 ul/well, and incubate at room temperature for 15 minutes
14. Add stop solution 100 ul/well and read immediately with spectrophotometric plate reader at 450 nm

5.4.4.2.2 Sandwich Elisa, spectrophotometric

1. Dilute capture antibody to planned concentration in PBS, and ensure enough volume to coat the required number of wells in 96-well plate, 100 ul/well.
2. Cover the plate with cling film and store overnight at 4º C
3. Discard the solution from the overnight incubation and blot the plate well on tissue, then wash x 3 with 150 ul washing buffer (PBS-0.05% Tween-20). Blot off excess liquid post each wash
4. Add blocking buffer 100 ul/well and incubate the plate at room temperature for 1 hour on a shaker @ 100 rpm
5. Discard the solution and blot the plate well on tissue, then wash x 3 with 150 ul washing buffer per well. Blot off excess liquid post each wash
6. Dilute antigen to planned concentration in PBS, and ensure enough volume to coat the required number of wells in 96-well plate, 100 ul/well
7. Add antigen 100 ul/well, cover the plate with cling film and incubate at room temperature for 1 hour on a shaker @ 100 rpm
8. Repeat step 5

9. Dilute detection antibody to planned concentration in blocking buffer, and ensure enough volume to coat the required number of wells in 96-well plate, 100 ul/well
10. Add detection antibody, 100 ul/well cover the plate with cling film and incubate at room temperature for 1 hour on a shaker @ 100 rpm
11. Repeat step 5
12. Dilute secondary antibody to the planned concentration in blocking buffer
13. Add secondary antibody, 100 ul/well cover the plate with cling film and incubate at room temperature for 2 hours on a shaker @ 100 rpm
14. Place TMP solution at room temperature 15 minutes before end of incubation period
15. Repeat step 5
16. Add TMP, 100 ul/well, and incubate at room temperature for 15 minutes
17. Add stop solution 100 ul/well and read immediately with spectrophotometric plate reader at 450 nm

5.4.4.2.3 Sandwich Elisa, electrochemiluminescence

1. Dilute capture antibody to planned concentration in PBS, and ensure enough volume to coat the required number of wells in MSD Quickplex 96-well plate, 30 ul/well
2. Cover the plate with cling film and store overnight at 4^{o} C
3. Discard the solution from the overnight incubation and blot the plate well on tissue, then wash x 3 with 150 ul washing buffer (PBS-0.1% Tween-20) per well. Blot off excess liquid post each wash

4. Add blocking buffer (1 % BSA/washing buffer) 100 ul/well and incubate the plate at room temperature for 1 hour on a shaker @ 100 rpm
5. Prepare standards and samples: during the blocking step, the standards and samples can be prepared. COC2 standard is stored at -80ºC. our standard curve has an upper limit value of 80 ng/ml, so dilute the stock to this value and then dilute serially (usually by 4, taking the lowest concentration down to 0.1953 ng/ml). Serum samples can be diluted 1:2 with MSD diluent, accounting for this dilution in assay analysis.
6. Discard the solution and blot the plate well on tissue, then wash x 3 with 150 ul washing buffer per well. Blot off excess liquid post each wash
7. Add the standards and samples (30 ul/well) and MSD diluent only to relevant rows.
8. Cover the plate with cling film and incubate at room temperature for 1 hour on a shaker @ 100 rpm
9. Repeat step 6
10. Dilute detection antibody to planned concentration in washing buffer, and ensure enough volume to coat the required number of wells in 96-well plate, 30 ul/well
11. Add detection antibody, 30 ul/well cover the plate with cling film and incubate at room temperature for 2 hours on a shaker @ 100 rpm
12. Repeat step 6
13. Add 1 X MSD read buffer (dilute higher concentrations in dH2O). Gently add 150 ul/well, and read immediately

5.4.4.2.4 Protein concentration quantification

Easy-Titer™ Mouse IgG Assay Kit (ThermoFisher Scientific)

Manufacturer's protocol

https://www.thermofisher.com/order/catalog/product/23300?SID=srch-srp-23300

5.4.4.2.5 Labelling mAbs with ruthenium (Sulfo-tagging)

Kit: MSD GOLD ™ SULFO-TAG NHS-Ester

Method:

Standard manufacturer protocol (link below)

https://www.mesoscale.com/~/media/files/handout/msd%20gold%20sulfotag%20conjugation%20quick%20guide.pdf

Conjugation ratio X:1

- Equilibrate the antibodies in room temperature 15 minutes
- Calculate the amount of MSD GOLD SULFO-TAG NHS Ester stock solution required for the conjugation reaction using the formula below

1,000 x [Protein conc. (mg/mL)/ Protein MW (Da)] x Challenge ratio x Vol. of protein solution (µL) = nmol of SULFO-TAG reagent required

Volume of MSD SULFO-TAG stock solution=

nmol of SULFO-TAG reagent required/ Conc. of SULFO-TAG stock solution (nmol/µL) = µL of SULFO-TAG stock solution required for conjugation reaction

- Tap the vial gently, dissolve in cold water, 150 nmol in 50 ul to generate 3 nmol/ul, gently votex
- Add the calculated volume to protein solution and discard the rest
- Incubate for 2 hours at 23°C in dark box
- Chose right ZEBRA column volume (5 ml for 300 ul)
- Prepare the centrifuge, cool down to 4ºC
- 20 minutes before the end of the 2h incubation, take two Zebra columns (one for conjugation and one for balance), place both in collection 15 ml tubes, remove the bottom and loosen the cap to remove the storage buffer
- Spin for 6 minutes
- wash three time with conjugate storage buffer-by centrifuging (add 2.5 mls each time) and spin for 6 minutes.
- Add the protein and stacker volume (100 ul), refer to table
- Spin for 5 minutes
- Collect the conjugated proteins
- Wrap in foil and keep in a dark box at 4°C
- Measure the concentration of the conjugated antibodies using Pierce™ BCA Protein Assay Kit (protocol x)

5.4.4.2.6 Generation of mouse N-terminal mAbs (1A4 and 3H8) and mouse C-terminal anti-C5 mAbs (2G12, 2B6, 8C1, 8E5, 7H12, 5F12 and 7H12)

Details of the generation of mouse anti-cMyC mAbs are as described in Baker et al.[52] Mice were immunised with recombinant C0C2 or synthetic peptide corresponding to the

28aa cardiac-specific insert within the C5 domain of cMyC. With two booster injections with the relevant antigen before hybridization on day 82.

Mouse spleen cells and mouse myeloma cells were then fused and cultured for the production of high quantities of antibodies.

N-terminal mAbs were then ranked by surface plasmon resonance, best ranking antibodies were selected and then filtered based on biophysical characteristics and binding affinity, and two clones: 1A4 and 3H8 were selected.

1A4 and 3H8 exhibited high affinity to COC2 and were mapped to sequences A125AELGESAPSPK and A149PDDPIGLFVM respectively around the interface between C0 and the proline/alanine-rich linker between C0 and C1.

For testing the C-terminal anti-c5 antibodies, the 28-residue cardiac specific insert within the C5 domain was synthesised as a single peptide for antigen recognition ELISA.

Antibodies (in supernatant form) with high affinity to the cardiac specific C5 insertions were selected.

5.4.4.2.7 generation of sheep N-terminal monoclonal antibodies

N-terminal sheep monoclonal antibodies SMAs were generated by Bioventix (Romans Business Park, Farnham, UK).

The reported advantages of sheep monoclonal antibodies over rodent antibodies are a higher affinity of up to several orders of magnitude, wider epitope recognition, and improved sensitivity and specificity towards the antigen.

The antibodies were tested against 25 overlapping 20-mers covering C0C1f.

Antibodies were available as supernatants when initial testing was performed before purified antibodies were made available for further testing.

The available antibodies are:

F1783.2A4.1E4, F1786.3F5.1A2, F1789.2D2.1D3, F1789.3D12.2A3, F1793.5G2.F5D4

F1794.3C2.F6B2, F1794.3C2.F6B7, F1806.1H10.1B11, F1809.3H2.2D11

5.4.4.3 Experiments and results

The creation of the FL-cMyC assay will be performed using the following method

1- Antigen recognition by mouse C-terminal anti-C5 mAbs
2- Antigen recognition by sheep N-terminal mAbs
3- Sandwich ELISA for the detection of full-length cMyC (FL-cMyC)
    - Mouse C-terminal anti-C5 mAbs paired with Sheep N-terminal mAbs
    - Mouse C-terminal anti-C5 mAbs paired with mouse N-terminal mAbs 1A4 and 3H8
    - ATLAS rabbit C-terminal polyclonal antibodies paired with mouse N-terminal mAbs 1A4 and 3H8

Both mouse C-terminal anti-C5 mAbs and sheep N-terminal mAbs were available in supernatant form during the exploratory phase, and while it is not expected for them to be perform adequately well in the designed assays, they remain valuable for exploring the feasibility of developing a selective FL-cMyC assay and to support the the request for purified mAbs for further testing.

5.4.4.3.1 Antigen (FL-cMyC, C0C1f and C0C2) recognition by supernatant mouse C-terminal anti-C5 mAbs

This was performed systematically in three steps

1- Measuring the concentration of supernatant C-terminal anti-C5 mAbs

2- Testing Goat-anti-mouse secondary antibodies for the determination of the ideal secondary antibody concentration to use in ELISA experiments

4- Antigen recognition experiments to confirm the specificity of the mouse anti-C5 mAbs to full-length cMyC

5.4.4.3.1.1 Measuring the concentration of mouse anti-C5 monoclonal antibodies (supernatant)

Concentrations of supernatant anti-C5 monoclonal antibodies were measured to inform custom dilutions to ensure equal final concentrations of all antibodies in estimation of binding affinity.

Tools and protocols

Easy-Titer™ Mouse IgG Assay Kit (ThermoFisher Scientific), see 5.4.4.2.4.

Results

Antibodies concentrations are shown in the table below

Antibody	Concentration ug/ml
2G12	35
2B6	36
8C1	58.5
8C5	12.7

5.4.4.3.1.2 Testing Goat-anti-mouse secondary antibodies for the determination of the ideal concentration to use in ELISA experiments

(Peroxidase-conjugated AffiniPure Goat anti-mouse Fc_y fragment specific -Jackson Immuno Research)

Selecting the optimal secondary antibody concentration is crucial for generating the best detection reading with minimum/negligible direct interaction with antigen.

It is recommended that the optimal concentration is determined experimentally for each assay; while this is ideal, it is time and resource consuming to perform for every experiment. However, exploratory experiments would help inform the desired concentrations.

The reasons why secondary antibodies can interact directly with antigens and other reagents is not discussed or explored during this research.

Methods:

- Experiment (a):

Standard Indirect ELISA protocol (see 5.4.4.2.1)

Antigen FL-cMyC, C0C1f (200 ng/ml) and negative antigen control (no antigen)

Primary antibody 1A4 N-terminal mAb 100 ng/ml

Goat anti-mouse secondary antibody (GaM-HRP) in serial dilution 1:10000-1:160000

Reading technique: spectrophotometric, absorbance at 450 nm wavelength quantifying the interaction between HRP and its substrate, 3,3',5,5'-tetramethylbenzidine.

- Experiment (b): Direct interaction between GaM-HRP secondary antibody and antigen FL-cMyC, and C0C1f

Protocol as above with the exclusion of primary antibodies.

Results and discussion:

There was a clear linearity between the concentration of GaM-HRP and the absorbance signal for both antigens when detected by 1A4, with no significant inter-antigen signal variation with GaM -HRP dilutions between 1:10000-1:40000 ($p > 0.5$), figure 5.7 (A)

Relative to negative control, direct secondary antibody-antigen interaction disappeared at 1:40000 dilution ($p = 0.8$, for difference in absorbance signal between GaM-HRP--control and GaM-HRP--FL-cMyC/C0C1f), figure 5.7 (B)

However, these experiments were performed with a fixed concentration of the antigen and also of primary antibody, therefore, the results might not apply more widely, adjustment of the concentration of GaM might be required in future experiments.

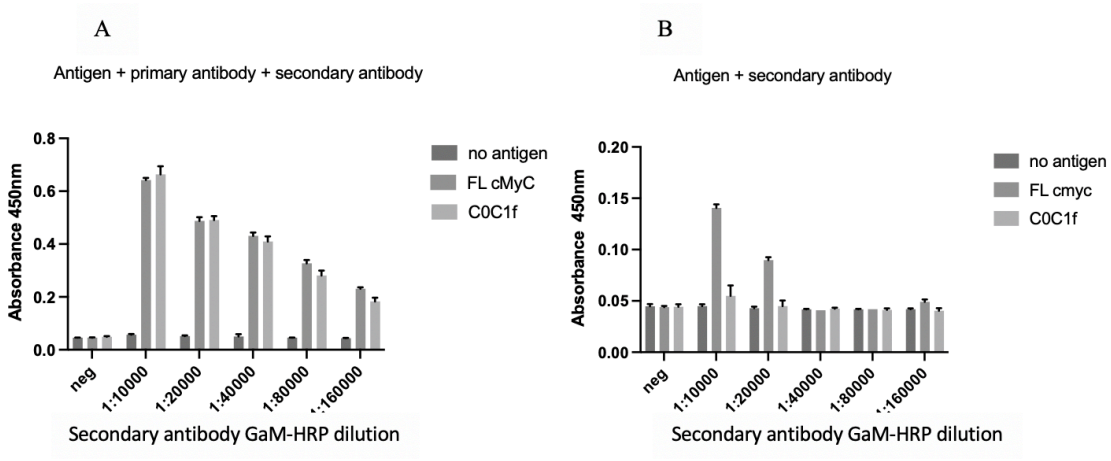


Figure 5.7. A: Direct antigen (FL-cMyC, and C0C1f) detection with 1A4 N-terminal mAb and serially diluted secondary antibody GaM-HRP. B: Direct interaction between antigen (FL-cMyC and C0C1F) and serially diluted secondary antibody GaM-HRP.

5.4.4.3.1.3 Antigen recognition with the C-terminal anti-C5 mAbs

Having identified the concentrations of the anti-C5 antibodies and also the suitable dilution of the GaM-HRP secondary antibody, I then performed an antigen recognition experiment with the anti-C5 antibodies at a standard antigen concentration of 100 ng/ml using the 1A4 N-terminal antibody as the benchmark for affinity.

Aim

To ensure that C-terminal anti-C5 mAbs selectively bind to FL-cMyC which contains the C5 domain, and to compare the affinity for FL-cMyC of the C-terminal anti-C5 mAbs with N-terminal mAb 1A4.

Methods

Standard Indirect ELISA protocol (see 5.4.4.2.1)

Antigen FL-cMyC, C0C1f and C0C2 (200 ng/ml) and negative antigen control (no antigen)

Primary antibodies:

1. Anti-C5 mAbs (2G12, 2B6, 8C1 and 8E5) 100 ng/ml
2. N-terminal mAbs 1A4 at 100 ng/ml (positive antibody control)

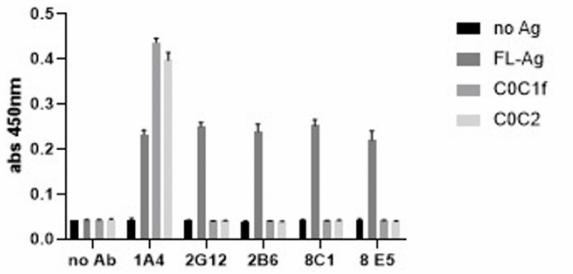
Secondary antibody: Goat anti-mouse secondary antibody (GaM-HRP) 1:40000

Reading technique: spectrophotometric, absorbance at 450 nm wavelength

Results

1A4 recognised FL-cMyC and the two N-terminal fragments C0C1f and C0C2. Only FL-cMyC was detected by the anti-C5 mAbs with a comparable affinity to 1A4 N-terminal mAb. Figure 5.8 and attached table.

At 1:40000, the GaM secondary antibody doesn't react directly with the antigens, confirming the suitability of this concentration with all mouse monoclonal primary antibodies used in ELISAs.



	FL-cMyC			C0C1f			C0C2		
	Mean of control	Mean of FL-cMyC	p value	Mean of control	Mean of C0C1f	p value	Mean of control	Mean of C0C2	p value
No Ab	0.0437	0.043	0.67	0.0437	0.0456	0.327	0.0437	0.044	0.64
1A4	0.043	0.248	<0.001	0.043	0.042	<0.001	0.043	0.038	<0.001
2G12	0.046	0.254	<0.001	0.046	0.043	0.08	0.046	0.045	0.65
2B6	0.041	0.234	<0.001	0.041	0.042	0.158	0.041	0.042	0.158
8C1	0.0413	0.237	<0.001	0.0413	0.042	0.116	0.0413	0.042	0.23
8E5	0.0423	0.216	<0.001	0.0423	0.041	0.348	0.0423	0.041	0.54

Figure 5.8 Antigen (FL-cMyC and N-terminal fragments C0C1f and C0C2) recognition by N-terminal mAb (1A4) and C-terminal mAbs (2G12, 2B6, 8C1 and 8E5).

Values represent spectrophotometric absorbance signal with statistical significance determined using the Holm-Sidak method, with alpha = 0.05, Each row was analysed individually, without assuming a consistent SD.

Discussion

Compared to the mouse N-terminal mAb 1A4, mouse anti-C5 (C-terminal) mAbs bind selectively to FL-cMyC only, with a comparable affinity to 1A4 N-terminal mAb, despite being in supernatant form.

5.4.4.3.2 Antigen recognition with supernatant Sheep N-terminal mAbs (2A3, 1D3, 1E4, 1A2, 6B7, 6B2, 1B12, 2D12 and 2C9)

This will be tested in two steps

1- Measuring the concentration of sheep N-terminal mAbs in each supernatant

2- Antigen recognition experiments

5.4.4.3.2.1 Measuring the concentrations of sheep N-terminal mAbs in each supernatant

Aims:

Concentrations of supernatant antibodies were measured to inform custom dilutions to ensure equal final concentrations of all antibodies in estimation of binding affinity

Tools and protocol:

Easy-Titer™ Mouse IgG Assay Kit (ThermoFisher Scientific), see 5.4.4.2.4.

Results:

Sheep mAb supernatant concentrations are presented in the table below.

Antibody	Concentration ug/ml
1E4	45
1A2	39
1D3	36
2A3	52
5D4	39
6B2	54
6B7	44
2D11	43

5.4.4.3.2.2 Antigen recognition with the sheep N-terminal mAbs

Aims:

1. To test the affinity of the N-terminal sheep monoclonal antibodies to cMyC

2. To select the antibodies with high affinity for potential pairing with anti-C5 mAbs for FL-cMyC assay and the mouse N-terminal mAbs 1A4 and 3H8 for total cMyC assay

Methods:

Standard Indirect ELISA (antigen recognition) protocol (see 5.4.3.1.2)

Antigen: FL-cMyC, C0C1f and C0C2 (200 ng/ml) and negative antigen control (no antigen)

Primary antibodies:

- Supernatant N-terminal sheep mAb (2A3, 1D3, 1E4, 1A2, 6B7, 6B2, 5D4, 1B12, 2D12 and 2C9) serially diluted

Secondary antibody:

- Donkey anti-sheep HRP conjugated secondary antibody 1:50000

Reading technique: spectrophotometric, absorbance at 450 nm wavelength

Results:

Results are shown in figure 5.9. Best performing antibodies at the tested dilutions are 2A3, 1D3, 5D4 and 6B7 (absorbance signal > 0.2 at 450 nm)

1:100 dilution of the supernatant (~400-500 ng/ml) appeared reasonable for further testing.

All Sheep monoclonal antibodies detected FL-cMyC as well as N-terminal fragments, except for two antibodies (2A3 and 2D11) which appear to be C0C2 specific.

Discussion:

The supernatant sheep anti-cMyC N-terminal mAbs (2A3, 1D3, 5D4 and 6B7) have good affinity to cMyC at 1:100 dilution (~400-500 ng/ml), however, 2A3 and 2D11 only recognised C0C2 and was blind to FL and C0C1f.

It is possible the target site of 2A3 and 2D11 is the C-terminal end of C0C2, therefore, it is able to detect C0C2 in its free (fragment) form with an exposed carboxy terminus but not when forming a peptide bond within FL-cMyC. Equally, it doesn't detect C0C1f since it doesn't contain the antibody's target site.

Purified monoclonal antibodies were requested for further testing, but while purified antibodies (sheep N-terminal and mouse anti-C5) were awaited, I continued to experiment with supernatant.

