HIGH SENSITIVITY TROPONIN - ITS USE IN DIAGNOSIS OF CARDIAC DYSFUNCTION

A thesis submitted in the fulfilment of the requirements for the degree of Doctor of Philosophy
University of Canberra

Gary Lloyd Koerbin
ABSTRACT

Troponins are regulatory proteins and part of the contractile apparatus that is integral to muscle contraction in skeletal and cardiac muscle but not smooth muscle and are important clinically because cardiac troponins (cTn) are sensitive indicators of myocyte injury and have become integral to the definition of myocardial infarction. There are several issues surrounding the significance of troponin and how it should be used, both for the assessment of cardiac disease and in settings of non-cardiac illness. This thesis examines a number of these areas of uncertainty.

This thesis focuses initially on the analytical validation of troponin assays and I offer guidelines for a standardised approach to undertaking the verification of these analytical characteristics. I report on these characteristics for 2 highly sensitive assays and their application to a cardio-healthy population.

In the second part of this thesis I focus on the physiology of troponin in the normal population. I describe studies undertaken with a cohort of healthy children and demonstrate the significance of population coning when determining the 99\textsuperscript{th} percentile of the upper reference limit using 2 highly sensitive troponin assays.

The final part of this thesis investigates the significance of troponin in the acute coronary syndrome (ACS) and non ACS setting. I offer a hypothesis suggesting that bleb formation is a mechanism for troponin release. I describe how improvements in sensitivity of troponin T assays allow better prognostic information regarding all cause mortality in end stage renal disease patients, demonstrate troponin release after strenuous exercise in elite cyclists and I describe a cross-sectional study looking at troponin concentrations in subjects with non cardiac illness and the general community. Using data mining techniques I demonstrate how
the use of a new high sensitivity troponin I assay can offer greater assistance to the clinician in stratifying patients at risk of a major adverse cardiac event (MACE). I provide evidence that suggests the use of a multi-marker approach to identifying patients at risk is potentially viable.
Undertaking a PhD requires a lot from many people to achieve the ultimate outcome. It requires a lot of time, assistance, guidance, support and encouragement from mentors, colleagues and friend. It is almost impossible to thank everyone who helps in these ways but there are those who must be thanked for without the ongoing support and guidance the task of undertaking and completing this thesis would have been less enjoyable and far more onerous.

I would like to thank my supervisors and advisors Peter Hickman, Julia Potter, Brett Lidbury, Alice Richardson and Luby Simson. Their support, encouragement, advice and mentoring ultimately has allowed me to be able to submit this work.

To Peter Hickman and Julia Potter a special thank you, over and above their advice and supervision, for their friendship, encouragement and support over the past 15 years.

To my friends and clinical colleagues, Walter Abhyaratna, Girish Talaulikar, Daryl McGill and Louise Cullen thank you for access to the clinical samples and clinical outcome data used in these research studies.

Undertaking the many analyses performed in these studies would only have been possible with the technical assistance, access to analytical instrumentation, support and the gentle “encouragement” of staff in the clinical chemistry department of ACT Pathology - Jaya Canard, Suzi Apostoloska, Di Talsma, Carmen Oakman and Corrina Newman. Also to Peter Talsma, thanks for the continued supply of journal articles to read. Thank you all.

My colleagues, Nicole Chia and Kerrie Andriolo, both of whom are also undertaking post graduate studies, provided encouragement at times when it was most needed. It was and is very much appreciated.
DECLARATION

In this thesis I detail the findings from research carried out between July 2009 and August 2013. The research studies described in Chapters 3-5 were carried out in collaboration with my co-authors, the names of whom are listed at the start of each chapter. For each of these studies I took a lead role in the experimental design, subject recruitment, data collection and analysis, with all authors contributing to final submitted version of the manuscripts. I obtained assistance with these concepts from my supervisory panel members A/Professor Peter E Hickman, Professor Julia M Potter, A/Professor Brett Lidbury and Dr Alice Richardson.