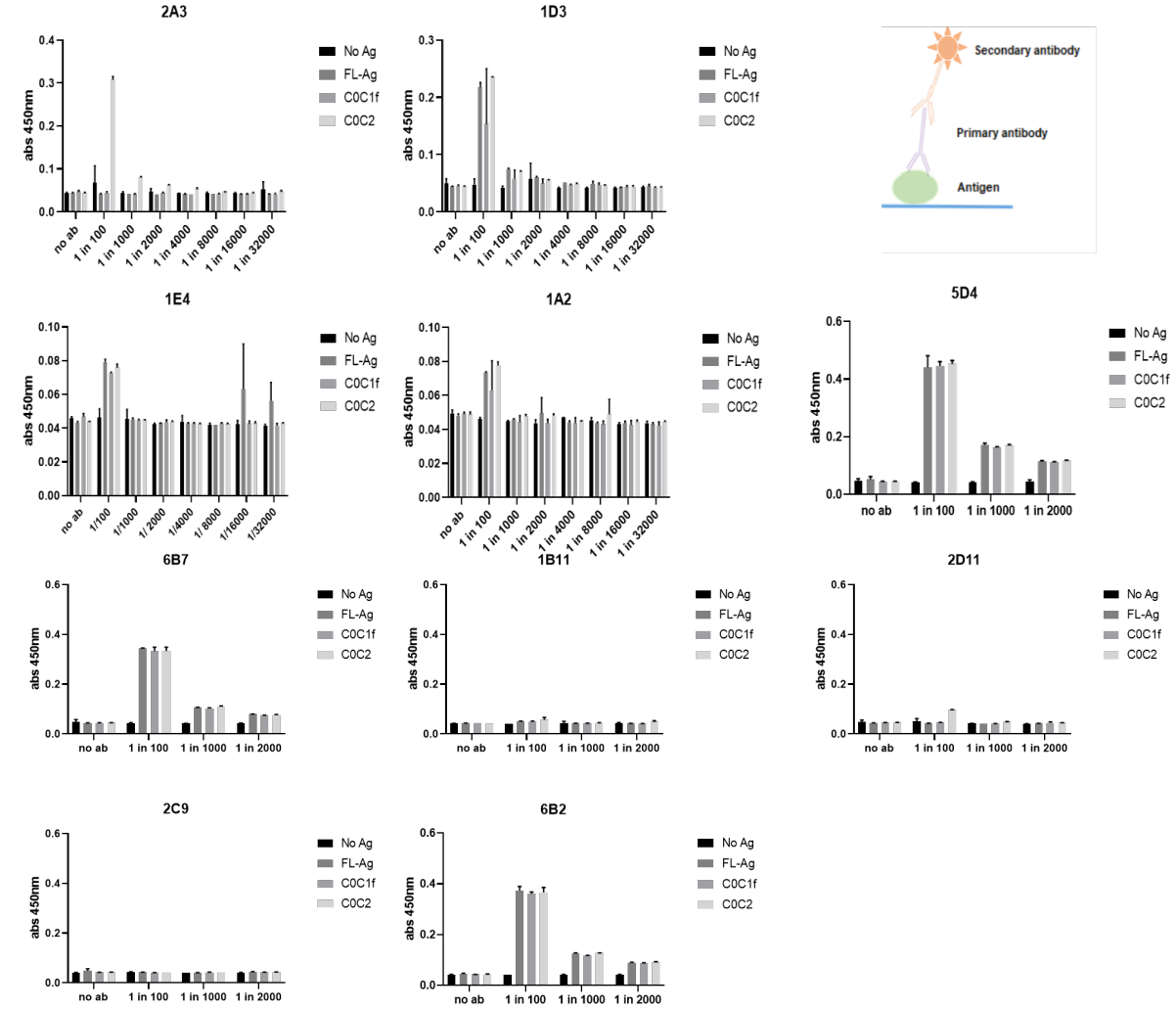


Figure 5.9 Indirect ELISA for the detection of FL, C0C1f and C0C2 cMyC with serially diluted Sheep N-terminal mAbs.

2A3, 1D3, 1E4, 1A2, 5D4, 6B7, 1B11, 2D11, 2C9, 6B2: sheep N-terminal mAbs. FL-Ag: Full length cMyC. C0C1f and C0C2: N-terminal cMyC fragments.

5.4.4.3.3 Pairing N-terminal and C-terminal mAbs for the selective detection of FL-cMyC

5.4.4.3.3.1 Sandwich ELISA, capturing with C-terminal anti-C5 mAb and detecting with sheep N-terminal mAb

Aim:

To test the pairing of C-terminal anti-C5 mAbs and sheep N-terminal mAbs for the selective detection of FL-cMyC

Methods:

- Standard sandwich ELISA cMyC quantification protocol (see 5.4.4.2.2)
- Capture antibody: supernatant C-terminal anti-C5 mAb 100 ng/ml, 1A4 100 ng/ml for (positive antibody control) and no antibody for negative antibody control
- Antigen FL-cMyC and COC2 (100 ng/ml) and negative antigen control (no antigen)
- Detection antibodies: Supernatant sheep N-terminal mAbs (2A3, 6B7, 6B2, and 5D4) 1:100
- Donkey anti-sheep HRP conjugated secondary antibody 1:50000
- Reading technique: spectrophotometric, absorbance at 450 nm

Results:

As demonstrated in figure 5.10, pairing C-terminal anti-C5 mAbs with N-terminal sheep mAbs (5D4, 6B7 and 6B2) selectively detects FL-cMyC at 200 ng/ml ($p < 0.01$ for FL-cMyC vs control. $P < 0.01$ for FL-cMyC vs C0C2, $p > 0.1$ for C0C2 vs negative control

for all mAbs), with 2G12 and 2B6 producing a higher signal than 8C1 and 8E5 at the used concentrations ($p < 0.05$).

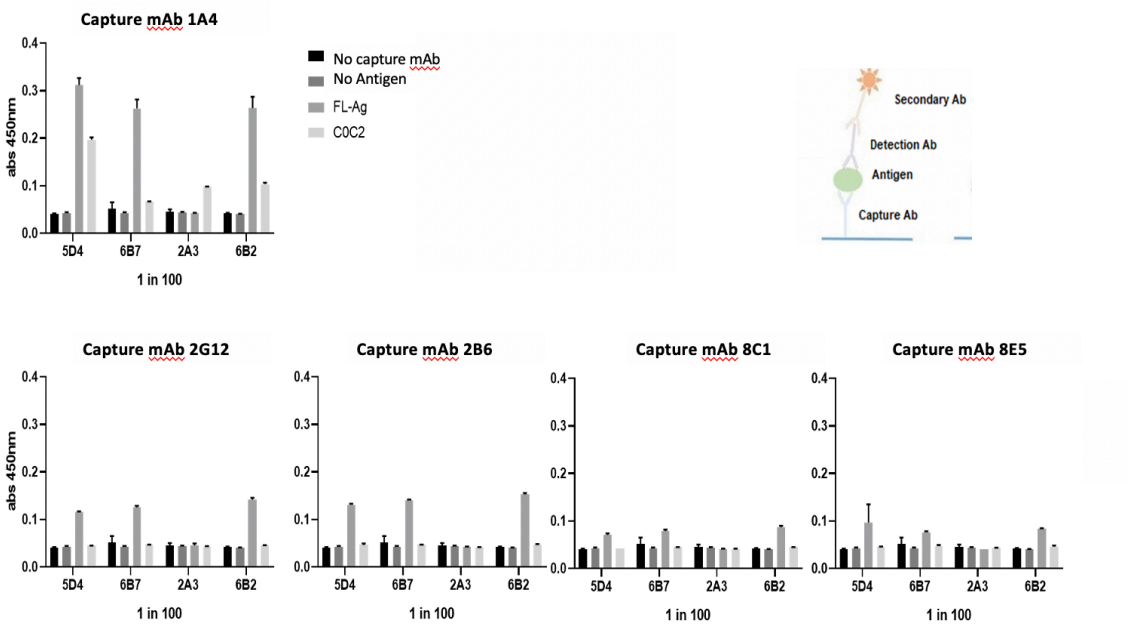


Figure 5.10 Sandwich ELISA capturing with C-terminal Anti-C5 mAbs and detecting with sheep N-terminal mAbs for the detection of FL-cMyC and C0C2 fragment.
2G12, 2B6, 8C1 and 8E5: C-terminal Anti-C5 mAbs. 1A4: mouse N-terminal mAb, 5D4, 6B7, 2A3 and 6B2: sheep N-terminal mAbs

Anti-C5 antibodies were all outperformed by 1A4 for detecting FL-cMyC captured by sheep N-terminal mAbs. The sheep N-terminal antibody 2A3 preserved its selectivity to C0C2 fragment and could not detect the captured FL and did not pair with the anti-C5 mAbs.

Discussion

Pairing C-terminal anti-C5 mAbs with sheep N-terminal antibodies selectively detects 100 ng/ml of FL-cMyC. Further testing with serially diluted FL is required.

However, despite comparable affinity to FL-cMyC between C-terminal anti-C5 mAbs and 1A4 when the antigen is plate-bound, 1A4 seems to perform better for capturing the antigen in sandwich ELISA setting.

Amongst the anti-C terminal mAbs, 2G12 and 2B6 were the best performing antibodies as capture antibodies when paired with the sheep N-terminal mAbs.

Note that both mouse anti-C5 and sheep N-terminal antibodies are in supernatant form, therefore, they might perform/pair better when purified and used at higher concentrations, further testing can be performed once purified forms of these antibodies are available.

The sheep monoclonal antibody 2A3 remains C0C2 selective, and although not helpful for the detection of FL-cMyC, it might be a key antibody for developing a specific C0C2 fragment assay.

At this stage, purified sheep N-terminal mAbs became available and were included in further testing

5.4.4.3.3.2 Sandwich ELISA, capturing with purified sheep N-terminal mAbs and detecting with supernatant C-terminal anti-C5 mAbs

Aim:

To test the pairing of purified sheep N-terminal mAbs as capture antibodies with anti C5 C-terminal mAbs for the selective detection of serially diluted FL-cMyC

Methods:

- Standard sandwich ELISA cMyC quantification protocol (see 5.4.4.2.2)
- Capture antibodies: purified sheep N-terminal mAbs (6B2, 5D4 and 2A3) at 1 ug/ml
- Antigen FL-cMyC, C0C1f and COC2 50 ng/ml, and negative antigen control (no antigen)
- Detection antibodies: supernatant Anti-C5 C-terminal mAbs (2G12, 2B6, 5D4 and 2A3) 100 ng/ml, 3H8 and 1A4 100 ng/ml for (positive antibody control) and no antibody for negative antibody control
- GaM-HRP conjugated secondary antibody 1:40 000
- Reading technique: spectrophotometric, absorbance at 450 nm

Results:

C-terminal anti-C5 monoclonal mAbs selectively detect 50 ng/ ml FL-cMyC captured by all sheep monoclonal antibodies including 2A3 (previously deemed C0C2 fragment

specific), but with a relatively low signal relative to the signal of the non-selective detection of FL-cMyC with 1A4 and 3H8. Figure 5.11.

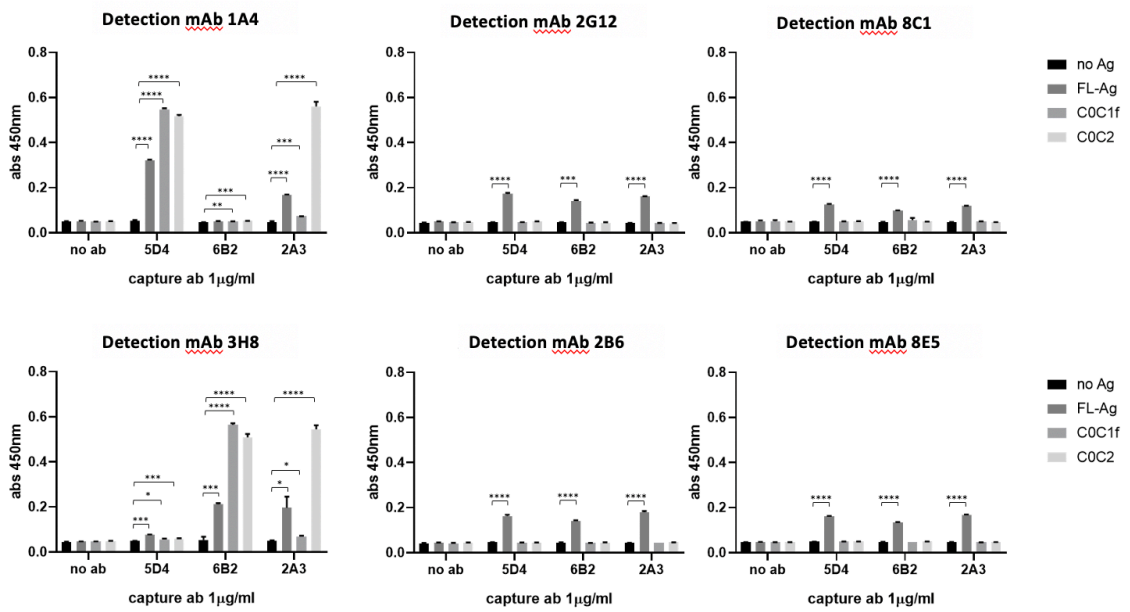


Figure 5.11 Sandwich ELISA, capturing with purified sheep N-terminal mAbs and detecting with supernatant C-terminal anti C5 mAbs for the detection of FL-cMyC and N-terminal cMyC fragments (C0C2 and C0C1f).

2G12, 2B6, 8C1 and 8E5: C-terminal Anti-C5 mAbs (supernatant). 1A4 and 3H8: mouse N-terminal mAb, 5D4, 6B2 and 2A3: sheep N-terminal mAbs. Statistical significance determined using the Holm-Sidak method, with alpha = 0.05 (* <0.05, ** <0.01, *** <0.001, **** <0.0001)

Discussion:

When capturing with sheep N-terminal mAbs, C-terminal anti-C5 mAbs selectively detect 50 ng/ml of FL-cMyC.

The reading signal is low at this antigen concentration, this might adversely affect the sensitivity of assay for detecting FL-cMyC at lower concentrations, this needs to be tested with serial dilution of FL-cMyC (purified anti-C5 antibodies were awaited).

It is unclear why 2A3 sheep monoclonal antibody captured FL-antigen in this experiment despite its selectivity for the C0C2-fragment in previous experiments. It is possible that the tested recombinant FL antigen has undergone some degree of degradation in vitro however, anti-C5 mAbs should not recognise the C0C2 fragment.

Overall, the spectrophotometric signal for FL-cMyC detection appears low and it might be difficult to optimise the sensitivity to the level that would allow clinical usage/translation of the assay. Therefore, I will test the pairing of the antibodies using the more sensitive electrochemiluminescence ELISA platform.

5.4.4.3.3.3 Electrochemiluminescence sandwich ELISA Pairing supernatant mouse C-terminal anti-C5 mAbs with mouse N-terminal mAbs

With both antibody groups belonging to the same species (mouse). Anti-mouse secondary antibodies will bind to the capture as well as the detection antibody. One way to overcome this foreseen issue is by performing direct sandwich ELISA experiment by pre-tagging the detection antibody. Our in-house MSD electrochemiluminescence reader detects with Ruthenium-tagged mAbs.

Mouse C-terminal anti-C5 mAbs in supernatant form are not suitable for Ruthenium-tagging due to matrix and low concentration. While waiting for purified antibodies to arrive, experiments will be confined to capturing with C-terminal anti-C5 antibodies and detecting with Ruthenium-tagged mouse N-terminal mAb 3H8.

Methods:

- Standard ECL sandwich ELISA cMyC quantification protocol (see 5.4.4.2.3)
- MSD 96 wells MULTI-ARRAY plate
- Capture: C-terminal anti-C5 monoclonal antibody (2G12, 2B6 and 8C1) 500 ng/ml (supernatant)
- Antigen: FL-cMyC serially diluted from 80 ng/ml to 20 ng/ml
- Detection: 3H8-Ruthenium 500 ng/ml, tagged as per standard protocol (5.4.4.2.5): conjugation ratio 10:1
- Reader: MSD Sector Imager 2400

Results:

As displayed in figure 5.12, 3H8-Ruthenium can detect 20 ng/ml of FL-cMyC captured by anti-C5 mouse monoclonal antibody in MSD ECL sandwich ELISA (p value < 0.005 for 20 ng/ml vs 0 ng/ml for all anti-C5 antibodies)

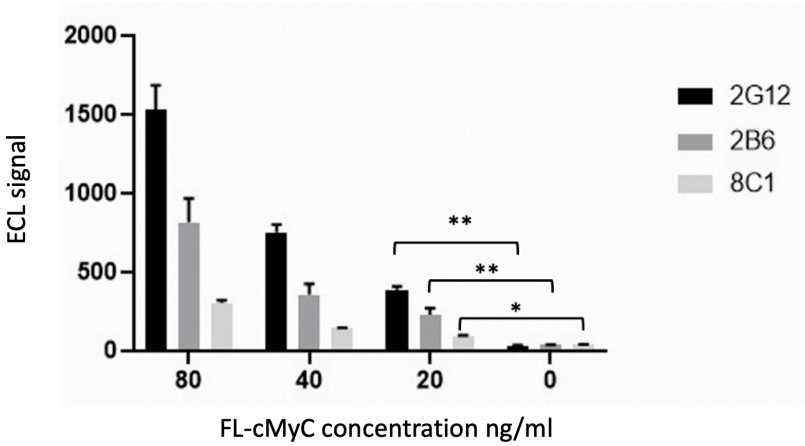


Figure 5.12 ECL sandwich ELISA for the detection of FL-cMyC captured with supernatant C-terminal anti-C5 mAb antibodies and detected with mouse 3H8-Ruthenium N-termianl mAb.

2G12, 2B6 and 8C1: mouse C-terminal anti-C5 mAbs. ECL signal with statistical significance determined using the Holm-Sidak method, with alpha = 0.05, Each row was analysed individually, without assuming a consistent SD. * $p < 0.05$, ** $p < 0.01$

Discussion:

This experiment confirms the feasibility of pairing mouse anti-C5 mAbs with mouse N-terminal 3H8-Ruthenium mAbs for the detection of FL-cMyC.

2G12 outperformed 2B6 and 8C1 and might be the best C-terminal anti-C5 antibody for pairing with 3H8-Ruthenium in a potential FL assay.

In the next experiment I will pair 2G12 C-terminal mAb with 3H8-Ruthenium for the selective detection of serially diluted FL-cMyC.

5.4.4.3.3.4 ECL sandwich ELISA for the detection of serially diluted FL-cMyC captured with 2G12 C-terminal anti-C5 mAb and detected with N-terminal 3H8-Ruthenium mAb

Methods:

- Standard ECL sandwich ELISA cMyC quantification protocol (see 5.4.4.2.3)
- MSD 96 wells MULTI-ARRAY plate
- Capture: supernatant C-terminal anti-C5 mAb 2G12, 500 ng/ml (supernatant)
- Antigen: FL-cMyC, serially diluted from 50 ng/ml to 20 ng/ml
- Detection: N-terminal mAb 3H8-Ruthenium, 500 ng/ml
- Reader: MSD Sector Imager 2400

Results:

N-terminal mAb 3H8-Ruthenium can detect 0.781 ng/ml of FL-cMyC captured by 2G12 anti-C5 C-terminal mAb in an ECL sandwich ELISA platform (p value 0.009 for 0.781 ng/ml vs control), figure 5.13.

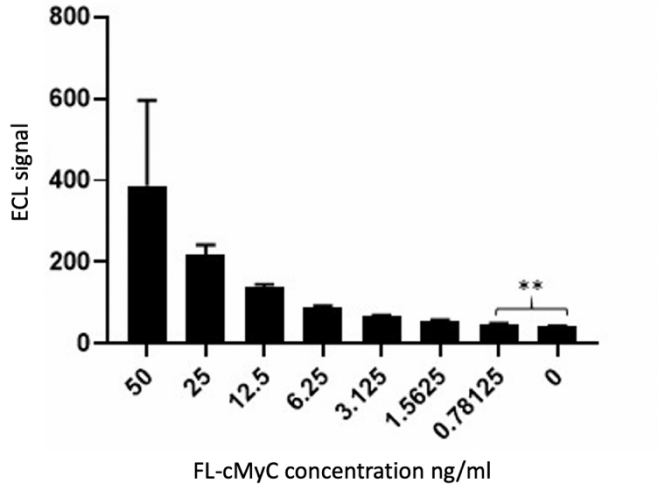


Figure 5.13 ECL sandwich ELISA for the detection of serially diluted FL-cMyC captured with supernatant C-terminal anti-C5 mAb 2G12 and detected with 3H8-Ruthenium N-terminal mAb

ECL signal with statistical significance determined using the Holm-Sidak method, with alpha = 0.05, Each row was analysed individually, without assuming a consistent SD. ** $p < 0.01$.

Discussion:

There is linearity between the signal and antigen concentration. Rapid attenuation of the signal is observed towards lower concentrations (from 3.125 ng/ml), further dilution of the antigen would help to establish the lower limit of detection using this combination.

Experiments so far demonstrate that detection of FL-cMyC is achievable in sandwich ELISA setting with three combinations of antibody groups

1- Mouse C-terminal anti-C5 mAbs (supernatant) paired with sheep N-terminal mAbs (spectrophotometric sandwich ELISA platform)

2- Mouse C-terminal anti-C5 mAbs paired with N-terminal Ruthenium-tagged 3H8 (electrochemiluminescence sandwich ELISA platform)

With most promising results achieved with the latter.

Further experiments to optimise the sensitivity are needed. This could be attempted by using purified C-terminal mAbs antibodies, different concentrations of detection, capture and secondary antibodies, different reagents, incubation and reaction times and different labelling of secondary and detection antibodies.

Purified mouse C-terminal anti-C5 mAbs have now become available.

In the next set of experiments, I will conduct the following (electrochemiluminescence sandwich ELISA platform)

1- Ruthenium (Sulfo)-tagging of best performing purified mouse C-terminal anti-C5 and N-terminal sheep mAbs

2- Pairing C-terminal anti-C5 and N-terminal mouse mAb, and switching the orientation of the antibodies (capture/detection) to identify the best performing FL-cMyC protocol

3- Pairing C-terminal anti-C5 and N-terminal sheep mAb, and switching the orientation of the antibodies (capture/detection) to identify the best performing FL-cMyC protocol

4- Compare the best performing protocols from 2 and 3

5- Pairing mouse N-terminal and sheep N-terminal mAbs, and switching the orientation of the antibodies (capture/detection) to identify the best performing in-house total (all species) cMyC assay

There are several considerations to take into account in relation to the aimed sensitivity of the FL-cMyC assay.

Spiking FL-cMyC antigen in human serum might yield different results to the experiments with recombinant analyte in buffer. This step is necessary to determine the true sensitivity of the assay.

It is also worth noting that the true concentration of the recombinant FL-cMyC used in the experiments might not match that recorded using a protein assay since the analyte spontaneously degrades pre-analysis due to temperature sensitivity, thaw-freeze cycles and also potential degradation within the analysis wells due to incubation at room temperature during the measurement protocol.

It is also possible that the folding of the FL-cMyC in-vivo and in-vitro might prevent optimal exposure of the target epitopes to the binding antibodies.

5.4.4.3.3.5 Labelling purified mouse C-terminal anti-C5 and sheep N-terminal mAbs with Ruthenium (Sulfo-tagging)

Methods:

MSD GOLD ™ SULFO-TAG NHS-Ester

Standard manufacturer protocol (5.4.4.2.5)

Antibodies: 2G12 mouse C-terminal anti-C5 mAb 1mg/mL, 2B6 mouse C-terminal anti-C5 mAb 1mg/mL, 3H8 mouse N-terminal mAb 1mg/mL, and 3C2 sheep N-terminal mAb 2.45 mg/mL.

Volume :300 ul

Conjugation ratio 10:1

Buffer exchange with supplied ZEBA columns to remove sodium azide additives was performed for 2B6 and 3C2 before conjugation.

2B6-Ruthenium 0.069 mg/ml

2G12-Ruthenium 0.735 mg/ml

3C2-Ruthenium 0.097 mg/ml

3H8-Ruthenium 0.697 mh/ml

The following pairings were tested for creating selective FL-cMyC assays:

1- Mouse N-terminal mAb (Capture) + Ruthenium-tagged mouse C-terminal anti-C5 mAb (detection)
2- Mouse C-terminal anti-C5 mAb (Capture) + Ruthenium-tagged mouse N-terminal mAb (detection)
3- Sheep N-terminal mAb (Capture) + Ruthenium-tagged mouse C-terminal anti-C5 mAb (detection)
4- Mouse C-terminal anti-C5 mAb (Capture) + Ruthenium-tagged sheep N-terminal mAb (detection)

In the following section, I will demonstrate the designs and the results of the best performing assays

5.4.4.3.3.6 ECL sandwich ELISA for the detection of FL-cMyC captured with 3H8 N-terminal mAb and detected with Ruthenium-tagged 2G12 C-terminal anti-C5 mAb

Methods:

- Standard ECL sandwich ELISA cMyC quantification protocol **(**5.4.4.2.3)
- MSD 96 wells MULTI-ARRAY plate
- Capture: N-terminal 3H8 mAb 1ug/ml
- Antigen: FL-cMyC serially diluted by 4 from 80 ng/ml to 0.0195 ng/ml
- Detection: C-terminal 2G12-Ruthenium 500 ng/ml
- MESO QuickPlex SQ 120 MM Reader, AI1AA-0, Meso Scale Discovery, USA)

Results:

Rapid attenuation in the ECL signal was seen with FL-cMyC dilutions below 20 ng/ml, with loss of statistically significant difference in the ECL signal at 0.3125 ng/ml and below of FL-cMyC against negative control, figure 5.14.

Discussion:

This assay is not sensitive enough for clinical testing in patients with low to moderate concentrations of cMyC.

Adjusting the concentrations of the detection and capture antibodies did not yield any improvement in the sensitivity of the assay.

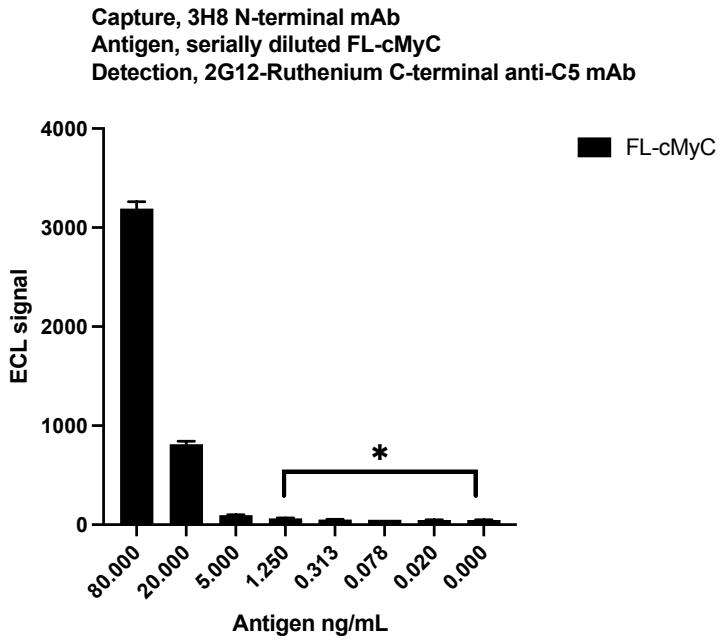


Figure 5.14 ECL Sandwich ELISA for the quantification of FL-cMyC captured with 3H8 N-terminal mAb and detected with 2G12-Ruthenium C-terminal anti-C5 mAb.

Statistical significance determined using the Holm-Sidak method, with alpha = 0.05 (* <0.05)

In the next experiment I will replace the detection antibody with 2B6 anti-C5 C-terminal antibody which was subjected to buffer exchange to remove sodium azide additives before tagging with Ruthenium.

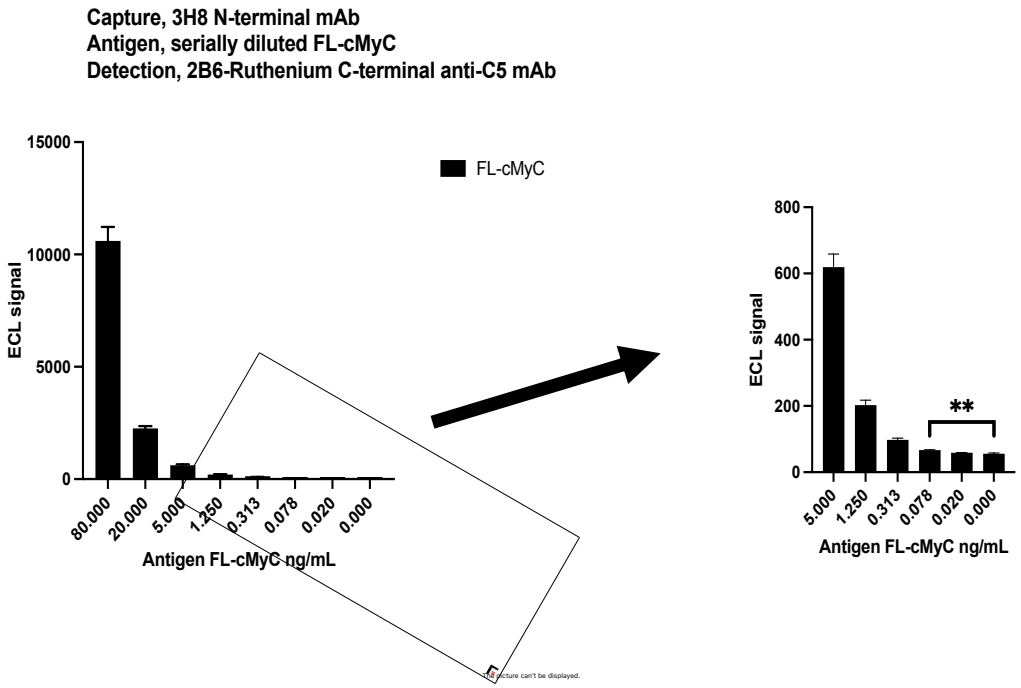
5.4.4.3.3.7 ECL sandwich ELISA for the detection of FL-cMyC captured with 3H8 N-terminal mAb and detected with 2B6- Ruthenium C-terminal anti-C5 mAb

Methods:

- Standard ECL sandwich ELISA cMyC quantification protocol (5.4.4.2.3)
- MSD 96 wells MULTI-ARRAY plate
- Capture: 3H8 N-terminal mAb 1ug/ml
- Antigen: FL-cMyC serially diluted by 4 from 80 ng/ml to 0.0195 ng/ml
- Detection: 2B6-Ruthenium C-terminal mAb 200 ng/ml
- MESO QuickPlex SQ 120 MM Reader, AI1AA-0, Meso Scale Discovery, USA

Results:

This assay can discriminate between negative control and low concentrations of FL-cMyC between 0.0195 and 0.078 ng/mL. Figure 5.15. and attached table.



FL-cMyC	Discovery?	P value	Mean of FL-cMyC signal	Mean of Control	Difference	SE of difference	t ratio	df	q value
80	Yes	< 0.001	10599	56	10543	362.9	29.05	4	< 0.001
20	Yes	< 0.001	2255	56	2199	62.24	35.34	4	< 0.001
5	Yes	< 0.001	619	56	563	22.88	24.6	4	< 0.001
1.25	Yes	< 0.001	202.3	56	146.3	8.913	16.42	4	< 0.001
0.3125	Yes	< 0.001	97.67	56	41.67	3.127	13.33	4	< 0.001
0.078	Yes	< 0.01	67	56	11	1.291	8.521	4	< 0.001
0.0195	No	0.090733	58.67	56	2.667	1.202	2.219	4	< 0.01

Figure 5.15 ECL Sandwich ELISA for the quantification of FL-cMyC capturing with 3H8 N-terminal monoclonal antibody and detecting with Ruthenium-tagged C-terminal anti-C5 mAb 2B6.

Statistical significance determined using the Holm-Sidak method, with alpha = 0.05 (** p <0.01)

Discussion:

Removal of the sodium azide preservative from the detection antibodies appears to have enhanced their conjugation/ affinity resulting in an assay that is capable of quantifying FL-cMyC concentrations below the 99th percentile of myocardial injury for cMyC (≈0.078 ng/mL)[52]. However, the 99th percentile of cMyC was measured with an assay that detects all circulating species of cMyC which include the C0C1f N-terminal fragment which has a molecular weight of ~35 kDa compared to ~135 kDa for FL-cMyC, therefore, the sensitivity of this assay for measuring intact or fuller length FL-cMyC is likely to be

underestimated, after adjusting for molecular weight differences. The sensitivity of this assay for FL-cMyC is potentially adequate for clinical applications.

The experiment above was performed *in vitro* with recombinant FL-cMyC in MSD dilution buffer as matrix, therefore, it is important to test the performance and validity of the assay in detecting FL-cMyC spiked in human serum with very low cMyC concentration obtained from healthy volunteers.

5.4.4.3.3.8 ECL sandwich ELISA for the detection of FL-cMyC spiked in human serum captured with 3H8 N-terminal mAb and detected with 2B6-Ruthenium C-terminal mAb

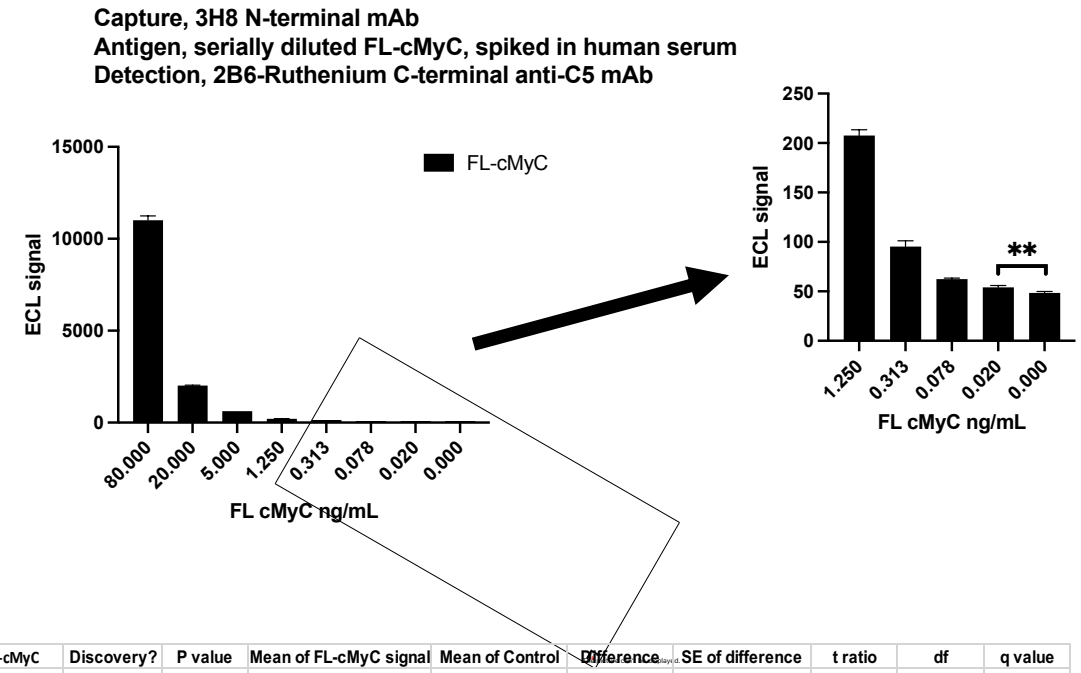
Methods:

- Standard ECL sandwich ELISA cMyC quantification protocol (see 5.4.4.2.3)
- MSD 96 wells MULTI-ARRAY plate
- Capture: 3H8 N-terminal mAb 1ug/ml
- Antigen: FL-cMyC spiked in human serum from healthy volunteer with cMyC concentration 0.0051 ng/L, and serially diluted by 4 from 80 ng/ml to 0.0195 ng/ml
- Detection: 2B6-Ruthenium C-terminal mAb 200 ng/ml
- MESO QuickPlex SQ 120 MM Reader, AI1AA-0, Meso Scale Discovery, USA

Results:

This assay has an acceptable signal linearity for FL-cMyC concentrations between 80 and 0.019 ng/mL, spiked in human serum from a healthy volunteer with a low cMyC

concentration (0.0051 ng/ml) and capable of discriminating between negative control and very low concentrations of FL-cMyC below 0.0195 ng/mL, Figure 5.16 and attached table.



FL-cMyC	Discovery?	P value	Mean of FL-cMyC signal	Mean of Control	Difference	SE of difference	t ratio	df	q value
80	Yes	< 0.001	11008	48	10959	137.2	79.89	4	< 0.001
20	Yes	< 0.001	2016	48	1968	11.0	178.8	4	< 0.001
5	Yes	< 0.001	625.7	48	577.3	1.7	339.7	4	< 0.001
1.25	Yes	< 0.001	207.7	48	159.3	3.4	46.21	4	< 0.001
0.3125	Yes	< 0.001	95.33	48	47	3.4	13.63	4	< 0.001
0.078	Yes	< 0.001	62.33	48	14	1.1	12.66	4	< 0.001
0.0195	Yes	< 0.01	54	48	5.667	1.5	3.9	4	< 0.01

Figure 5.16 ECL Sandwich ELISA for the quantification of FL-cMyC spiked in human serum from a healthy volunteer capturing with 3H8 N-terminal mAb and detecting with 2B6-Ruthenium C-terminal mAb.

Statistical significance determined using the Holm-Sidak method, with alpha = 0.05 (** p <0.01)

Discussion:

The results are promising and suggest that I have formulated an assay capable of quantifying low concentrations of intact cMyC relevant to clinical settings.

In the next experiment I will examine the stability of my best performing FL-cMyC assay (3HG + 2B6-Ruthenium) and calculate the within-run and between-runs coefficients of variation.

5.4.4.3.3.9 Within-and between-plate coefficient of variation of ECL sandwich ELISA for the detection of FL-cMyC spiked in human serum, captured with 3H8 N-terminal mAb and detected with 2B6 C-terminal mAb

Method:

- Standard ECL sandwich ELISA cMyC quantification protocol (see 5.4.4.2.3)
- 3 X MSD 96 wells MULTI-ARRAY plates
- Capture: 3H8 N-terminal mAb 1ug/ml
- Antigen: FL-cMyC spiked in human serum from healthy volunteer with cMyC concentration 0.006 ng/ml, and serially diluted by 4 from 80 ng/ml to 0.0195 ng/ml, assayed in duplicate (columns 1 and 2)
- Detection: 2B6-Ruthenium C-terminal anti-C5 mAb 200 ng/ml
- MESO QuickPlex SQ 120 MM Reader, AI1AA-0, Meso Scale Discovery, USA

Results:

Within plate (between duplicate) coefficient of variation CV% for ECL signal of FL-cMyC assay are depicted in tables 4, 5 and 6. CV % remained low (below 10) for all concentrations between 80 ng/mL and 0.0195 ng/mL and for negative control (background). Tables 5.4, 5.5 and 5.6.

Plate 1					
FL-cMyC ng/mL	**Duplicate 1**	**Duplicate 2**	**Mean**	**SD**	**CV%**
80	10766	11016	10891	176.8	1.6
20	2000	2037	2018.5	26.2	1.3
5	626	623	624.5	2.1	0.3
1.25	201	211	206	7.1	3.4
0.3125	102	92	97	7.1	7.3
0.078	61	63	62	1.4	2.3
0.0195	52	54	53	1.4	2.7
0	47	47	47	0.0	0.0

Table 5.4 Within plate (between duplicate) CV% for ECL signal by FL-cMyC assay (run 1)

Plate 2					
FL-cMyC ng/mL	**Duplicate 1**	**Duplicate 2**	**Mean**	**SD**	**CV%**
80	11201	11760	11480.5	395.3	3.4
20	2200	2176	2188	17.0	0.8
5	652	634	643	12.7	2.0
1.25	210	230	220	14.1	6.4
0.3125	93	88	90.5	3.5	3.9
0.078	65	69	67	2.8	4.2
0.0195	56	60	58	2.8	4.9
0	51	52	51.5	0.7	1.4

Table 5.5 Within plate (between duplicate) CV% for ECL signal by FL-cMyC assay (run 2)

Plate 3					
FL-cMyC ng/mL	**Duplicate 1**	**Duplicate 2**	**Mean**	**SD**	**CV%**
80	12131	11982	12056.5	105.4	0.9
20	2187	2301	2244	80.6	3.6
5	654	701	677.5	33.2	4.9
1.25	212	234	223	15.6	6.9
0.3125	123	124	123.5	0.7	0.6
0.078	68	66	67	1.4	2.1
0.0195	57	57	57	0.0	0.0
0	49	49	49	0.0	0.0

Table 5.6 Within plate (between duplicate) CV% for ECL signal by FL-cMyC assay (run 3)

Between assay CV% was also low, table 5.7.

	Mean of ECL signals of duplicates					
FL-cMyC ng/mL	Assay 1	Assay 2	Assay 3	Mean	SD	CV%
80	10891	11480	12056.5	11475.8	582.8	3.7
20	2018.5	2188	2244	2150.2	117.4	5.7
5	624.5	643	677.5	648.3	26.9	2.1
1.25	206	220	223	216.3	9.1	4.6
0.3125	97	90.5	123.5	103.7	17.5	4.9
0.078	62	67	67	65.3	2.9	5.5
0.0195	53	58	57	56.0	2.6	6.4
0	47	51.5	49	49.2	2.3	6.5

Table 5.7. Between plate CV% for ECL signal by FL-cMyC assay.

Discussion:

The assay appears to be stable with low CV (<10%) at low concentrations and reproducible. The thaw-freeze cycle effect on the integrity of the standard and serum antigen should be taken into account for any future calibration or utility of the assay. I have previously demonstrated auto-proteolysis/degradation of cMyC with prolonged incubation at room temperature (see 5.4.3.2).