I obtained assistance with and analysis with the mathematical approach to data mining from A/Professor Brett Lidbury and Dr Alice Richardson.

I obtained assistance with the administrative and scientific components of this thesis from A/Professor Luby Simson.
DEDICATION

To my family, it was my mother’s wish to see her two sons receive “the floppy hat”.

Unfortunately she passed away before both my brother, Paul, and I completed our studies.

My dad will complete that wish for her.

To Anne, Liesel and Scott, thank you for putting up with the “Grumpy Gus” when he reared his head over the past few years and for the unconditional support.

shukran kabeer.
PEER REVIEWED JOURNAL ARTICLES


**Koerbin G**, Tate JR, Hickman, PE. The analytical characteristics of the Roche hs-TnT assay and its application to a cardio-healthy population. Ann Clin Biochem. 2010;47:524-8


Tate JR, Panteghini M, **Koerbin G**, Hickman PE, Schneider HG, Jaffe A. Verification of the analytical characteristics of troponin assays in the laboratory – a how to guide. Clin Biochem Reviews Troponin Monograph 2012 69-85


Potter JM, Simpson A, Koerbin G, Kerrigan J, Southcott E, Hickman PE. Cardiac troponin and non-cardiac illness: high sensitivity cardiac troponins in a cross-sectional study in a general hospital and a community population. (submitted to Clin Chim Acta)
PEER REVIEWED CONFERENCE PROCEEDINGS


Hickman PE, Koerbin G, Potter JM, Talaulikar G, McGill D. 5 Year Outcomes in renal Dialysis Patients: New hsTnI assays are as informative as hsTnT. Clin Biochem Rev 2011;34:S26


SCIENTIFIC CONFERENCE AND MEETING PRESENTATIONS

2010  SW AIMS meeting, Canberra
       “Troponin Past, Present and Future ?”

2010  AACB NSW/ACT Branch Meeting
       “Evaluation of the Roche hs-TnT Assay”

2010  AACB SES, Sydney
       “hs-TnT which reference intervals ?”

2010  AACB/AIMS Combined Annual Scientific Meeting, Perth
       “Highly Sensitive TnT – An opening to a whole new world”

2011  Roche Cardiac Symposium, Heidelberg, Germany
       “hs-Tn and Healthy Populations”

2011  Abbott New Zealand Architect User Symposium, Rotorua, NZ
       “Highly Sensitive Troponin”

2012  Abbott Scientific Symposium. Sydney
       “High Sensitivity troponin – its use in diagnosis of cardiac dysfunction

2012  Abbott Scientific Symposium. Melbourne
       “High Sensitivity troponin – its use in diagnosis of cardiac dysfunction
AWARDS

The Roche Diagnostics Australia Award
“Best Poster Presentation prize for the 2012 AACB Scientific Conference, Melbourne 2012”
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>CERTIFICATE OF AUTHOURSHIP OF THESIS</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>vi</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>vii</td>
</tr>
<tr>
<td>PUBLICATIONS AND PRESENTATIONS RELEVENT TO THIS THESIS</td>
<td></td>
</tr>
<tr>
<td>Peer reviewed journal articles</td>
<td>viii</td>
</tr>
<tr>
<td>Peer reviewed conference proceedings</td>
<td>xiii</td>
</tr>
<tr>
<td>Scientific conference and meeting presentations</td>
<td>xvi</td>
</tr>
<tr>
<td>Awards</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xxi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xxiv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xxv</td>
</tr>
<tr>
<td>CHAPTER 1</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Troponin release only occurs in the presence of necrosis</td>
<td>1</td>
</tr>
<tr>
<td>How should we use troponin in the investigation of the Acute Coronary syndrome (ACS)?</td>
<td>2</td>
</tr>
<tr>
<td>What is the significance of troponin in the pathological non-ACS setting?</td>
<td>2</td>
</tr>
<tr>
<td>Thesis outline</td>
<td></td>
</tr>
<tr>
<td>Are the high sensitivity troponin assays fit for purpose?</td>
<td>3</td>
</tr>
<tr>
<td>What is the distribution of troponin in healthy people and how do we determine appropriate decision points?</td>
<td>4</td>
</tr>
<tr>
<td>What information DOES the troponin concentration provide to us in the ACS and non ACS setting?</td>
<td>4</td>
</tr>
<tr>
<td>CHAPTER 2</td>
<td>6</td>
</tr>
<tr>
<td>Literature review</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>6</td>
</tr>
<tr>
<td>Normal physiology of troponin and its application to the ACS</td>
<td>6</td>
</tr>
<tr>
<td>Cardiac troponin complex</td>
<td>8</td>
</tr>
<tr>
<td>Normal myocyte cell turnover</td>
<td>9</td>
</tr>
<tr>
<td>Definition of the Acute Coronary Syndrome (ACS)</td>
<td>10</td>
</tr>
<tr>
<td>Development of ACS</td>
<td>10</td>
</tr>
<tr>
<td>Acute Myocardial Infarction (AMI)</td>
<td>13</td>
</tr>
<tr>
<td>Pathophysiology of Myocardial infarction</td>
<td>14</td>
</tr>
<tr>
<td>Clinical features of myocardial ischemia and infarction</td>
<td>15</td>
</tr>
<tr>
<td>Spontaneous myocardial infarction (MI type 1)</td>
<td>16</td>
</tr>
<tr>
<td>Myocardial infarction secondary to an ischemic imbalance (MI type 2)</td>
<td>16</td>
</tr>
<tr>
<td>Biomarker use in the detection of myocardial injury with necrosis</td>
<td>18</td>
</tr>
<tr>
<td>The cardiospecificity of troponin: evidence of skeletal muscle release of cTnT</td>
<td>19</td>
</tr>
<tr>
<td>Troponin and non Acute Coronary Syndrome (ACS)</td>
<td>20</td>
</tr>
<tr>
<td>Troponin and renal disease</td>
<td>21</td>
</tr>
<tr>
<td>Topic</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Troponin and sepsis</td>
<td>23</td>
</tr>
<tr>
<td>Irreversible damage</td>
<td>23</td>
</tr>
<tr>
<td>Reversible damage</td>
<td>24</td>
</tr>
<tr>
<td>Formation and release of membranous blebs.</td>
<td>26</td>
</tr>
<tr>
<td>Troponin and exercise</td>
<td>27</td>
</tr>
<tr>
<td>Proteolytic troponin degradation products and increased cellular wall permeability</td>
<td>29</td>
</tr>
<tr>
<td>Troponin assays</td>
<td>30</td>
</tr>
<tr>
<td>Troponin assay issues - Standardisation</td>
<td>34</td>
</tr>
<tr>
<td>Troponin assay issues - interference</td>
<td>35</td>
</tr>
<tr>
<td>Troponin assay issues - Reference values</td>
<td>37</td>
</tr>
<tr>
<td>Troponin Issues – changes in testing protocols</td>
<td>38</td>
</tr>
<tr>
<td>Use of the troponin 99th percentile URL for the diagnosis of ACS and in the assessment of cardiac risk?</td>
<td>39</td>
</tr>
<tr>
<td>Other biochemical cardiac markers</td>
<td>40</td>
</tr>
<tr>
<td>B type natriuretic peptide (BNP) and C-reactive protein (CRP)</td>
<td>40</td>
</tr>
<tr>
<td>Heart Type fatty acid binding protein and copeptin</td>
<td>42</td>
</tr>
<tr>
<td>Growth-differentiation factor-15</td>
<td>43</td>
</tr>
<tr>
<td>MicroRNAs</td>
<td>43</td>
</tr>
</tbody>
</table>

**CHAPTER 3.1**
The determination of the performance characteristics of highly sensitive troponin assays and validation of their fitness for purpose in the clinical laboratory