Overall, pairing 3H8 N-terminal mAb with 2B6-Ruthenium C-terminal mAb on an ECL sandwich ELISA platform produced a sensitive and stable assay for the detection of FL-cMyC.

It is crucial, to ensure the selectivity of the assay to FL-cMyC. In the next experiment, I will use N-terminal fragment C0C2 as control

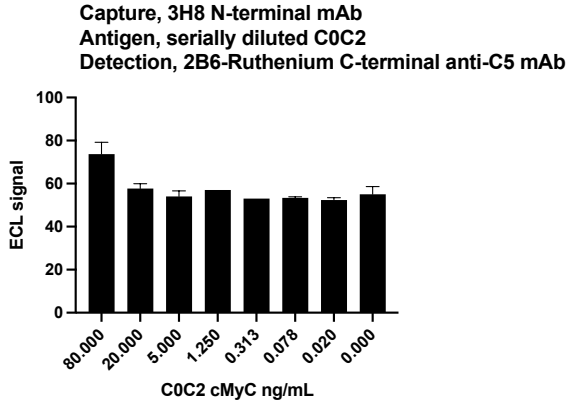
5.4.4.3.3.10 The specificity of ECL sandwich ELISA assay (3H8 + 2B6-Ruthenium) to FL-cMyC

Methods:

- Standard ECL sandwich ELISA cMyC quantification protocol (see 5.4.4.2.3)
- 3 X MSD 96 wells MULTI-ARRAY plates
- Capture: 3H8 N-terminal mAb 1ug/ml
- Antigen: C0C2 fragment spiked in human serum from healthy volunteer with cMyC concentration 5 ng/L (0.005 ng/ml), and serially diluted by 4 from 80 ng/ml to 0.0195 ng/ml, assayed in duplicate.
- Detection: 2B6-Ruthenium C-terminal mAb 200 ng/ml
- MESO QuickPlex SQ 120 MM Reader, AI1AA-0, Meso Scale Discovery, USA

Results:

The assay is unable to discriminate between C0C2 and negative control. The assay is blind to C0C2. Figure 5.17 and attached table.



C0C2 ng/ml	Discovery?	P value	Mean of C0C2 signal	Mean of Control	Difference	SE of difference	t ratio	df
80.000	No	0.01	73.67	55	18.67	3.801	4.912	4
20.000	No	0.34	57.67	55	2.667	2.472	1.079	4
5.000	No	0.72	54	55	-1	2.582	0.3873	4
1.250	No	0.39	57	55	2	2.082	0.9608	4
0.313	No	0.39	53	55	-2	2.082	0.9608	4
0.078	No	0.47	53.33	55	-1.667	2.108	0.7906	4
0.020	No	0.29	52.33	55	-2.667	2.186	1.22	4

Figure 5.17 The N-fragment C0C2 detection with ECL sandwich ELISA assay capturing with 3H8 and detecting with 2B6-Ruthenium.

Statistical significance determined using the Holm-Sidak method, with alpha = 0.05.

Discussion:

This FL-cMyC immunoassay (3H8 + 2B6-Ruthenium) is blind to the N-terminal fragment C0C2 which does not contain the C5 domain for the binding of the detection antibody 2B6, and therefore, it can selectively detect FL-cMyC by straddling the calpain cleavage site within the M domain.

To study the proportion of FL-cMyC within the circulating species of cMyC in the serum, I needed to develop a sensitive in-house total (all species) cMyC assay by pairing two N-terminal antibodies for the detection of C0C2 and C0C1f on the same ECL analysis platform.

Our traditional total cMyC assay was created by pairing 3H8 and 1A4 N-terminal mAb on an ECL sandwich ELISA platform. The limit of quantification of this assay was in the region of 80 pg/mL [52], which was not sensitive enough to match the developed FL-cMyC assay and to quantify cMyC at low concentrations. [53] I have therefore attempted to reformulate the assay using several combinations of N-terminal antibodies from two species, mouse and sheep including:

1A4 and 3H8: mouse N-terminal mAb

3C2: sheep N-terminal mAb

- 1A4 + 3H8-Ruthenium (our traditional assay)
- 3H8 + 1A4-Ruthenium
- 3C2 + 3H8-Ruthenium
- 3H8 + 3C2 Ruthenium

Below I will show the results of the best performing assays.

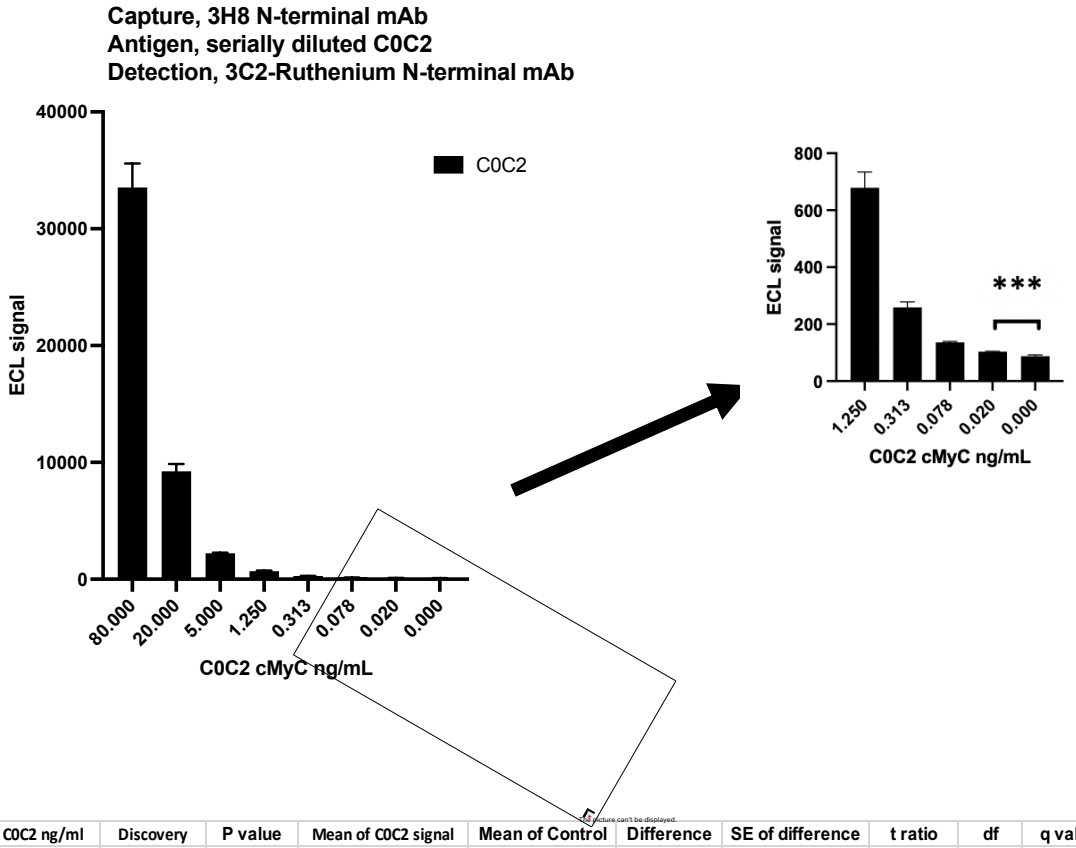
5.4.4.3.3.11 ECL sandwich ELISA for the detection of C0C2 spiked in human serum, captured with 3H8 N-terminal mAb and detected with 3C2 N-terminal-Ruthenium mAb

Methods:

- Standard ECL sandwich ELISA cMyC quantification protocol (see 5.4.4.2.3)
- MSD 96 wwells MULTI-ARRAY plate
- Capture: mouse 3H8 N-terminal mAb 1ug/ml
- Antigen: C0C2 spiked in human serum from healthy volunteer with low cMyC concentration of 4.8 ng/mL, and serially diluted by 4 from 80 ng/ml to 0.0195 ng/ml
- Detection: sheep 3C2-Ruthenium N-terminal mAb200 ng/ml
- MESO QuickPlex SQ 120 MM Reader, AI1AA-0, Meso Scale Discovery, USA

Results:

The assay is able to detect low levels of C0C2 below 19.5 pg/mL. Figure 5.18 and attached table.



C0C2 ng/ml	Discovery	P value	Mean of C0C2 signal	Mean of Control	Difference	SE of difference	t ratio	df	q value
80	Yes	< 0.001	33534	88	33446	1182	28.29	4	< 0.001
20	Yes	<0.001	9239	88	9151	357.1	25.63	4	<0.001
5	Yes	< 0.001	2215	88	2127	32	66.45	4	< 0.001
1.25	Yes	< 0.001	679.7	88	591.7	32.12	18.42	4	< 0.001
0.3125	Yes	< 0.001	259.3	88	171.3	11.62	14.74	4	< 0.001
0.078	Yes	< 0.001	137	88	49	3.512	13.95	4	< 0.001
0.0195	Yes	< 0.01	104.3	88	16.33	3.127	5.223	4	< 0.01

Figure 5.18 ECL Sandwich ELISA for the quantification of C0C2 capturing with mouse 3H8 N-terminal mAb and detecting with sheep 3C2 Ruthenium N-terminal mAb.

Statistical significance determined using the Holm-Sidak method, with alpha = 0.05 (* <0.05, ** <0.01, *** <0.001, **** <0.0001)

Discussion:

This assay is more sensitive than our traditional in-house C0C2 assay which uses a combination of 1A4 and 3H8-Ruthenium (both mouse N-terminal mAbs) and has a limit of detection ~80 ng/mL.

The assay is able to detect low levels of cMyC (below 0.0195 ng/mL) which is suitable for complementing the FL-cMyC assay for the in-house determination of

[FL-cMyC]:[total (all species) cMyC] ratio.

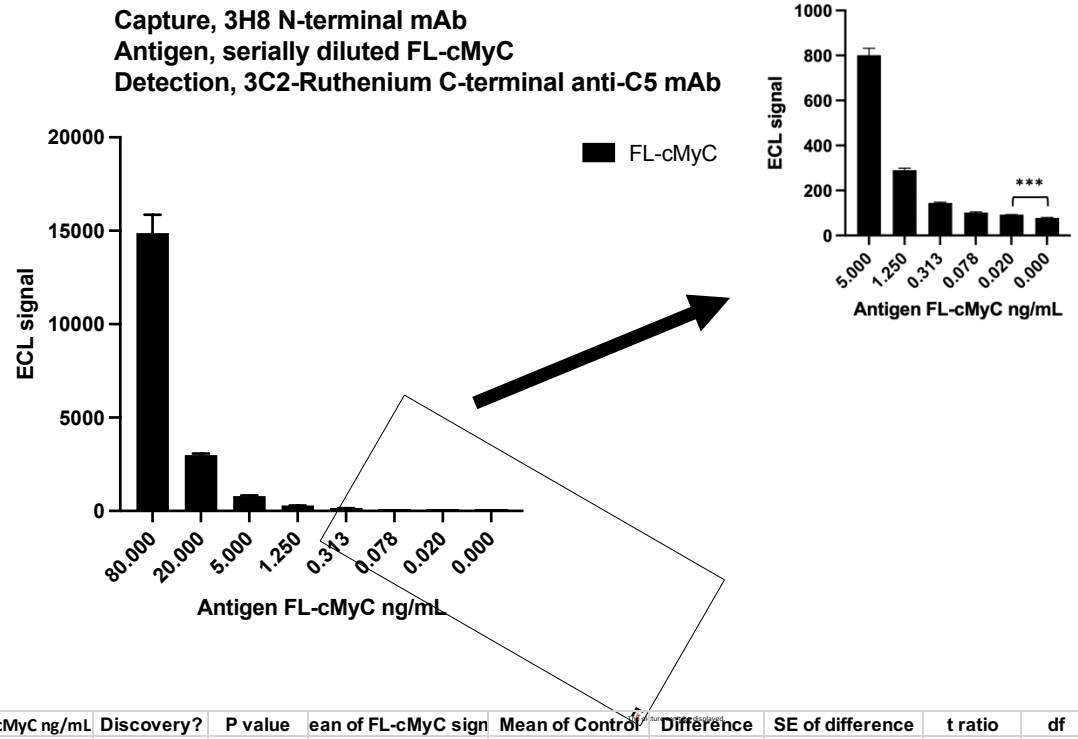
5.4.4.3.3.12 ECL sandwich ELISA for the non-selective detection of FL-cMyC spiked in human serum, captured with 3H8 N-terminal mAb and detected with 3C2 N-terminal-Ruthenium mAb

Methods:

- Standard ECL sandwich ELISA cMyC quantification protocol (see 5.4.4.2.3)
- MSD 96 wells MULTI-ARRAY plate
- Capture: 3H8 N-terminal mAb 1ug/ml
- Antigen: FL-cMyC spiked in human serum from healthy volunteer with cMyC concentration 0.0048 ng/ml, and serially diluted by 4 from 80 ng/ml to 0.0195 ng/ml
- Detection: 3C2-Ruthenium N-terminal mAb 200 ng/ml
- MESO QuickPlex SQ 120 MM Reader, AI1AA-0, Meso Scale Discovery, USA

Results:

The assay has good linearity and a limit of detection below 0.0195 ng/mL. Figure 5.19 and attached table



FL-cMyC ng/mL	Discovery?	P value	ean of FL-cMyC sign	Mean of Control	Difference	SE of difference	t ratio	df
80	Yes	< 0.001	14869	78	14791	569.6	25.97	4
20	Yes	< 0.001	2986	78	2908	52.41	55.48	4
5	Yes	< 0.001	801	78	723	17.99	40.19	4
1.25	Yes	< 0.001	290	78	212	5.416	39.14	4
0.3125	Yes	< 0.001	144.7	78	66.67	2.261	29.49	4
0.078	Yes	< 0.001	102	78	24	2.517	9.537	4
0.0195	Yes	< 0.001	92.67	78	14.67	1.563	9.381	4

Figure 5.19 ECL Sandwich ELISA for the non-selective quantification of FL-cMyC capturing with 3H8 N-terminal monoclonal antibody and detecting with 3C2-Ruthenium N-terminal mAb.

Statistical significance determined using the Holm-Sidak method, with alpha = 0.05 (* <0.05, ** <0.01, *** <0.001, **** <0.0001)

Discussion:

The total cMyC assay is able to quantify low concentrations of FL-cMyC below 0.0195 ng/ml. This non-selective assay is more sensitive for the quantification of FL-cMyC than

the specific FL-cMyC assay created by pairing an N-terminal antibody and C-terminal antibody. This might be the result of a higher affinity of the N-terminal antibodies than the C-terminal antibodies to their respective target epitopes, or a degree of in-vitro degradation/cleavage of FL-cMyC.

To summarise, to straddle the cleavage site of cMyC and create a selective fuller-length FL (intact) circulating cMyC assay, high affinity anti-N-terminal and anti-C-terminal cMyC antibodies were paired to create a sensitive electrochemiluminescence sandwich ELISA assay. I also created a sensitive total cMyC assay that can quantify all circulating species of cMyC that contain the N-terminus (N-terminal fragments and fuller-intact-cMyC).

In the next experiment I will immunoassay cMyC in the serum samples from the phenylephrine and MI swine models that I used to determine the phosphorylation and fragmentation of cMyC within the myocardium, to determine the proportion of FL-cMyC within the total circulating species of the protein.

5.5 Fragmentation pattern of circulating cMyC in pigs subjected to phenylephrine-induced myocardial injury and Left Anterior Descending ligation-induced myocardial infarction

5.5.1 Methods

Animal models

Animal models and sample collection are described in (5.3.1.1)

Serial blood sampling was performed at baseline and at 1-hour after myocardial injury in all animals. Additional blood sampling at 24-hours was performed in animals that were euthanised 24-hours post injury.

Blood samples were collected under sterile laboratory conditions, centrifuged, and stored as frozen plasma (-80 °C) at the University at Buffalo. Samples were transferred to KCL on dry ice and stored immediately upon arrival at -80 °C.

Immunoassays:

Standard ECL cMyC quantification protocol was followed.

Samples were immunoassayed in triplicate at two dilutions for the quantification of FL-cMyC and total (all species) cMyC using the assays described above.

5.5.2 Results:

In the PE model, a significant increase in total cMyC ECL signal was observed at 1-h and 24-h post injury, compared to baseline (188±3.6, 197±29 and 227±53, for baseline, 1-h and 24-h, respectively; p=0.013 for 0-h vs 1-h, p= 0.018 for 0-h vs 24-h). With the FL-cMyC assay, a significant increase in the ECL signal was observed 1-h post injury compared to baseline 58±6 vs 132±33 for 0-h vs 1-h respectively; p=0.019, followed by a reduction in the signal back to baseline at 24-h; 58±6.

PE model	**FL-cMyC assay ECL signal**			**Total-cMyC assay ECL signal**			**FL-cMyC / Total-cMyC**		
Sample ID	**0-h**	**1-h**	**24-h**	**0-h**	**1-h**	**24-h**	**0-h**	**1-h**	**24-h**
1	54	101	53	114	230	208	0.47	0.44	0.25
2	65	128	65	121	191	186	0.54	0.67	0.35
3	55	167	56	119	172	287	0.46	0.97	0.20

Table 5.8 ECL signal for FL-cMyC and total (all species) cMyC from the PE model at different time points post injury (0-h, 1-h and 24-h) quantified by the respective assays.

The ratio between FL-cMyC and total cMyC ECL signal was mean ±SD: 0.69 ± 0.26 and 0.26 ± 0.07, at 1-h and 24-h, respectively, p=0.05 for difference between the two groups, table 5.8, figure 5.20.

Table 5.8. ECL signal with FL-cMyC and total-cMyC immunoassay in the PE model, and ratios.

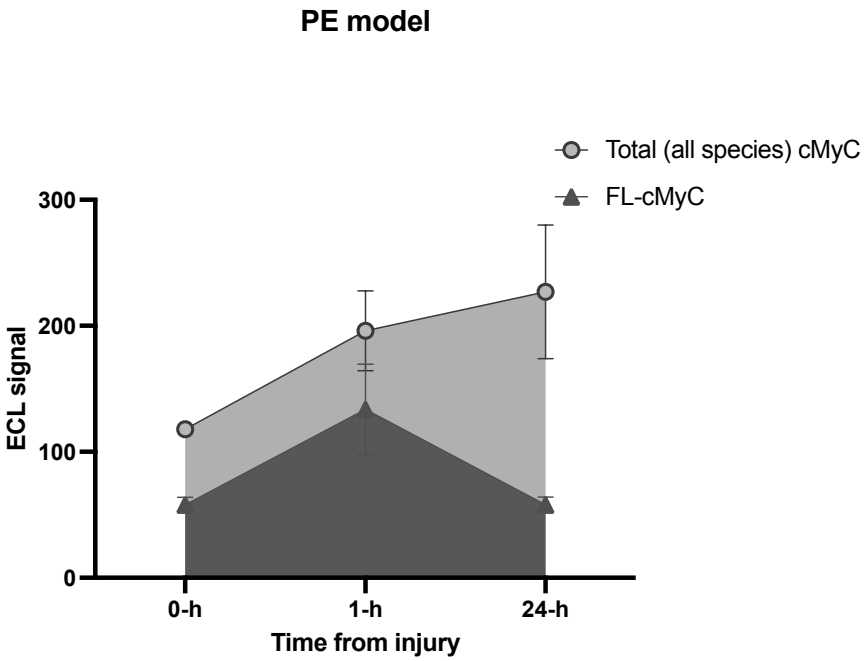


Figure 5.20 Temporal change in total (all species) cMyC (light grey) and FL-cMyC (dark grey) quantified by ECL signal in the PE model.

In the MI model, a significant increase in total cMyC ECL signal was observed at 1-h and 24-h post injury, compared to baseline (124±12, 1478±298 and 274±58, for baseline, 1-h and 24-h, respectively; p=0.001 for 0-h vs 1-h, p= 0.012 for 0-h vs 24-h). With the FL-cMyC assay, no significant increase in the ECL signal was observed 1-h or 24-h post injury compared to baseline 58±6 vs 56±4 and 55±2 for 0-h, 1-h and 24-h, respectively; p=0.69 for 0-h vs 1-h; p=0.47 for 0-1 vs 24-h.

The ratio of FL-cMyC to total cMyC ECL signal was (mean ±SD): 0.043 ± 0.05 and 0.2 ± 0.03, at 1-h and 24-h, respectively, p=0.001 for difference between the two groups.

On pairwise comparison between the two models, there was significant difference in the ratio of FL-cMyC to total-cMyC between the PE model and MI model at 1-h (0.69 ± 0.26 vs 0.043 ± 0.05, respectively, p= 0.013, but no significant difference was observed at 24-h (0.26 ± 0.07 vs 0.2 ± 0.03, p=0.278). Table 5.9 and figure 5.21.