**CHAPTER 3.2**
Verification of the analytical characteristics of troponin assays in the laboratory – a how to guide

**Abstract**

**Introduction**

**Verification and Validation Studies**

**Troponin Assays**

**Assay principles**

**Antibody specificity**

**Troponin plasma forms and definition of the measurand**

**Troponin I**

**Troponin T**

**Standardisation**

**Troponin I**

**Troponin T**

**Limit of blank, limit of detection, and limit of quantitation**

**Imprecision and limit of quantitation**

**Deriving basic information on imprecision**

**Imprecision profiling**

**Controversial issues about imprecision**

**Interferences**

**Haemolysis Testing**

**Heterophile antibodies and HAMA**
CHAPTER 5.4
Cardiac electrical conduction, autonomic activity and biomarker release during recovery from prolonged strenuous exercise in trained male cyclists.

Abstract

Introduction

Materials and Methods

Subjects

Study design

Incremental exercise test

Prolonged constant-load exercise test

Electrocardiograph recordings

Data analysis

Blood sample collection and biochemical analysis

Statistics

Results

Subject characteristics

Prolonged constant-load cycle test

Heart rate variability

Cardiac cycle dynamics

Biochemical analysis

Discussion

Conclusions

CHAPTER 5.5
Cardiac troponin and non-cardiac illness: high sensitivity cardiac troponins in a cross-sectional study in a general hospital and a community population

Abstract

Introduction

Materials and Methods

Patient samples

Laboratory analyses

Data handling

Results

Discussion

CHAPTER 5.6
A data mining approach for the prognostic efficacy of troponin I and other biomarkers for predicting a coronary event within 30 days in emergency department patients.

Abstract

Introduction

Decision tree

Random Forest

Support vector machine (SVM)

Principal components analysis (PCA)

Classical Multidimensional Scaling (MDS)
<table>
<thead>
<tr>
<th>Chapter and Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials and Methods</td>
<td>290</td>
</tr>
<tr>
<td>Participants</td>
<td>290</td>
</tr>
<tr>
<td>Procedures</td>
<td>291</td>
</tr>
<tr>
<td>Data mining</td>
<td>292</td>
</tr>
<tr>
<td>Results</td>
<td>293</td>
</tr>
<tr>
<td>Discussion</td>
<td>305</td>
</tr>
<tr>
<td>Conclusions</td>
<td>306</td>
</tr>
<tr>
<td><strong>CHAPTER 6</strong></td>
<td></td>
</tr>
<tr>
<td>Summary and future directions</td>
<td>309</td>
</tr>
<tr>
<td>Practical recommendations provided by the studies in this thesis</td>
<td>311</td>
</tr>
<tr>
<td>The future</td>
<td>311</td>
</tr>
</tbody>
</table>
LIST OF TABLES

CHAPTER 2
Table 2.1  Classifications of Myocardial Infarction. 17
Table 2.2  Non-coronary conditions that may cause elevated troponin 21
Table 2.3  99th percentile and imprecision levels for troponin assays 33
Table 2.4  Scorecard designation of troponin assays 34
Table 2.5  Association between biomarker and serious cardiac outcome after 72 hours. 41

CHAPTER 3.2
Table 3.2.1  Analytical characteristics of commercial cardiac troponin I and T assays declared by the manufacturer 64
Table 3.2.2  Requirements for the applicability of EQA results to evaluation of the performance of individual laboratories in the measurement of cTn 81
Table 3.2.3  Analytical performance goals for cTnI measurements using routine methods based on data of biological variability 82

CHAPTER 3.3
Table 3.3.1  Measured mean cardiac troponin (cTn) concentrations, recovery and imprecision for nine daily measurements of thirteen linearly related plasma samples. 95
Table 3.3.2  Median cardiac troponin T concentrations and 99th percentile values in men and women younger and older than 60 years. 101

CHAPTER 4.2
Table 4.2.1  Number of LOOK children from each year of the study who had cTnI concentrations above the indicated cut-point. 144
Table 4.2.2  Variable 99th percentiles based upon whether highest or lowest cTnI concentration used, where multiple blood samples collected from the one child. 146
Table 4.2.3  Median, 2.5th and 97.5th percentiles for both cTnI concentration in the different groups, and the biological variation of cTnI in these groups. 148
Table 4.2.4  Index of Individuality and RCV data for hs-cTnI in healthy children. 149

CHAPTER 4.4
Table 4.4.1  Proportion of TnT-positive results relating to the number of times a child was bled. 172

CHAPTER 4.5
Table 4.5.1  Characteristics of the healthy controls, after coning based on biomarker, clinical and echocardiographic screening. 190
Table 4.5.2  The effect of coning with both laboratory and clinical indices on the 99th percentile for the Abbott ARCHITECT hs-cTnI assay. 191
Table 4.5.3  The effect of coning with both laboratory and clinical indices on the 99th percentile for the Roche hs-cTnT assay. 192
<table>
<thead>
<tr>
<th>Table 4.5.4</th>
<th>Shapiro – Wilk probability of a Gaussian distribution. $P &lt; 0.05$ indicates a normal distribution.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 4.5.5</td>
<td>Major population studies looking at 99th percentiles for hs-cTnI and hs-cTnT</td>
</tr>
</tbody>
</table>

**CHAPTER 5.3**

Table 5.3.1 | Descriptive statistics for high risk variables. |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 5.3.2</td>
<td>Area under curve analysis for all cause mortality prediction</td>
</tr>
</tbody>
</table>

**CHAPTER 5.4**

Table 5.4.1 | Heart rate variability parameters before and during recovery from prolonged strenuous exercise. |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 5.4.2</td>
<td>Biochemical analyte concentrations before and during recovery from prolonged strenuous exercise.</td>
</tr>
</tbody>
</table>

**CHAPTER 5.5**

Table 5.5.1 | TnI concentration measured with a high-sensitivity assay, in a hospital and community practice population. |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 5.5.2</td>
<td>TnT concentration measured with a high-sensitivity assay, in a hospital and community practice population.</td>
</tr>
<tr>
<td>Table 5.5.3</td>
<td>Summary of Emergency Department requests</td>
</tr>
<tr>
<td>Table 5.5.4</td>
<td>Clinical assessment and mortality in all patients with cTnI above the 99th percentile</td>
</tr>
</tbody>
</table>

**CHAPTER 5.6**

Table 5.6.1 | Combined and gender specific sensitivity, specificity, PPV and NPV values obtained when comparing patients with no cardiac condition and those patients who had a confirmed MACE |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 5.6.2</td>
<td>Descriptive statistical analysis showing event rates at specific hsTnI concentration decision points.</td>
</tr>
<tr>
<td>Table 5.6.3</td>
<td>Combined and gender specific sensitivity, specificity, PPV and NPV values obtained for those patients who had a confirmed MACE using manufacturer defined hsTnI concentration decision points</td>
</tr>
<tr>
<td>Table 5.6.4</td>
<td>Combined and gender specific sensitivity, specificity, PPV and NPV values obtained for those patients with a stable cardiac condition using manufacturer defined decision points:</td>
</tr>
<tr>
<td>Table 5.6.5</td>
<td>Combined gender sensitivity, specificity, PPV and NPV values obtained for those patients with MACE and stable cardiac condition using decision tree defined cutpoints</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

### CHAPTER 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Schematic of troponin complex</td>
<td>9</td>
</tr>
<tr>
<td>2.2</td>
<td>The 7 stages of development of an atherosclerotic plaque</td>
<td>11</td>
</tr>
<tr>
<td>2.3</td>
<td>Atheromatous plaque development – preclinical and clinical phases</td>
<td>12</td>
</tr>
<tr>
<td>2.4</td>
<td>ACS as a continuum of disease</td>
<td>13</td>
</tr>
<tr>
<td>2.5</td>
<td>Differentiation between MI types 1 and 2</td>
<td>17</td>
</tr>
<tr>
<td>2.6</td>
<td>Mechanism of troponin release</td>
<td>27</td>
</tr>
<tr>
<td>2.7</td>
<td>Schematic model illustrating the release mechanisms of cTnI and cTnT from cardiomyocytes following reversible or irreversible damage.</td>
<td>30</td>
</tr>
</tbody>
</table>