MI model	**FL-cMyC assay ECL signal**			**Total-cMyC assay ECL signal**			**FL-cMyC / Total-cMyC**		
Sample ID	**0-h**	**1-h**	**24-h**	**0-h**	**1-h**	**24-h**	**0-h**	**1-h**	**24-h**
3	53	58	55	130	1164	249	0.41	0.05	0.22
4	65	62	57	110	1758	341	0.59	0.04	0.17
5	56	53	53	134	1514	232	0.42	0.04	0.23

Table 5.9 ECL signal for FL-cMyC and total (all species) cMyC from the MI model at different time points post injury (0-h, 1-h and 24-h) quantified by the respective assays.

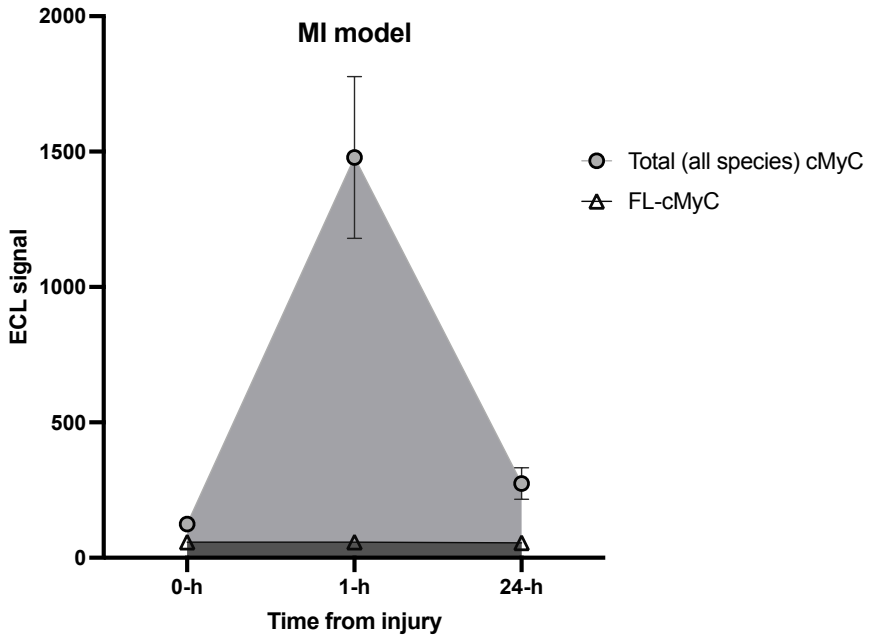


Figure 5.21 Temporal change in total (all species) cMyC (light grey) and FL-cMyC (dark grey) quantified by ECL signal in the MI model.

5.5.3 Discussion:

The study demonstrates the release and kinetics of FL-cMyC relative to total cMyC in swine subjected to two distinct myocardial injury protocols: phenylephrine (PE)-induced myocardial injury and LAD ligation-induced MI.

There was a significant increase in both total and FL-cMyC in the PE model one hour post myocardial injury relative to baseline. In contrast, in the MI model only total cMyC increased significantly one hour post injury and there was no apparent increase in FL-cMyC. This led to a discrepancy in the [FL-cMyC]:[total-cMyC] ratio between the two models, with significantly higher ratio in the PE model compared to MI model (0.69 ± 0.26 vs 0.043 ± 0.05, respectively, p= 0.013).

At 24-h, compared to 1-h, the total cMyC signal was comparable in the PE model (197±29 vs 227±53, respectively, p= 0.45), but a significant drop in the signal was observed in the MI model (1478±298 vs 274±58, p=0.012). Different kinetics were observed for FL-cMyC signal between the two time points, whilst it remained static in the MI model it returned towards baseline in the PE model. Consequently, no significant difference in the [FL-cMyC]:[total-cMyC] ratio was observed at 24-h (0.26 ± 0.07 vs 0.2 ± 0.03, p=0.278).

The kinetic pattern of total-cMyC in the MI model is similar to that observed after iatrogenic MI (intracoronary ablation of septal hypertrophy) in humans with an earlier peak and earlier fall in cMyC concentration compared to cTnT. [24] But it is not known why a different pattern of cMyC concentration was observed in the PE model where total-cMyC signal was comparable between 1-h and 24-h timepoints. Histopathological examination and hematoxylin and eosin-staining of myocardial tissue from the PE model performed by our collaborators at the University at Buffalo demonstrated absence of contraction band necrosis, nuclear loss, and inflammatory cell infiltration, and 6-fold increase in apoptosis 3 h after intravenous PE that returned to normal value 24 h later.[168] Whereas in myocardial infarction, necrosis is a predominant histopathological finding. Additionally, it is possible that other factors contribute to cMyC release in various proportions in the two models, such as release of cytosolic cMyC and, potentially, increased cell membrane permeability in LV stretch-induced myocardial injury compared to MI.

FL-cMyC signal peaked 1-h after PE induced myocardial injury and returned to baseline 24-h later but remained at baseline level through to 24-h post injury in the MI model.

The increase in the circulating [FL-cMyC]:[total-cMyC] ratio in the PE model aligns well with the fragmentation pattern that was observed in the myocardium (5.2.2.2) and might mirror the phosphorylation status of the released circulating cMyC reflecting that in the myocardial tissue.

Phenylephrine is an alpha-adrenergic receptor agonist that directly promotes kinase mediated protein phosphorylation, predominantly through PKA, as part of the canonical signaling pathway. As well as directly phosphorylating cMyC there will be indirect effects on cMyC through other biochemical and physiological pathways. These likely lead to a multifaceted cause of cMyC release in the PE model which in turn could contribute to the appearance of FL-cMyC.

Further, the reduction in the FL-cMyC signal back to baseline at 24-h might reflect a degree of circulatory cMyC degradation and/or renal clearance.

This study has several limitations, the number of animals included is small, both FL-cMyC and total cMyC assays were developed and optimised against human cMyC, and therefore it would be inaccurate to extrapolate the cMyC concentrations using the standard curves generated with human recombinant protein; this is why the analysis was limited to the ECL signal. The phosphorylation status of circulating cMyC was not determined in this study.

In the next study, I will determine whether differences in the fragmentation pattern of the circulating forms of cMyC are present in human serum from patients who also have two distinct types of myocardial injury.

5.6 The fragmentation pattern of circulating human cMyC in Type 1 and Type 2 AMI

- -

5.6.1 Introduction:

Previously, in porcine models with two distinct types of myocardial injury, I have demonstrated that the phosphorylation and fragmentation pattern of cMyC within the myocardium are dependent on the type of myocardial injury. At one hour after myocardial injury, a similarly divergent fragmentation pattern of cMyC was found in the circulation, likely reflecting findings in myocardial tissue, with significantly higher [FL-cMyC]:[total cMyC] ratio in the PE model compared to the LAD occlusion model. To determine if these patterns translate to human serum, I examined the fragmentation pattern of cMyC in serum drawn from patients with two types of myocardial injury: Type 1 AMI (STEMI) and Type 2 AMI (supply-demand mismatch). cMyC in the serum samples will be immunoassayed using the finalised FL-cMyC and total-cMyC assays to determine the [FL-cMyC]:[total cMyC] ratio.

5.6.2 Methods:

5.6.2.1 *Ethics:*

The study was carried out according to the principles of the Declaration of Helsinki.

All participants gave informed consent

Ethical approval IRAS 102914...add REC number

5.6.2.2 *Participants:*

Eligible patients presenting to St Thomas' Hospital between 09:00 and 16:00 from 01/03/2021 to 17/07/2021 were prospectively recruited.

Potential participants within 12 hours of admission were identified by screening the Electronic Patient Record for new admissions to the Acute Medical and Coronary Care Unit.

5.6.2.3 *Inclusion/exclusion criteria:*

Type 1 AMI

Inclusion criteria:

1- Age > 18
2- Onset of symptoms < 12 hours
3- STEMI requiring activation of primary percutaneous coronary intervention pathway
4- Evidence of culprit acute coronary artery obstruction on invasive coronary angiography

Exclusion criteria:

1- Cardiogenic shock
2- eGFR <60 mL/min/1.73m2
3- Positive for COVID-19

Type 2 AMI

Inclusion criteria:

1- Age > 18
2- onset of symptoms < 12 hours
3- Non-sinus tachyarrhythmia with ventricular rate > 120 BPM
4- Evidence of myocardial injury with hs-cTnT > 2 X 99th percentile for myocardial injury (> 28 pg/mL)
5- Adjudicated clinical diagnosis of Type 2 AMI

Exclusion criteria:

1- New or known obstructive coronary artery disease
2- RWMA on cardiac imaging
3- eGFR <60 mL/min/1.73m2
4- Positive for COVID-19

5.6.2.4 *Sample collection, processing, and storage*

Blood was collected into 5 mL plastic serum-separation Vacutainer SST II Advance gel tubes (Becton Dickinson) using a 21 Gauge winged blood collection set with flexible tube needle (Becton Dickinson). Samples were allowed to clot for 30 minutes at room temperature and then centrifuged at 2200 x g for 10 minutes at room temperature. Separated acellular serum (0.9 mL) was then aliquoted into matching cryovial tubes (1.5 ml Mikroröhre PCR-PT, SARSTEDT AG & Co. KG) before being frozen at -80 °C within 1 hour from phlebotomy.

5.6.2.5 *Sample analysis:*

Samples were analysed in triplicate using the selective FL-cMyC and total cMyC assays that I created, characterised and described in this thesis.

5.6.2.6 *Data analysis:*

Data were analysed using R version 3.6.1.

Baseline characteristics were described using percentage, means or medians (standard deviation and first quartile-third quartile where applicable).

Student's t test and Mann-Whitney U test were used for comparing groups as appropriate.

5.6.3 Results:

Nine patients with type 1 AMI and 5 patients with type 2 AMI were recruited and included in the final analysis.

Baseline characteristics are shown in table 5.10.

Patients with Type 1 AMI were significantly younger than those with Type 2 (p=0.03).

No significant differences in other baseline characteristics were observed between the groups.

	Type 1 (n=9)	Type 2 (n=5)	p value
Age (median, 1st Q, 3rd Q)	52 (47, 56)	68 (61, 79)	0.03
BMI (mean ± SD)	30 ± 4.6	26 ± 2	0.21
Onset of symptoms to blood draw (hr)	6 ± 1.2	9.4 ± 3.5	0.26
Smoking history n (%)	7 (77)	3 (60)	0.58
Diabetes n (%)	2 (0.22)	0 (0)	0.5
Tablets controlled diabetes n (%)	1 (0.11)	0	0.99
Insulin controlled diabetes n (%)	1 (0.11)	0	0.99
Hypertension n (%)	5 (0.55)	1 (0.2)	0.33
Systolic BP (mean ± SD)	136 ±24	131 ± 17	0.79
Diastolic BP (mean ± SD)	86 ± 18	84 ± §7	0.82
Oxygen saturation (mean ± SD)	98 ± 2	95 ± 9	0.35
Temperature n (%)	36 ± 0.4	36 ± 0.5	0.98
eGFR (mean ± SD)	90 ± 24	86 ± 1.4	0.46
Peak troponin (cTnT, mean ± SD) *	3349 ± 3285	788 ± 913	0.12

Table 5.10 Baseline characteristics.

* The highest troponin level recorded during admission as requested for clinical purposes only.

There is a significantly higher ratio of FL (intact) to total (all species) cMyC in the tachyarrhythmia-induced myocardial injury subtype of Type 2 AMI compared to Type 1-AMI, mean ± SD: 35.4 ± 15.2 vs 4.7 ± 4.1, respectively; p=0.0016, figure 5.22.

ECL signals as measured by the FL-cMyC and total cMyC assays and the ratios between both signals per patient are presented in table 5.11.

There was no significant difference in peak troponin between the two groups (p= 0.12).

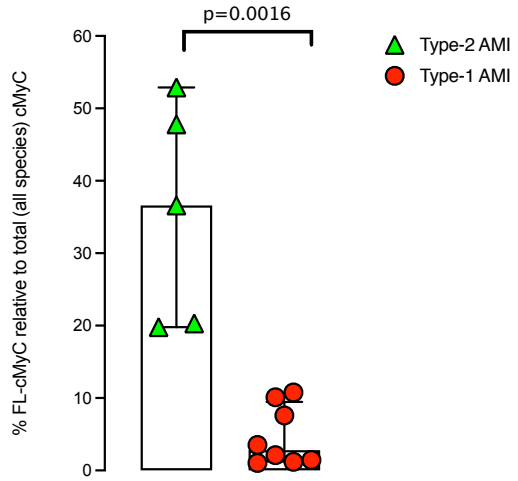


Figure 5.22 FL-cMyC relevant to total cMyC in type 1 and type 2-AMI.

FL-cMyC and total (all species) cMyC quantified by ECL signal produced by the respective assay.

Type 1-AMI			
	ECL signal		
ID	FL-cMyC	Total cMyC	% FL-cMyC / total cMyC
2	696	9152	7.6
5	58	5346	1.05
6	123	5802	2.12
7	52	482	10.1
9	139	9670	1.44
10	74	6292	1.18
16	127	3621	3.51
17	54	501	10.78
Type 2-AMI			
	ECL signal		
ID	FL-cMyC	Total cMyC	% FL-cMyC / total cMyC
8	75	157	47.8
11	80	593	20.3
18	76	383	19.8
19	70	191	36.6
20	64	121	52.89

Table 5.11. ECL signal as measured by FL-cMyC and total (all species) cMyC assays in Type 1- and Type 2-AMI

5.6.4 Discussion:

The study shows that the ratio of intact (FL-cMyC) to total cMyC is significantly higher in carefully adjudicated patients with a supply-demand mismatch subtype of type 2 AMI compared to patients with a STEMI subtype of Type 1 AMI. 35.4 ± 15.2 vs 4.7 ± 4.1, respectively; p=0.0016

This might have been the result of an increased phosphorylation of cMyC in the selected Type 2 subgroup to above the baseline, protecting the protein against calpain mediated cleavage.

The inclusion/exclusion criteria used to assign patients to type 1 or type 2 AMI were chosen to minimise the overlap between the two groups. However, the presence of a fixed coronary stenosis on angiography does not exclude Type 2 AMI,[39] similarly the absence of coronary obstruction on angiography does not exclude Type 1. Additionally, we acknowledge that type 2 AMI as defined by the UDMI is a heterogeneous group,[39] with varying stem pathological processes that cause the myocardial injury, and possible varied pathophysiological changes at the cellular/molecular level.

Therefore, the results of this study cannot be extrapolated to other subtypes of type 2 AMI as classified by the UDMI.

Additionally, heterogeneity in the cellular/molecular expression within the same subgroup is also expected. Several factors such as the preconditioning status of the heart, the length of ischaemia, reperfusion injury, and presence of collateral circulation might alter the phosphorylation of the cardiac proteins and therefore the calpain mediated cleavage event.

Additionally, several pharmacological confounders such as inotrope support, adrenergic blockade, could affect kinase activity and the phosphorylation status of MyC, and subsequently its fragmentation pattern.

It is not known if the fragmentation of cMyC in the studied models occurred predominantly within or outside the myocardium (in the circulation). My work on the animal model suggests that the fragmentation of cMyC originates, at least in part, from within the myocardium. However, the possibility remains that an additional calpain mediated extra-myocardial (post release) fragmentation of circulating cMyC occurs; additionally, the renal clearance profile of FL-cMyC is not known.

It would therefore be useful to determine the temporal change of the circulating FL-cM cMyC concentrations after presentation.

Baker, et al immunoblotted circulating cMyC in serially collected serum samples over 24 hours from a patient with STEMI, using low affinity polyclonal antibodies. In this patient the C0C1f fragment dominates at all time points apart from at 3hrs, when cMyC first appears predominantly as full length. From 4 hours onwards, both full length and fragmented cMyC appear to diminish gradually, figure 4.23.[52]

It is unclear whether full length cMyC degrades into shorter fragments or is simply cleared from the circulation.

The dominance of C0C1f supports the hypothesis that it differentiates Type 1 AMI.

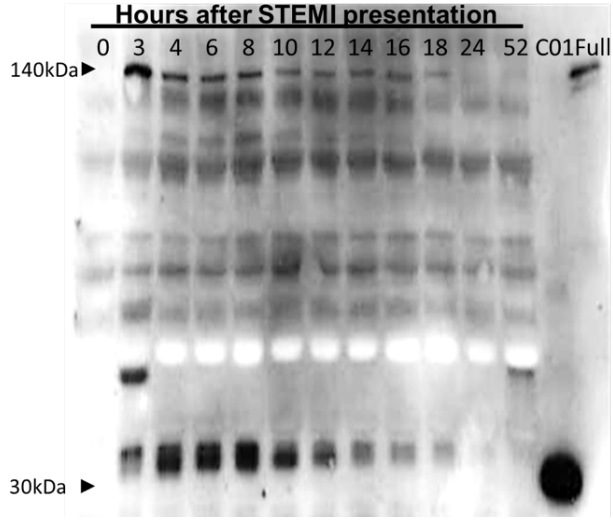


Figure 5.23 cMyC immunoblot of serum collected serially from an individual patient with STEMI.

"C01" and "Full" are recombinant C0C1f and FL-cMyC, respectively (From Baker at al[52])

cMyC is susceptible to a variety of endopeptidases/proteases including dominant serum proteases such as Factor X. Full-length cMyC has been exposed to Factor Xa in-vitro (personal communication with Dr Thomas Kampourakis, BHF Research Fellow and Lecturer in the Randall Centre for Cell & Molecular Biophysics, King's College London)

It was clear Factor Xa is also capable of cleaving cMyC at a site very close to the calpain cleavage site to generate C0C1f. Unlike calpain, Factor Xa seemed relatively agnostic as to whether the M domain is, or is not, phosphorylated. It is, therefore, possible that intact cMyC maybe released early during type 1 AMI and then converted to C0C1f, either in the circulation in vivo or after collection, in vitro, during serum separation. Furthermore, this conversion can occur irrespective of whether the M-domain is phosphorylated or not.

To establish whether cMyC fragmentation occurs *ex vivo* during the processing or assaying of samples, we could spike recombinant full-length cMyC into whole blood

freshly collected from volunteers (with low native cMyC) in different types of collection tubes. These samples can then be decanted at room temperature for 1, 2, 4 and 6 hours before centrifugation for collection of serum and plasma, respectively.

Protease inhibitors can also be added to try to stabilise cMyC and prevent *ex vivo* fragmentation.

Minimising / eliminating *ex vivo* fragmentation would allow us to accurately examine the temporal change in the ratio of [FL-cMyc]:[total cMyC] in serially collected blood samples from patients with different types of myocardial injury.

Fragmentation of cardiac troponin has been studied. Madsen et al reported that in patients with STEMI, intact cTnI and a single degradation product were detectable on immunoblot as early as 90 minutes after onset of symptoms with further degradation after 165 minutes.[170]

Similar findings were observed in the serum of patients with hypertrophic cardiomyopathy undergoing percutaneous septal ablation.[171] However, the fragmentation of cardiac troponin has not been studied in other types of myocardial injury and has not led to clinical utility.

In summary, there are several unanswered questions and limitations that are worth addressing in future research work.

Conclusion:

Phosphorylation and fragmentation pattern of cMyC are dependent on the type of myocardial injury and might aid the differentiation between different types of myocardial injury. Large cohorts with carefully adjudicated participants with different types of myocardial injury are required to further test the hypothesis.

Chapter 6. Summary and Future Directions

The research projects included in this thesis provide further validation of cMyC as a biomarker of acute and chronic myocardial injury and explore its potential role in the vital differentiation between different types of myocardial injury.

The long- and short-term biological variation studies show that cMyC exhibits acceptable weekly RCV, low hourly RCV, and low weekly and hourly Index of Individuality. In combination these properties suggest that when measure serially cMyC could be suitable for the diagnosis, monitoring and risk stratification of chronic and acute myocardial injury. Additionally, the analytical quality specifications based on biological variation are similar to those for cardiac troponin and should be achievable at clinically relevant concentrations.

Testing the RCV in cohorts with acute and chronic cardiac disease and reported/measured outcomes is necessary to verify the ability of cMyC to monitor disease activity and predict outcomes, and also to determine if analytical quality specifications should be based on clinical outcomes or biological variation.

The diurnal variation study revealed a strong circadian rhythmicity for the concentration of cMyC and cTnT with a 5-hour phase difference, cMyC peaking 5 hours earlier than cTnT, a very interesting phenomenon.

It is important to establish whether the circadian rhythm and predictable fluctuation of the biomarker concentration around the homeostatic set point does impair the diagnostic performance of cMyC and cTnT. Limited studies suggested that this isn't the case for cTnT, but it is yet to be established for cMyC. This could be addressed by testing the

diagnostic performance of cMyC in cohorts of patients presenting with suspected NSTEMI, stratified by the onset of chest pain around the acrophase and bathophase or during the ascending or descending phase of the biomarkers concentration as predicted by the circadian pattern.

Additionally, it would be interesting to establish whether the disturbance of the circadian rhythm, if it exists, could be a sign of worsening or decompensation of chronic cardiac conditions, and also whether shift workers have different diurnal variation patterns.

Further, examining the biological variation in specific cohorts that are less "healthy" and at increased risk of myocardial injury would also add to our knowledge about the diagnostic and prognostic value of RCV and the optimal analytical performance specifications in special clinical groups. One important group would be patients with chronic renal dysfunction.

In patients with renal dysfunction, using pre-determined adjudicated cohort-derived diagnostic cut-off thresholds, I studied the diagnostic performance of cMyC-guided ESC 0/1-h triage algorithm for suspected NSTEMI, and demonstrated that it was comparable to that of cardiac troponins, however, while the safety of the algorithm is high, the accuracy and overall efficacy is reduced. It was not possible to optimise accuracy by adjusting the cut-off thresholds without a parallel reduction in the overall efficacy.

The diagnostic cut-off thresholds used in this study were pre-derived from an adjudicated cohort using machine learning and not from the RCVs calculated in this thesis. The latter could be validated in this cohort. Additionally, as mentioned above, RCVs could be derived from patients without acute myocardial injury and then validated in patients with

NSTEMI in this cohort, to establish if the performance of the derived RCV is different to that of the machine-learning derived cut-off thresholds.

There are known unknowns including the release and clearance of the biomarkers in patients with renal dysfunction with and without-myocardial infarction. Relevant studies on troponin have been inconclusive. There is an ongoing collaboration with colleagues at Herlev and Gentofte Hospital, Copenhagen, Denmark to study the effect of haemodialysis on cMyC concentration in patients with end-stage renal failure and the prognostic performance of cMyC and cTns in patients with end-stage renal failure.

Additionally, it is unknown whether the circulating cMyC in the blood is fragmented or intact, which if established, could help differentiate between elevated cMyC in the blood due to acute and chronic cardiac injury.

Another special clinical group was the cohort of human participants who were subjected to brief ischaemia, where changes in the concentration of the large molecule cMyC paralleled changes in the smaller molecule cTnI, indicating similar cardiomyocyte release of protein biomarkers of injury in response to brief ischemia, regardless of molecular size and co-location within the sarcomere.

Biomarker concentration increased to above the 99th percentile in several participants, however, which was observed in fewer patients with cMyC than with cTns.

These findings have potential impact on our understanding of ischaemia and raise the need to revisit the traditional diagnostic criteria of unstable angina using high-sensitive assays.

Proposed release mechanisms in-response to ischemia include exocytosis or ischemia-induced disruption of cell membrane integrity allowing the release of large molecular weight proteins such as intact cMyC without need for exocytosis. However, our study doesn't provide evaluation of whether cMyC is present as a whole protein in the circulation or as N-terminal fragment C0C1f (~35 kDa), which is the form detected in the circulation after MI and detected by our standard immunoassay. Therefore, the exact mechanism of cellular release of these biomarkers after induced ischemia remain speculative. The fragmentation pattern of cMyC after short durations of ischaemia might be different to that after spontaneous myocardial infarction and could therefore aid the differentiation between pathologies. Our collaborators at Herlev and Gentofte Hospital, Copenhagen, Denmark are in the process of repeating the study with larger number of participants and a longer duration of blood sampling. We will re-study the temporal release of cMyC and cTns in the new cohort and examine the fragmentation of cMyC to help understand the release mechanism and its ability to differentiate between ischaemia and infarction.

My studies so far suggested that cMyC is suitable for the diagnosis, monitoring and risk stratification of acute and chronic cardiac injury, performs comparably to cardiac troponins in patients with renal dysfunction and suspected NSTEMI and has similar kinetic profile to that of cardiac troponin in patients with induced ischaemia.

However, the work presented within the thesis also exposes some gaps in our understanding of the release mechanism of cMyC and cTns, and their circulating species in different types of myocardial injury. Additionally, cMyC-guided triage of

patients with suspected NSTEMI exhibited similar specificity to cTn-guided triage in patients with and without renal dysfunction, which is less than ideal

I hypothesised that calpain mediated cleavage of cMyC is dependent on its phosphorylation status and can therefore aid the differentiation between Type 1 AMI and other types of myocardial injury

I tested this hypothesis systematically in-heart and in-circulation. The most challenging task was to create an immunoassay for the selective quantification of full-length (intact) cMyC.

This was successfully achieved by pairing high affinity anti-N-terminal and anti-C-terminal cMyC antibodies, to straddle the cleavage site of cMyC, and create a sensitive FL-cMyC specific electrochemiluminescence sandwich ELISA assay.

Immunoblotting showed reduced phosphorylation and increased fragmentation of cMyC in porcine myocardium subjected to ischaemic, compared to LV stretch-induced, injury. There was a tendency in the latter for an increase in cMyC phosphorylation above the baseline (control) 24 hours after PE infusion.

Immunoassay of cMyC in the porcine serum sampled at 1-h post injury showed an increase in the circulating FL (intact) cMyC in parallel with total (all species) cMyC in the PE model, whereas no detectable FL (intact) cMyC was observed in the MI model (only fragmented cMyC was present in the circulation).

Immunoassay of cMyC in the human serum showed significantly higher ratio of FL (intact) to total (all species) cMyC in the tachyarrhythmia-induced myocardial injury subtype of Type 2 AMI compared to Type 1-AMI.

The results of this research work are very promising and suggest that the phosphorylation and fragmentation patterns of cMyC are dependent on the type of myocardial injury and might aid in the differentiation between different types of myocardial injury. This finding, if validated, might be a breakthrough in the biomarker-guided triage and classification of myocardial injury, and might improve our understanding of the release mechanism of cMyC in different types of injury.

It is not known if the fragmentation of cMyC in the studied models occurred predominantly within or outside the myocardium (in the circulation). My work on the animal model suggests that the fragmentation of cMyC originates, at least in part, from within the myocardium. However, it is still possible that an additional calpain mediated extra-myocardial (post release) fragmentation of circulating cMyC occurs.

We have started a collaboration with Professor Kasper Iverson, Herlev and Gentofte Hospital, Copenhagen, Denmark to establish the half-life and also the temporal fragmentation of cMyC.

It is also important to establish whether in-vitro fragmentation of cMyC occurs during processing or assaying the samples. For this, I propose spiking recombinant full-length cMyC into whole blood freshly collected from volunteers (with low native cMyC) in different types of collection tubes. These samples can then be decanted at room

temperature for 1, 2, 4 and 6 hours before centrifugation for collection of serum and plasma.

The aim is to establish the ideal pre-analytical approach to maximise the utility of the fragmentology of cMyC in the differentiation between types of injury, followed by a prospective observational study to further establish the role of the fragmentology in refining the triage and diagnosis of myocardial injury.

In the meantime, we will proceed with retrospective assessment of the fragmentation pattern of cMyC in readily available external cohorts with different types of myocardial injury, as part of established and ongoing academic collaborations

1- Patients with end-stage renal failure pre- and after dialysis
2- Serially collected blood from patients with STEMI
3- Adjudicated large cohort with different types of myocardial injury

I will continue to lead and contribute to the research work listed above and further related work at postdoctoral level, under the supervision and guidance of Professor Mike Marber, and will continue to pursue academia as an integral part of my future career.

6 REFERENCE LIST

1. Kaier TE, Twerenbold R, Lopez-Ayala P, Nestelberger T, Boeddinghaus J, Alaour B, Huber IM, Zhi Y, Koechlin L, Wussler D, et al. A 0/1h-algorithm using cardiac myosin-binding protein C for early diagnosis of myocardial infarction. *Eur Heart J Acute Cardiovasc Care*. 2022. doi: 10.1093/ehjacc/zuac007

2. Katus HA, Remppis A, Neumann FJ, Scheffold T, Diederich KW, Vinar G, Noe A, Matern G, Kuebler W. Diagnostic efficiency of troponin T measurements in acute myocardial infarction. *Circulation*. 1991;83:902-912. doi: 10.1161/01.cir.83.3.902

3. Katus HA, Remppis A, Scheffold T, Diederich KW, Kuebler W. Intracellular compartmentation of cardiac troponin T and its release kinetics in patients with reperfused and nonreperfused myocardial infarction. *Am J Cardiol*. 1991;67:1360-1367. doi: 10.1016/0002-9149(91)90466-x

4. Westermann D, Neumann JT, Sorensen NA, Blankenberg S. High-sensitivity assays for troponin in patients with cardiac disease. *Nat Rev Cardiol*. 2017;14:472-483. doi: 10.1038/nrcardio.2017.48

5. Apple FS, Jaffe AS, Collinson P, Mockel M, Ordonez-Llanos J, Lindahl B, Hollander J, Plebani M, Than M, Chan MH, et al. IFCC educational materials on selected analytical and clinical applications of high sensitivity cardiac troponin assays. *Clin Biochem*. 2015;48:201-203. doi: 10.1016/j.clinbiochem.2014.08.021

6. Apple FS, Sandoval Y, Jaffe AS, Ordonez-Llanos J, Bio-Markers ITFoCAoC. Cardiac Troponin Assays: Guide to Understanding Analytical Characteristics and Their Impact on Clinical Care. *Clin Chem*. 2017;63:73-81. doi: 10.1373/clinchem.2016.255109

7. Aw TC, Huang WT, Le TT, Pua CJ, Ang B, Phua SK, Yeo KK, Cook SA, Chin CWL. High-Sensitivitycardiac Troponinsin Cardio-Healthy Subjects: A Cardiovascular Magnetic Resonance Imaging Study. *Sci Rep*. 2018;8:15409. doi: 10.1038/s41598-018-33850-9

8. Alaour B, Liew F, Kaier TE. Cardiac Troponin - diagnostic problems and impact on cardiovascular disease. *Ann Med*. 2018;50:655-665. doi: 10.1080/07853890.2018.1530450

9. Lan NSR, Bell DA. Revisiting the Biological Variability of Cardiac Troponin: Implications for Clinical Practice. *Clin Biochem Rev*. 2019;40:201-216. doi: 10.33176/AACB-19-00032

10. Vasile VC, Saenger AK, Kroning JM, Klee GG, Jaffe AS. Biologic variation of a novel cardiac troponin I assay. *Clin Chem*. 2011;57:1080-1081. doi: 10.1373/clinchem.2011.162545

11. Wu AH, Lu QA, Todd J, Moecks J, Wians F. Short- and long-term biological variation in cardiac troponin I measured with a high-sensitivity assay: implications for clinical practice. *Clin Chem*. 2009;55:52-58. doi: 10.1373/clinchem.2008.107391

12. Fraser CG. Reference change values: the way forward in monitoring. *Ann Clin Biochem*. 2009;46:264-265. doi: 10.1258/acb.2009.009006

13. Jaffe AS, Ordonez-Llanos J. High-sensitivity cardiac troponin: from theory to clinical practice. *Rev Esp Cardiol (Engl Ed)*. 2013;66:687-691. doi: 10.1016/j.rec.2013.04.020

14. Reichlin T, Schindler C, Drexler B, Twerenbold R, Reiter M, Zellweger C, Moehring B, Ziller R, Hoeller R, Rubini Gimenez M, et al. One-hour rule-out and rule-in of acute myocardial infarction using high-sensitivity cardiac troponin T. *Arch Intern Med*. 2012;172:1211-1218. doi: 10.1001/archinternmed.2012.3698

15. Collet JP, Thiele H, Barbato E, Barthelemy O, Bauersachs J, Bhatt DL, Dendale P, Dorobantu M, Edvardsen T, Folliguet T, et al. 2020 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation. *Eur Heart J*. 2020. doi: 10.1093/eurheartj/ehaa575

16. Reichlin T, Twerenbold R, Reiter M, Steuer S, Bassetti S, Balmelli C, Winkler K, Kurz S, Stelzig C, Freese M, et al. Introduction of high-sensitivity troponin assays: impact on myocardial infarction incidence and prognosis. *Am J Med*. 2012;125:1205-1213 e1201. doi: 10.1016/j.amjmed.2012.07.015

17. Mueller C. Biomarkers and acute coronary syndromes: an update. *Eur Heart J*. 2014;35:552-556. doi: 10.1093/eurheartj/eht530

18. Shah ASV, Anand A, Strachan FE, Ferry AV, Lee KK, Chapman AR, Sandeman D, Stables CL, Adamson PD, Andrews JPM, et al. High-sensitivity troponin in the evaluation of patients with suspected acute coronary syndrome: a stepped-wedge, cluster-randomised controlled trial. *Lancet*. 2018;392:919-928. doi: 10.1016/S0140-6736(18)31923-8

19. Haaf P, Reichlin T, Twerenbold R, Hoeller R, Rubini Gimenez M, Zellweger C, Moehring B, Fischer C, Meller B, Wildi K, et al. Risk stratification in patients with acute chest pain using three high-sensitivity cardiac troponin assays. *Eur Heart J*. 2014;35:365-375. doi: 10.1093/eurheartj/eht218

20. Mueller C, Giannitsis E, Mockel M, Huber K, Mair J, Plebani M, Thygesen K, Jaffe AS, Lindahl B, Biomarker Study Group of the ESCACCA. Rapid rule out

of acute myocardial infarction: novel biomarker-based strategies. *Eur Heart J Acute Cardiovasc Care*. 2017;6:218-222. doi: 10.1177/2048872616653229

21. Mockel M, Giannitsis E, Mueller C, Huber K, Jaffe AS, Mair J, Plebani M, Thygesen K, Lindahl B, Biomarker Study Group of the European Society of Cardiology Acute Cardiovascular Care A. Editor's Choice-Rule-in of acute myocardial infarction: Focus on troponin. *Eur Heart J Acute Cardiovasc Care*. 2017;6:212-217. doi: 10.1177/2048872616653228

22. Neumann JT, Twerenbold R, Blankenberg S. Application of High-Sensitivity Troponin in Suspected Myocardial Infarction. Reply. *N Engl J Med*. 2019;381:2483. doi: 10.1056/NEJMc1913971

23. Boeddinghaus J, Nestelberger T, Twerenbold R, Neumann JT, Lindahl B, Giannitsis E, Sorensen NA, Badertscher P, Jann JE, Wussler D, et al. Impact of age on the performance of the ESC 0/1h-algorithms for early diagnosis of myocardial infarction. *Eur Heart J*. 2018;39:3780-3794. doi: 10.1093/eurheartj/ehy514

24. Boeddinghaus J, Twerenbold R, Nestelberger T, Badertscher P, Wildi K, Puelacher C, du Fay de Lavallaz J, Keser E, Rubini Gimenez M, Wussler D, et al. Clinical Validation of a Novel High-Sensitivity Cardiac Troponin I Assay for Early Diagnosis of Acute Myocardial Infarction. *Clin Chem*. 2018;64:1347-1360. doi: 10.1373/clinchem.2018.286906

25. Crea F, Jaffe AS, Collinson PO, Hamm CW, Lindahl B, Mills NL, Thygesen K, Mueller C, Patrono C, Roffi M, et al. Should the 1h algorithm for rule in and rule out of acute myocardial infarction be used universally? *Eur Heart J*. 2016;37:3316-3323. doi: 10.1093/eurheartj/ehw282

26. Frankenstein L, Wu AH, Hallermayer K, Wians FH, Jr., Giannitsis E, Katus HA. Biological variation and reference change value of high-sensitivity troponin T in healthy individuals during short and intermediate follow-up periods. *Clin Chem*. 2011;57:1068-1071. doi: 10.1373/clinchem.2010.158964

27. Vorlat A, Van Hoof VO, Hammami R, van Kerckhoven S, Van der Heijden CM, Coenen D, Bosmans JM, Haine S, Vandendriessche TR, Vrints CJ, et al. Usefulness of early rule-in and rule-out biomarker protocols to estimate ischemia-induced myocardial injury in early chest pain presenters. *Am J Cardiol*. 2015;115:1667-1671. doi: 10.1016/j.amjcard.2015.03.012

28. Lan NSR, Nguyen LT, Vasikaran SD, Wilson C, Jonsson J, Rankin JM, Bell DA. Short- and long-term biological variation of cardiac troponin I in healthy individuals, and patients with end-stage renal failure requiring haemodialysis or cardiomyopathy. *Clin Chem Lab Med*. 2020;58:1941-1949. doi: 10.1515/cclm-2020-0046

29. Pickering JW, Greenslade JH, Cullen L, Flaws D, Parsonage W, Aldous S, George P, Worster A, Kavsak PA, Than MP. Assessment of the European Society of

Cardiology 0-Hour/1-Hour Algorithm to Rule-Out and Rule-In Acute Myocardial Infarction. *Circulation*. 2016;134:1532-1541. doi: 10.1161/CIRCULATIONAHA.116.022677

30. Collinson P. Troponin, delta change and the evolution of cardiac biomarkers - back to the future (again). *Ann Clin Biochem*. 2018;55:626-629. doi: 10.1177/0004563218774809

31. Aakre KM, Roraas T, Petersen PH, Svarstad E, Sellevoll H, Skadberg O, Saele K, Sandberg S. Weekly and 90-minute biological variations in cardiac troponin T and cardiac troponin I in hemodialysis patients and healthy controls. *Clin Chem*. 2014;60:838-847. doi: 10.1373/clinchem.2013.216978

32. Boeddinghaus J, Nestelberger T, Koechlin L, Wussler D, Lopez-Ayala P, Walter JE, Troester V, Ratmann PD, Seidel F, Zimmermann T, et al. Early Diagnosis of Myocardial Infarction With Point-of-Care High-Sensitivity Cardiac Troponin I. *J Am Coll Cardiol*. 2020;75:1111-1124. doi: 10.1016/j.jacc.2019.12.065

33. Boeddinghaus J, Nestelberger T, Twerenbold R, Wildi K, Badertscher P, Cupa J, Burge T, Machler P, Corbiere S, Grimm K, et al. Direct Comparison of 4 Very Early Rule-Out Strategies for Acute Myocardial Infarction Using High-Sensitivity Cardiac Troponin I. *Circulation*. 2017;135:1597-1611. doi: 10.1161/CIRCULATIONAHA.116.025661

34. Andruchow JE, Kavsak PA, McRae AD. Contemporary Emergency Department Management of Patients with Chest Pain: A Concise Review and Guide for the High-Sensitivity Troponin Era. *Can J Cardiol*. 2018;34:98-108. doi: 10.1016/j.cjca.2017.11.012

35. Jaffe AS, Moeckel M, Giannitsis E, Huber K, Mair J, Mueller C, Plebani M, Thygesen K, Lindahl B. In search for the Holy Grail: suggestions for studies to define delta changes to diagnose or exclude acute myocardial infarction: a position paper from the study group on biomarkers of the Acute Cardiovascular Care Association. *Eur Heart J Acute Cardiovasc Care*. 2014;3:313-316. doi: 10.1177/2048872614541906

36. NICE. High-sensitivity troponin tests for the early rule out of NSTEMI. 2020.

37. Jaffe AS, White H. Ruling-In Myocardial Injury and Ruling-Out Myocardial Infarction With the European Society of Cardiology 1-Hour Algorithm. *Circulation*. 2016;134:1542-1545. doi: 10.1161/CIRCULATIONAHA.116.024687

38. Thygesen K, Alpert JS, White HD, Joint ESCAAHAWHFTFftRoMI. Universal definition of myocardial infarction. *Eur Heart J*. 2007;28:2525-2538. doi: 10.1093/eurheartj/ehm355

39. Thygesen K, Alpert JS, Jaffe AS, Chaitman BR, Bax JJ, Morrow DA, White HD, Executive Group on behalf of the Joint European Society of Cardiology

/American College of Cardiology /American Heart Association /World Heart Federation Task Force for the Universal Definition of Myocardial I. Fourth Universal Definition of Myocardial Infarction (2018). *Circulation*. 2018;138:e618-e651. doi: 10.1161/CIR.0000000000000617

40. Kaier TE, Alaour B, Marber M. Cardiac troponin and defining myocardial infarction. *Cardiovasc Res*. 2021. doi: 10.1093/cvr/cvaa331

41. Sandoval Y, Thordsen SE, Smith SW, Schulz KM, Murakami MM, Pearce LA, Apple FS. Cardiac troponin changes to distinguish type 1 and type 2 myocardial infarction and 180-day mortality risk. *Eur Heart J Acute Cardiovasc Care*. 2014;3:317-325. doi: 10.1177/2048872614538411

42. Chapman AR, Adamson PD, Mills NL. Assessment and classification of patients with myocardial injury and infarction in clinical practice. *Heart*. 2017;103:10-18. doi: 10.1136/heartjnl-2016-309530

43. Szymanski FM, Karpinski G, Platek AE, Majstrak F, Hrynkiewicz-Szymanska A, Kotkowski M, Puchalski B, Filipiak KJ, Opolski G. Clinical characteristics, aetiology and occurrence of type 2 acute myocardial infarction. *Kardiologia polska*. 2014;72:339-344. doi: 10.5603/KP.a2013.0284

44. Hazim E-H, Elijah R, Katrina S, Gretchen LW. Prognostic implications of type 2 myocardial infarctions. *World Journal of Cardiovascular Diseases*. 2012;Vol.02No.04:5. doi: 10.4236/wjcd.2012.24039

45. Smith SW, Diercks DB, Nagurney JT, Hollander JE, Miller CD, Schrock JW, Singer AJ, Apple FS, McCullough PA, Ruff CT, et al. Central versus local adjudication of myocardial infarction in a cardiac biomarker trial. *American heart journal*. 2013;165:273-279 e271. doi: 10.1016/j.ahj.2012.12.012

46. Stone GW, Sabik JF, Serruys PW, Simonton CA, Genereux P, Puskas J, Kandzari DE, Morice MC, Lembo N, Brown WM, 3rd, et al. Everolimus-Eluting Stents or Bypass Surgery for Left Main Coronary Artery Disease. *N Engl J Med*. 2016;375:2223-2235. doi: 10.1056/NEJMoa1610227

47. Cutlip DE. Procedural Myocardial Infarction: Definitions Everywhere, But Not Any That May Fit. *J Am Coll Cardiol*. 2020;76:1640-1643. doi: 10.1016/j.jacc.2020.08.024

48. Hara H, Serruys PW, Takahashi K, Kawashima H, Ono M, Gao C, Wang R, Mohr FW, Holmes DR, Davierwala PM, et al. Impact of Peri-Procedural Myocardial Infarction on Outcomes After Revascularization. *J Am Coll Cardiol*. 2020;76:1622-1639. doi: 10.1016/j.jacc.2020.08.009

49. Gregson J, Stone GW, Ben-Yehuda O, Redfors B, Kandzari DE, Morice MC, Leon MB, Kosmidou I, Lembo NJ, Brown WM, 3rd, et al. Implications of Alternative Definitions of Peri-Procedural Myocardial Infarction After Coronary

Revascularization. *J Am Coll Cardiol*. 2020;76:1609-1621. doi: 10.1016/j.jacc.2020.08.016

50. Serruys PW, Morice MC, Kappetein AP, Colombo A, Holmes DR, Mack MJ, Stahle E, Feldman TE, van den Brand M, Bass EJ, et al. Percutaneous coronary intervention versus coronary-artery bypass grafting for severe coronary artery disease. *N Engl J Med*. 2009;360:961-972. doi: 10.1056/NEJMoa0804626

51. Valentine CM, Tcheng JE, Waites T. Translating the Translation: What Clinicians Should Know About the Fourth Universal Definition of Myocardial Infarction. *J Am Coll Cardiol*. 2018;72:2668-2670. doi: 10.1016/j.jacc.2018.10.015

52. Baker JO, Tyther R, Liebetrau C, Clark J, Howarth R, Patterson T, Mollmann H, Nef H, Sicard P, Kailey B, et al. Cardiac myosin-binding protein C: a potential early biomarker of myocardial injury. *Basic Res Cardiol*. 2015;110:23. doi: 10.1007/s00395-015-0478-5

53. Marjot J, Liebetrau C, Goodson RJ, Kaier T, Weber E, Heseltine P, Marber MS. The development and application of a high-sensitivity immunoassay for cardiac myosin-binding protein C. *Transl Res*. 2016;170:17-25 e15. doi: 10.1016/j.trsl.2015.11.008

54. Marjot J, Kaier TE, Martin ED, Reji SS, Copeland O, Iqbal M, Goodson B, Hamren S, Harding SE, Marber MS. Quantifying the Release of Biomarkers of Myocardial Necrosis from Cardiac Myocytes and Intact Myocardium. *Clin Chem*. 2017;63:990-996. doi: 10.1373/clinchem.2016.264648

55. Kaier TE, Anand A, Shah AS, Mills NL, Marber M. Temporal Relationship between Cardiac Myosin-Binding Protein C and Cardiac Troponin I in Type 1 Myocardial Infarction. *Clin Chem*. 2016;62:1153-1155. doi: 10.1373/clinchem.2016.257188

56. Schulte C, Barwari T, Joshi A, Theofilatos K, Zampetaki A, Barallobre-Barreiro J, Singh B, Sorensen NA, Neumann JT, Zeller T, et al. Comparative Analysis of Circulating Noncoding RNAs Versus Protein Biomarkers in the Detection of Myocardial Injury. *Circ Res*. 2019;125:328-340. doi: 10.1161/CIRCRESAHA.119.314937

57. Kaier TE, Twerenbold R, Puelacher C, Marjot J, Imambaccus N, Boeddinghaus J, Nestelberger T, Badertscher P, Sabti Z, Gimenez MR, et al. Direct Comparison of Cardiac Myosin-Binding Protein C With Cardiac Troponins for the Early Diagnosis of Acute Myocardial Infarction. *Circulation*. 2017;136:1495-1508. doi: 10.1161/CIRCULATIONAHA.117.028084

58. Kaier TE, Stengaard C, Marjot J, Sorensen JT, Alaour B, Stavropoulou-Tatla S, Terkelsen CJ, Williams L, Thygesen K, Weber E, et al. Cardiac Myosin-Binding Protein C to Diagnose Acute Myocardial Infarction in the Pre-Hospital Setting. *J Am Heart Assoc*. 2019;8:e013152. doi: 10.1161/JAHA.119.013152

59. Kaier TE, Twerenbold R, Alaour B, Badertscher P, Puelacher C, Marjot J, Boeddinghaus J, Nestelberger T, Wildi K, Wussler D, et al. 1086Derivation and validation of a 0/1h-algorithm to diagnose myocardial infarction using cardiac myosin-binding protein c - direct comparison to hs-cTnI. *European Heart Journal*. 2018;39. doi: 10.1093/eurheartj/ehy565.1086

60. Dryer RL. Chemical Diagnosis of Disease. S. Brown, F. L. Mitchell, and D. S. Young, Eds. Elsevier/North Holland Biomedical Press, Amsterdam and New York. 1979. *Clinical Chemistry*. 1980;26:1639-1640.

61. Fraser CG, Harris EK. Generation and application of data on biological variation in clinical chemistry. *Crit Rev Clin Lab Sci*. 1989;27:409-437. doi: 10.3109/10408368909106595

62. Sandberg S RT, Asrsand A, Fraser C. Biological Variation. In: Rifai N HA, Wittwer C ed. *Tietz Fundamentals of Clinical Chemistry and Molecular Diagnostics*. 2019:51-64.

63. Burtis CA, Bruns DE, Sawyer BG, Tietz NW. *Tietz fundamentals of clinical chemistry and molecular diagnostics*. Seventh edition. ed. St. Louis, Missouri: Elsevier/Saunders; 2015.

64. Fraser CG, Petersen PH, Libeer J-C, Ricos C. Proposals for Setting Generally Applicable Quality Goals Solely Based on Biology. *Annals of Clinical Biochemistry*. 1997;34:8-12. doi: 10.1177/000456329703400103

65. Jones GRD, Albarede S, Kesseler D, MacKenzie F, Mammen J, Pedersen M, Stavelin A, Thelen M, Thomas A, Twomey PJ, et al. Analytical performance specifications for external quality assessment - definitions and descriptions. *Clin Chem Lab Med*. 2017;55:949-955. doi: 10.1515/cclm-2017-0151

66. Harris EK, Yasaka T. On the calculation of a "reference change" for comparing two consecutive measurements. *Clin Chem*. 1983;29:25-30.

67. Harris EK. Effects of intra- and interindividual variation on the appropriate use of normal ranges. *Clin Chem*. 1974;20:1535-1542.

68. Lacher DA, Hughes JP, Carroll MD. Estimate of biological variation of laboratory analytes based on the third national health and nutrition examination survey. *Clin Chem*. 2005;51:450-452. doi: 10.1373/clinchem.2004.039354

69. Fraser CG. Reference change values. *Clin Chem Lab Med*. 2011;50:807-812. doi: 10.1515/CCLM.2011.733

70. Sikaris K. Application of the stockholm hierarchy to defining the quality of reference intervals and clinical decision limits. *Clin Biochem Rev*. 2012;33:141-148.

71. Ritchie RF, Palomaki G. Selecting clinically relevant populations for reference intervals. *Clin Chem Lab Med*. 2004;42:702-709. doi: 10.1515/CCLM.2004.120

72. Ricos C, Iglesias N, Garcia-Lario JV, Simon M, Cava F, Hernandez A, Perich C, Minchinela J, Alvarez V, Domenech MV, et al. Within-subject biological variation in disease: collated data and clinical consequences. *Ann Clin Biochem*. 2007;44:343-352. doi: 10.1258/000456307780945633

73. Badrick T. Biological variation: Understanding why it is so important? *Pract Lab Med*. 2021;23:e00199. doi: 10.1016/j.plabm.2020.e00199

74. Burnett RW. Accurate estimation of standard deviations for quantitative methods used in clinical chemistry. *Clin Chem*. 1975;21:1935-1938.

75. Reed AH, Henry RJ, Mason WB. Influence of statistical method used on the resulting estimate of normal range. *Clin Chem*. 1971;17:275-284.

76. Fokkema MR, Herrmann Z, Muskiet FA, Moecks J. Reference change values for brain natriuretic peptides revisited. *Clin Chem*. 2006;52:1602-1603. doi: 10.1373/clinchem.2006.069369

77. Carlsen S, Petersen PH, Skeie S, Skadberg O, Sandberg S. Within-subject biological variation of glucose and HbA(1c) in healthy persons and in type 1 diabetes patients. *Clin Chem Lab Med*. 2011;49:1501-1507. doi: 10.1515/CCLM.2011.233

78. Horn PS, Feng L, Li Y, Pesce AJ. Effect of outliers and nonhealthy individuals on reference interval estimation. *Clin Chem*. 2001;47:2137-2145.

79. Scharnhorst V, Krasznai K, van 't Veer M, Michels RH. Variation of cardiac troponin I and T measured with sensitive assays in emergency department patients with noncardiac chest pain. *Clin Chem*. 2012;58:1208-1214. doi: 10.1373/clinchem.2011.179903

80. Mueller M, Biener M, Vafaie M, Doerr S, Keller T, Blankenberg S, Katus HA, Giannitsis E. Absolute and relative kinetic changes of high-sensitivity cardiac troponin T in acute coronary syndrome and in patients with increased troponin in the absence of acute coronary syndrome. *Clin Chem*. 2012;58:209-218. doi: 10.1373/clinchem.2011.171827

81. Fraser CG, Hyltoft Petersen P, Libeer JC, Ricos C. Proposals for setting generally applicable quality goals solely based on biology. *Ann Clin Biochem*. 1997;34 (Pt 1):8-12. doi: 10.1177/000456329703400103

82. Tonks DB. A study of the accuracy and precision of clinical chemistry determinations in 170 Canadian laboratories. *Clin Chem*. 1963;9:217-233.

83. Cotlove E, Harris EK, Williams GZ. Biological and analytic components of variation in long-term studies of serum constituents in normal subjects. 3. Physiological and medical implications. *Clin Chem*. 1970;16:1028-1032.

84. Stockl D, Baadenhuijsen H, Fraser CG, Libeer JC, Petersen PH, Ricos C. Desirable routine analytical goals for quantities assayed in serum. Discussion

paper from the members of the external quality assessment (EQA) Working Group A on analytical goals in laboratory medicine. *Eur J Clin Chem Clin Biochem*. 1995;33:157-169.

85. Fraser CG, Petersen PH. Desirable performance standards for imprecision and bias in alternate sites. The views of laboratory professionals. *Arch Pathol Lab Med*. 1995;119:909-913.

86. Gowans EM, Hyltoft Petersen P, Blaabjerg O, Horder M. Analytical goals for the acceptance of common reference intervals for laboratories throughout a geographical area. *Scand J Clin Lab Invest*. 1988;48:757-764. doi: 10.3109/00365518809088757

87. Biswas SS, Bindra M, Jain V, Gokhale P. Evaluation of imprecision, bias and total error of clinical chemistry analysers. *Indian J Clin Biochem*. 2015;30:104-108. doi: 10.1007/s12291-014-0448-y

88. Oosterhuis WP, Theodorsson E. Total error vs. measurement uncertainty: revolution or evolution? *Clinical Chemistry and Laboratory Medicine (CCLM)*. 2016;54:235-239. doi: doi:10.1515/cclm-2015-0997

89. D. Kenny CGFPHPAK. Consensus agreement. *Scandinavian Journal of Clinical and Laboratory Investigation*. 1999;59:585-585. doi: 10.1080/00365519950185409

90. Sandberg S, Fraser CG, Horvath AR, Jansen R, Jones G, Oosterhuis W, Petersen PH, Schimmel H, Sikaris K, Panteghini M. Defining analytical performance specifications: Consensus Statement from the 1st Strategic Conference of the European Federation of Clinical Chemistry and Laboratory Medicine. *Clin Chem Lab Med*. 2015;53:833-835. doi: 10.1515/cclm-2015-0067

91. Jones GR, Sikaris K, Gill J. 'Allowable Limits of Performance' for External Quality Assurance Programs - an Approach to Application of the Stockholm Criteria by the RCPA Quality Assurance Programs. *Clin Biochem Rev*. 2012;33:133-139.

92. Horvath AR, Bossuyt PM, Sandberg S, John AS, Monaghan PJ, Verhagen-Kamerbeek WD, Lennartz L, Cobbaert CM, Ebert C, Lord SJ, et al. Setting analytical performance specifications based on outcome studies - is it possible? *Clin Chem Lab Med*. 2015;53:841-848. doi: 10.1515/cclm-2015-0214

93. Roraas T, Stove B, Petersen PH, Sandberg S. Biological Variation: The Effect of Different Distributions on Estimated Within-Person Variation and Reference Change Values. *Clin Chem*. 2016;62:725-736. doi: 10.1373/clinchem.2015.252296

94. Ceriotti F, Diaz-Garzon Marco J, Fernandez-Calle P, Maregnani A, Aarsand AK, Coskun A, Jonker N, Sandberg S, Carobene A, European Federation of Clinical C, et al. The European Biological Variation Study (EuBIVAS): weekly biological

variation of cardiac troponin I estimated by the use of two different high-sensitivity cardiac troponin I assays. *Clin Chem Lab Med*. 2020;58:1741-1747. doi: 10.1515/cclm-2019-1182

95. Schindler EI, Szymanski JJ, Hock KG, Geltman EM, Scott MG. Short- and Long-term Biologic Variability of Galectin-3 and Other Cardiac Biomarkers in Patients with Stable Heart Failure and Healthy Adults. *Clin Chem*. 2016;62:360-366. doi: 10.1373/clinchem.2015.246553

96. Wu AH, Shea E, Lu QT, Minyard J, Bui K, Hsu JC, Agee SJ, Todd J. Short- and long-term cardiac troponin I analyte stability in plasma and serum from healthy volunteers by use of an ultrasensitive, single-molecule counting assay. *Clin Chem*. 2009;55:2057-2059. doi: 10.1373/clinchem.2009.128611

97. Meijers WC, van der Velde AR, Muller Kobold AC, Dijck-Brouwer J, Wu AH, Jaffe A, de Boer RA. Variability of biomarkers in patients with chronic heart failure and healthy controls. *Eur J Heart Fail*. 2017;19:357-365. doi: 10.1002/ejhf.669

98. Corte Z, Garcia C, Venta R. Biological variation of cardiac troponin T in patients with end-stage renal disease and in healthy individuals. *Ann Clin Biochem*. 2015;52:53-60. doi: 10.1177/0004563214545116

99. Fraser CG, Petersen PH, Ricos C, Haeckel R. Proposed quality specifications for the imprecision and inaccuracy of analytical systems for clinical chemistry. *Eur J Clin Chem Clin Biochem*. 1992;30:311-317.

100. Alaour B, Omland T, Torsvik J, Kaier TE, Sylte MS, Strand H, Quraishi J, McGrath S, Williams L, Meex S, et al. Biological variation of cardiac myosin-binding protein C in healthy individuals. *Clin Chem Lab Med*. 2021. doi: 10.1515/cclm-2021-0306

101. Klinkenberg LJ, van Dijk JW, Tan FE, van Loon LJ, van Dieijen-Visser MP, Meex SJ. Circulating cardiac troponin T exhibits a diurnal rhythm. *J Am Coll Cardiol*. 2014;63:1788-1795. doi: 10.1016/j.jacc.2014.01.040

102. van der Linden N, Hilderink JM, Cornelis T, Kimenai DM, Klinkenberg LJJ, van Doorn WP, Litjens EJR, van Suijlen JDE, van Loon LJC, Bekers O, et al. Twenty-Four-Hour Biological Variation Profiles of Cardiac Troponin I in Individuals with or without Chronic Kidney Disease. *Clin Chem*. 2017;63:1655-1656. doi: 10.1373/clinchem.2017.275107

103. Klinkenberg LJ, Wildi K, van der Linden N, Kouw IW, Niens M, Twerenbold R, Rubini Gimenez M, Puelacher C, Daniel Neuhaus J, Hillinger P, et al. Diurnal Rhythm of Cardiac Troponin: Consequences for the Diagnosis of Acute Myocardial Infarction. *Clin Chem*. 2016;62:1602-1611. doi: 10.1373/clinchem.2016.257485

104. Zaninotto M, Padoan A, Mion MM, Marinova M, Plebani M. Short-term biological variation and diurnal rhythm of cardiac troponin I (Access hs-TnI) in healthy subjects. *Clin Chim Acta*. 2020;504:163-167. doi: 10.1016/j.cca.2020.02.004

105. Vasile VC, Saenger AK, Kroning JM, Klee GG, Jaffe AS. Biologic Variation of a Novel Cardiac Troponin I Assay. *Clinical Chemistry*. 2011;57:1080-1081. doi: 10.1373/clinchem.2011.162545

106. Vasile VC, Saenger AK, Kroning JM, Jaffe AS. Biological and analytical variability of a novel high-sensitivity cardiac troponin T assay. *Clin Chem*. 2010;56:1086-1090. doi: 10.1373/clinchem.2009.140616

107. Koopman MG, Koomen GC, Krediet RT, de Moor EA, Hoek FJ, Arisz L. Circadian rhythm of glomerular filtration rate in normal individuals. *Clin Sci (Lond)*. 1989;77:105-111. doi: 10.1042/cs0770105

108. Cohen MC, Rohtla KM, Lavery CE, Muller JE, Mittleman MA. Meta-analysis of the morning excess of acute myocardial infarction and sudden cardiac death. *Am J Cardiol*. 1997;79:1512-1516. doi: 10.1016/s0002-9149(97)00181-1

109. Wildi K, Singeisen H, Twerenbold R, Badertscher P, Wussler D, Klinkenberg LJJ, Meex SJR, Nestelberger T, Boeddinghaus J, Miro O, et al. Circadian rhythm of cardiac troponin I and its clinical impact on the diagnostic accuracy for acute myocardial infarction. *Int J Cardiol*. 2018;270:14-20. doi: 10.1016/j.ijcard.2018.05.136

110. Reiter R, Swingen C, Moore L, Henry TD, Traverse JH. Circadian dependence of infarct size and left ventricular function after ST elevation myocardial infarction. *Circ Res*. 2012;110:105-110. doi: 10.1161/CIRCRESAHA.111.254284

111. Fournier S, Taffe P, Radovanovic D, Von Elm E, Morawiec B, Stauffer JC, Erne P, Beggah A, Monney P, Pascale P, et al. Myocardial infarct size and mortality depend on the time of day-a large multicenter study. *PLoS One*. 2015;10:e0119157. doi: 10.1371/journal.pone.0119157

112. Fournier S, Eeckhout E, Mangiacapra F, Trana C, Lauriers N, Beggah AT, Monney P, Cook S, Bardy D, Vogt P, et al. Circadian variations of ischemic burden among patients with myocardial infarction undergoing primary percutaneous coronary intervention. *Am Heart J*. 2012;163:208-213. doi: 10.1016/j.ahj.2011.11.006

113. Hilderink JM, Klinkenberg LJJ, Aakre KM, de Wit NCJ, Henskens YMC, van der Linden N, Bekers O, Rennenberg R, Koopmans RP, Meex SJR. Within-day biological variation and hour-to-hour reference change values for hematological parameters. *Clin Chem Lab Med*. 2017;55:1013-1024. doi: 10.1515/cclm-2016-0716

114. Reichlin T, Hochholzer W, Bassetti S, Steuer S, Stelzig C, Hartwiger S, Biedert S, Schaub N, Buerge C, Potocki M, et al. Early diagnosis of myocardial infarction with sensitive cardiac troponin assays. *N Engl J Med*. 2009;361:858-867. doi: 10.1056/NEJMoa0900428

115. Thygesen K, Mair J, Mueller C, Huber K, Weber M, Plebani M, Hasin Y, Biasucci LM, Giannitsis E, Lindahl B, et al. Recommendations for the use of natriuretic peptides in acute cardiac care: a position statement from the Study Group on Biomarkers in Cardiology of the ESC Working Group on Acute Cardiac Care. *Eur Heart J*. 2012;33:2001-2006. doi: 10.1093/eurheartj/ehq509

116. Group NW, Wu AH, Jaffe AS, Apple FS, Jesse RL, Francis GL, Morrow DA, Newby LK, Ravkilde J, Tang WH, et al. National Academy of Clinical Biochemistry laboratory medicine practice guidelines: use of cardiac troponin and B-type natriuretic peptide or N-terminal proB-type natriuretic peptide for etiologies other than acute coronary syndromes and heart failure. *Clin Chem*. 2007;53:2086-2096. doi: 10.1373/clinchem.2007.095679

117. Collet J-P, Thiele H, Barbato E, Barthélémy O, Bauersachs J, Bhatt DL, Dendale P, Dorobantu M, Edvardsen T, Folliguet T, et al. 2020 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: The Task Force for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC). *European Heart Journal*. 2020. doi: 10.1093/eurheartj/ehaa575

118. Morrow DA, Cannon CP, Jesse RL, Newby LK, Ravkilde J, Storrow AB, Wu AH, Christenson RH, National Academy of Clinical B. National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines: Clinical characteristics and utilization of biochemical markers in acute coronary syndromes. *Circulation*. 2007;115:e356-375. doi: 10.1161/CIRCULATIONAHA.107.182882

119. Wu AH, Jaffe AS. The clinical need for high-sensitivity cardiac troponin assays for acute coronary syndromes and the role for serial testing. *Am Heart J*. 2008;155:208-214. doi: 10.1016/j.ahj.2007.10.016

120. Eggers KM, Jaffe AS, Venge P, Lindahl B. Clinical implications of the change of cardiac troponin I levels in patients with acute chest pain - an evaluation with respect to the Universal Definition of Myocardial Infarction. *Clin Chim Acta*. 2011;412:91-97. doi: 10.1016/j.cca.2010.09.020

121. Apple FS, Pearce LA, Smith SW, Kaczmarek JM, Murakami MM. Role of monitoring changes in sensitive cardiac troponin I assay results for early diagnosis of myocardial infarction and prediction of risk of adverse events. *Clin Chem*. 2009;55:930-937. doi: 10.1373/clinchem.2008.114728

122. Reichlin T, Irfan A, Twerenbold R, Reiter M, Hochholzer W, Burkhalter H, Bassetti S, Steuer S, Winkler K, Peter F, et al. Utility of absolute and relative changes in cardiac troponin concentrations in the early diagnosis of acute

myocardial infarction. *Circulation*. 2011;124:136-145. doi: 10.1161/CIRCULATIONAHA.111.023937

123. Giannitsis E, Becker M, Kurz K, Hess G, Zdunek D, Katus HA. High-sensitivity cardiac troponin T for early prediction of evolving non-ST-segment elevation myocardial infarction in patients with suspected acute coronary syndrome and negative troponin results on admission. *Clin Chem*. 2010;56:642-650. doi: 10.1373/clinchem.2009.134460

124. Kavsak PA, Ko DT, Wang X, Macrae AR, Jaffe AS. 2007 universal myocardial infarction definition change criteria for risk stratification by use of a high-sensitivity cardiac troponin I assay. *Clin Chem*. 2010;56:487-489. doi: 10.1373/clinchem.2009.136689

125. Apple FS, Smith SW, Pearce LA, Murakami MM. Delta changes for optimizing clinical specificity and 60-day risk of adverse events in patients presenting with symptoms suggestive of acute coronary syndrome utilizing the ADVIA Centaur TnI-Ultra assay. *Clin Biochem*. 2012;45:711-713. doi: 10.1016/j.clinbiochem.2012.02.030

126. Storrow AB, Nowak RM, Diercks DB, Singer AJ, Wu AH, Kulstad E, LoVecchio F, Fromm C, Headden G, Potis T, et al. Absolute and relative changes (delta) in troponin I for early diagnosis of myocardial infarction: Results of a prospective multicenter trial. *Clin Biochem*. 2015;48:260-267. doi: 10.1016/j.clinbiochem.2014.09.012

127. Almashali M, McDowell G, Morris N, Body R, group BEoSTs. P821Absolute and relative changes (delta) cardiac troponin I using a contemporary point of care assay for early diagnosis of myocardial infarction. *European Heart Journal*. 2018;39. doi: 10.1093/eurheartj/ehy564.P821

128. Simpson P, Tirimacco R, Cowley P, Siew M, Berry N, Tate J, Tideman P. A comparison of cardiac troponin T delta change methods and the importance of the clinical context in the assessment of acute coronary syndrome. *Ann Clin Biochem*. 2019;56:701-707. doi: 10.1177/0004563219876671

129. Lippi G, Cervellin G, Sanchis-Gomar F. Critical appraisal to using relative or absolute cardiac troponins change for diagnosing acute myocardial infarction. *Journal of Laboratory and Precision Medicine*. 2018;3.

130. Biener M, Giannitsis E, Lamerz J, Mueller-Hennessen M, Vafaie M, Katus HA. Prognostic value of elevated high-sensitivity cardiac troponin T levels in a low risk outpatient population with cardiovascular disease. *Eur Heart J Acute Cardiovasc Care*. 2016;5:409-418. doi: 10.1177/2048872615610869

131. Szummer K, Lundman P, Jacobson SH, Schon S, Lindback J, Stenestrand U, Wallentin L, Jernberg T, Swedeheart. Relation between renal function, presentation, use of therapies and in-hospital complications in acute coronary

syndrome: data from the SWEDEHEART register. *J Intern Med*. 2010;268:40-49. doi: 10.1111/j.1365-2796.2009.02204.x

132. Noeller TP, Meldon SW, Peacock WF, Emerman CL, McErlean ES, Vanlente F, Nissen SE. Troponin T in elders with suspected acute coronary syndromes. *Am J Emerg Med*. 2003;21:293-297. doi: 10.1016/s0735-6757(03)00081-0

133. Nordenskjold AM, Ahlstrom H, Eggers KM, Frobert O, Jaffe AS, Venge P, Lindahl B. Short- and long-term individual variation in cardiac troponin in patients with stable coronary artery disease. *Clin Chem*. 2013;59:401-409. doi: 10.1373/clinchem.2012.191700

134. Roos A, Bandstein N, Lundback M, Hammarsten O, Ljung R, Holzmann MJ. Stable High-Sensitivity Cardiac Troponin T Levels and Outcomes in Patients With Chest Pain. *J Am Coll Cardiol*. 2017;70:2226-2236. doi: 10.1016/j.jacc.2017.08.064

135. Apple FS, Murakami MM, Pearce LA, Herzog CA. Predictive value of cardiac troponin I and T for subsequent death in end-stage renal disease. *Circulation*. 2002;106:2941-2945. doi: 10.1161/01.cir.0000041254.30637.34

136. Kozinski M, Krintus M, Kubica J, Sypniewska G. High-sensitivity cardiac troponin assays: From improved analytical performance to enhanced risk stratification. *Crit Rev Clin Lab Sci*. 2017;54:143-172. doi: 10.1080/10408363.2017.1285268

137. Hammarsten O, Mair J, Mockel M, Lindahl B, Jaffe AS. Possible mechanisms behind cardiac troponin elevations. *Biomarkers*. 2018;23:725-734. doi: 10.1080/1354750X.2018.1490969

138. Twerenbold R, Badertscher P, Boeddinghaus J, Nestelberger T, Wildi K, Puelacher C, Sabti Z, Rubini Gimenez M, Tschirky S, du Fay de Lavallaz J, et al. 0/1-Hour Triage Algorithm for Myocardial Infarction in Patients With Renal Dysfunction. *Circulation*. 2018;137:436-451. doi: 10.1161/CIRCULATIONAHA.117.028901

139. Liu M, Li XC, Lu L, Cao Y, Sun RR, Chen S, Zhang PY. Cardiovascular disease and its relationship with chronic kidney disease. *Eur Rev Med Pharmacol Sci*. 2014;18:2918-2926.

140. Anavekar NS, McMurray JJ, Velazquez EJ, Solomon SD, Kober L, Rouleau JL, White HD, Nordlander R, Maggioni A, Dickstein K, et al. Relation between renal dysfunction and cardiovascular outcomes after myocardial infarction. *N Engl J Med*. 2004;351:1285-1295. doi: 10.1056/NEJMoa041365

141. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med*. 2004;351:1296-1305. doi: 10.1056/NEJMoa041031

142. Roffi M, Patrono C, Collet JP, Mueller C, Valgimigli M, Andreotti F, Bax JJ, Borger MA, Brotons C, Chew DP, et al. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC). *Eur Heart J*. 2016;37:267-315. doi: 10.1093/eurheartj/ehv320

143. Kaier T. Derivation and validation of a 0/1h-algorithm to diagnose myocardial infarction using cardiac myosin-binding protein c - direct comparison to hs-cTnI. 2018.

144. Twerenbold R, Wildi K, Jaeger C, Gimenez MR, Reiter M, Reichlin T, Walukiewicz A, Gugala M, Krivoshei L, Marti N, et al. Optimal Cutoff Levels of More Sensitive Cardiac Troponin Assays for the Early Diagnosis of Myocardial Infarction in Patients With Renal Dysfunction. *Circulation*. 2015;131:2041-2050. doi: 10.1161/CIRCULATIONAHA.114.014245

145. Arnadottir A, Pedersen S, Bo Hasselbalch R, Goetze JP, Friis-Hansen LJ, Bloch-Munster AM, Skov Jensen J, Bundgaard H, Iversen K. Temporal Release of High-Sensitivity Cardiac Troponin T and I and Copeptin After Brief Induced Coronary Artery Balloon Occlusion in Humans. *Circulation*. 2021;143:1095-1104. doi: 10.1161/CIRCULATIONAHA.120.046574

146. Weil BR, Young RF, Shen X, Suzuki G, Qu J, Malhotra S, Canty JM, Jr. Brief Myocardial Ischemia Produces Cardiac Troponin I Release and Focal Myocyte Apoptosis in the Absence of Pathological Infarction in Swine. *JACC Basic Transl Sci*. 2017;2:105-114. doi: 10.1016/j.jacbts.2017.01.006

147. Jaffe AS. Another Unanswerable Question. *JACC Basic Transl Sci*. 2017;2:115-117. doi: 10.1016/j.jacbts.2017.03.002

148. deFilippi CR, Mills NL. Rapid Cardiac Troponin Release After Transient Ischemia: Implications for the Diagnosis of Myocardial Infarction. *Circulation*. 2021;143:1105-1108. doi: 10.1161/CIRCULATIONAHA.120.052649

149. Hasselbalch RB, Bundgaard H, Iversen KK. Response by Hasselbalch et al to Letter Regarding Article, "Temporal Release of High-Sensitivity Cardiac Troponin T and I and Copeptin After Brief Induced Coronary Artery Balloon Occlusion in Humans". *Circulation*. 2021;144:e169-e170. doi: 10.1161/CIRCULATIONAHA.121.055682

150. Gautel M, Zuffardi O, Freiburg A, Labeit S. Phosphorylation switches specific for the cardiac isoform of myosin binding protein-C: a modulator of cardiac contraction? *EMBO J*. 1995;14:1952-1960.

151. Mohamed AS, Dignam JD, Schlender KK. Cardiac myosin-binding protein C (MyBP-C): identification of protein kinase A and protein kinase C

phosphorylation sites. *Arch Biochem Biophys*. 1998;358:313-319. doi: 10.1006/abbi.1998.0857

152. Jia W, Shaffer JF, Harris SP, Leary JA. Identification of novel protein kinase A phosphorylation sites in the M-domain of human and murine cardiac myosin binding protein-C using mass spectrometry analysis. *J Proteome Res*. 2010;9:1843-1853. doi: 10.1021/pr901006h

153. Carrier L, Mearini G, Stathopoulou K, Cuello F. Cardiac myosin-binding protein C (MYBPC3) in cardiac pathophysiology. *Gene*. 2015;573:188-197. doi: 10.1016/j.gene.2015.09.008

154. Bardswell SC, Cuello F, Rowland AJ, Sadayappan S, Robbins J, Gautel M, Walker JW, Kentish JC, Avkiran M. Distinct sarcomeric substrates are responsible for protein kinase D-mediated regulation of cardiac myofilament Ca2+ sensitivity and cross-bridge cycling. *J Biol Chem*. 2010;285:5674-5682. doi: 10.1074/jbc.M109.066456

155. Sadayappan S, Gulick J, Osinska H, Martin LA, Hahn HS, Dorn GW, 2nd, Klevitsky R, Seidman CE, Seidman JG, Robbins J. Cardiac myosin-binding protein-C phosphorylation and cardiac function. *Circ Res*. 2005;97:1156-1163. doi: 10.1161/01.RES.0000190605.79013.4d

156. Messer AE, Gallon CE, McKenna WJ, Dos Remedios CG, Marston SB. The use of phosphate-affinity SDS-PAGE to measure the cardiac troponin I phosphorylation site distribution in human heart muscle. *Proteomics Clin Appl*. 2009;3:1371-1382. doi: 10.1002/prca.200900071

157. Martin-Garrido A, Biesiadecki BJ, Salhi HE, Shaifta Y, Dos Remedios CG, Ayaz-Guner S, Cai W, Ge Y, Avkiran M, Kentish JC. Monophosphorylation of cardiac troponin-I at Ser-23/24 is sufficient to regulate cardiac myofibrillar Ca(2+) sensitivity and calpain-induced proteolysis. *J Biol Chem*. 2018;293:8588-8599. doi: 10.1074/jbc.RA117.001292

158. Barefield DY, McNamara JW, Lynch TL, Kuster DWD, Govindan S, Haar L, Wang Y, Taylor EN, Lorenz JN, Nieman ML, et al. Ablation of the calpain-targeted site in cardiac myosin binding protein-C is cardioprotective during ischemia-reperfusion injury. *J Mol Cell Cardiol*. 2019;129:236-246. doi: 10.1016/j.yjmcc.2019.03.006

159. Govindan S, McElligott A, Muthusamy S, Nair N, Barefield D, Martin JL, Gongora E, Greis KD, Luther PK, Winegrad S, et al. Cardiac myosin binding protein-C is a potential diagnostic biomarker for myocardial infarction. *J Mol Cell Cardiol*. 2012;52:154-164. doi: 10.1016/j.yjmcc.2011.09.011

160. Decker RS, Decker ML, Kulikovskaya I, Nakamura S, Lee DC, Harris K, Klocke FJ, Winegrad S. Myosin-binding protein C phosphorylation, myofibril structure, and contractile function during low-flow ischemia. *Circulation*. 2005;111:906-912. doi: 10.1161/01.CIR.0000155609.95618.75

161. Sadayappan S, Osinska H, Klevitsky R, Lorenz JN, Sargent M, Molkentin JD, Seidman CE, Seidman JG, Robbins J. Cardiac myosin binding protein C phosphorylation is cardioprotective. *Proc Natl Acad Sci U S A*. 2006;103:16918-16923. doi: 10.1073/pnas.0607069103

162. Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD, Writing Group on behalf of the Joint ESCAAHAWHFTFftUDoMI. Third universal definition of myocardial infarction. *Global heart*. 2012;7:275-295. doi: 10.1016/j.gheart.2012.08.001

163. Chapman AR, Adamson PD, Mills NL. Assessment and classification of patients with myocardial injury and infarction in clinical practice. *Heart*. 2016. doi: 10.1136/heartjnl-2016-309530

164. Collinson P, Lindahl B. Type 2 myocardial infarction: the chimaera of cardiology? *Heart*. 2015;101:1697-1703. doi: 10.1136/heartjnl-2014-307122

165. Sandoval Y, Smith SW, Thordsen SE, Apple FS. Supply/demand type 2 myocardial infarction: should we be paying more attention? *Journal of the American College of Cardiology*. 2014;63:2079-2087. doi: 10.1016/j.jacc.2014.02.541

166. Stein GY, Herscovici G, Korenfeld R, Matetzky S, Gottlieb S, Alon D, Gevrielov-Yusim N, Iakobishvili Z, Fuchs S. Type-II myocardial infarction--patient characteristics, management and outcomes. *PloS one*. 2014;9:e84285. doi: 10.1371/journal.pone.0084285

167. Goyal A, Gluckman TJ, Tcheng JE. What's in a Name? *The New ICD-10 (10th Revision of the International Statistical Classification of Diseases and Related Health Problems) Codes and Type 2 Myocardial Infarction*. 2017;136:1180-1182. doi: 10.1161/circulationaha.117.030347

168. Weil BR, Suzuki G, Young RF, Iyer V, Canty JM, Jr. Troponin Release and Reversible Left Ventricular Dysfunction After Transient Pressure Overload. *J Am Coll Cardiol*. 2018;71:2906-2916. doi: 10.1016/j.jacc.2018.04.029

169. Techiryan G, Weil BR, Palka BA, Canty JM, Jr. Effect of Intracoronary Metformin on Myocardial Infarct Size in Swine. *Circ Res*. 2018;123:986-995. doi: 10.1161/CIRCRESAHA.118.313341

170. Madsen LH, Christensen G, Lund T, Serebruany VL, Granger CB, Hoen I, Grieg Z, Alexander JH, Jaffe AS, Van Eyk JE, et al. Time course of degradation of cardiac troponin I in patients with acute ST-elevation myocardial infarction: the ASSENT-2 troponin substudy. *Circ Res*. 2006;99:1141-1147. doi: 10.1161/01.RES.0000249531.23654.e1

171. Madsen LH, Lund T, Grieg Z, Nygaard S, Holmvang L, Jurlander B, Grande P, Christensen G, Atar D. Cardiac troponin I degradation in serum of patients with

hypertrophic obstructive cardiomyopathy undergoing percutaneous septal ablation. *Cardiology*. 2009;114:167-173. doi: 10.1159/000226596

END OF THESIS

7 Appendix

7.1 Ethical approvals

NHS
Health Research Authority

South Central - Berkshire Research Ethics Committee
Bristol REC Centre
Whitefriars
Level 3, Block B
Lewins Mead
Bristol
BS1 2NT

Telephone: (020) 71048043

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

30 May 2018

Professor Michael Marber
The Rayne Institute, St Thomas' Hospital
Lambeth Palace Road,
London SE1 7EH

Dear Professor Marber

Study title:	**Measurement of week-to-week biological variation of cardiac myosin-binding protein C (cMyC)**
REC reference:	**18/SC/0310**
Protocol number:	**N/A**
IRAS project ID:	**241895**

The Proportionate Review Sub-committee of the South Central - Berkshire Research Ethics Committee reviewed the above application on 12 June 2018.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact hra.studyregistration@nhs.net outlining the reasons for your request. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Ethical opinion

The Committee concluded that your research does not comprise NHS research because it does not involve patients, their carers or relatives as participants. Furthermore a favourable opinion of

the Committee was not required for the purposes of the Human Tissue Act given that samples will be provided with consent and the additional safeguard of anonymization will be in place. You will not be storing relevant material either, given your plans for early intervention to render blood acellular. In summary no NHS ethics review was required and the opinion of the Committee can only be considered as advisory.

The Committee was disappointed to note that participants would not be rewarded for the time and inconvenience involved. The study is reasonably well funded and the patent that might eventually be obtained will be of considerable financial value to the spin off company which will own it. At the very least the Committee would have expected the information sheet to make it clear that the burdens of participation would not be rewarded notwithstanding funding and commercialisation.

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA and HCRW Approval (England and Wales)/ NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion").

Approved documents

The documents reviewed and approved were:

Document	*Version*	*Date*	
Copies of advertisement materials for research participants [poster]	v1	06 February 2018	
Costing template (commercial projects) [R&D cost approval]	1	08 May 2018	
IRAS Application Form [IRAS_Form_17052018]		17 May 2018	
IRAS Checklist XML [Checklist_17052018]		17 May 2018	
Participant consent form [Consent form]	v1	06 February 2018	
Participant information sheet (PIS) [Participant Information sheet]	V3	26 April 2018	
Research protocol or project proposal [Study protocol: A prospective cohort study on the week-to-week biological variation of cMyC in healthy volunteers]	2	26 April 2018	
Summary CV for Chief Investigator (CI) [CV Professor Michael Marber]		16 January 2018	
Summary CV for student [Tom Kaier]	1	11 May 2018	
Summary CV for student [CV Bashir Alaour]		16 January 2018	

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

There were no declarations of interests

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

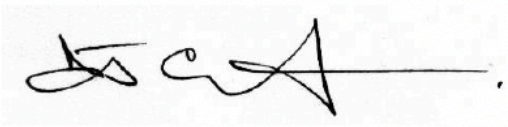
HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

18/SC/0310	**Please quote this number on all correspondence**

Yours sincerely



Mr David Carpenter

A Research Ethics Committee established by the Health Research Authority