### CHAPTER 3.2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.1</td>
<td>Imprecision profiles.</td>
<td>69</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Effect of haemolysis on Tn assays</td>
<td>71</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Bland Altman analysis of hsTnT and 4th generation TnT assays</td>
<td>76</td>
</tr>
<tr>
<td>3.2.4</td>
<td>hs-cTnT population distribution.</td>
<td>79</td>
</tr>
</tbody>
</table>

### CHAPTER 3.3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.1</td>
<td>Imprecision profile of hsTnT.</td>
<td>96</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Passing-Bablok regression analysis plot of cardiac troponin T for 96 plasma samples</td>
<td>97</td>
</tr>
<tr>
<td>3.3.3</td>
<td>Bland Altman analysis of cardiac troponin T for 96 plasma samples.</td>
<td>98</td>
</tr>
<tr>
<td>3.3.4</td>
<td>Expanded Bland Altman analysis of cardiac troponin T for 96 plasma samples.</td>
<td>98</td>
</tr>
<tr>
<td>3.3.5</td>
<td>Regression analysis, serum vs lithium heparin plasma</td>
<td>99</td>
</tr>
<tr>
<td>3.3.6</td>
<td>Distribution of serum troponin concentrations for the Roche hs-TnT method</td>
<td>100</td>
</tr>
<tr>
<td>3.3.7</td>
<td>Differences in cardiac troponin T concentrations in men and women younger and older than 60 years.</td>
<td>101</td>
</tr>
</tbody>
</table>

### CHAPTER 3.4

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4.1</td>
<td>Imprecision profile showing assay total CV versus log concentration for the Abbott hs-TnI assay</td>
<td>116</td>
</tr>
<tr>
<td>3.4.2A</td>
<td>Deming regression comparison between on market TnI assay and research prototype hsTnI assay over the range 10-950 ng/L.</td>
<td>118</td>
</tr>
<tr>
<td>3.4.2B</td>
<td>Difference plot showing comparison between on market TnI assay and research prototype hsTnI assay over the range 10-950 ng/L.</td>
<td>119</td>
</tr>
<tr>
<td>3.4.3A</td>
<td>Difference plot showing comparison between serum and lithium heparin plasma over the range 1-5400 ng/L.</td>
<td>120</td>
</tr>
<tr>
<td>3.4.3B</td>
<td>Difference plot showing comparison between serum and EDTA plasma over the range 1-5400 ng/L.</td>
<td>121</td>
</tr>
<tr>
<td>3.4.4</td>
<td>Effect of haemolysis</td>
<td>122</td>
</tr>
<tr>
<td>3.4.5A</td>
<td>Distribution of serum hs-TnI concentrations in cardio-healthy males</td>
<td>123</td>
</tr>
<tr>
<td>3.4.5B</td>
<td>Distribution of serum hs-TnI concentrations in cardio-healthy females</td>
<td>124</td>
</tr>
</tbody>
</table>
CHAPTER 4.2
Figure 4.2.1  hs-cTnI frequency distribution for 8 year old males.  139
Figure 4.2.2  hs-cTnI frequency distribution for 10 year old males.  139
Figure 4.2.3  hs-cTnI frequency distribution for 12 year old males.  140
Figure 4.2.4  hs-cTnI frequency distribution for 8 year old females.  140
Figure 4.2.5  hs-cTnI frequency distribution for 10 year old females.  141
Figure 4.2.6  hs-cTnI frequency distribution for 12 year old females.  141
Figure 4.2.7  Within- and between-child cTnI concentrations for 453 children who had more than 1 measurement made.  142
Figure 4.2.8  Within- and between-child cTnI concentrations for 453 children who had more than 1 measurement made (lowest to highest).  143
Figure 4.2.9  Change in results for the 11 children with at least one result above the 99th percentile and two measurements made.  145
Figure 4.2.10  Change in results for the 11 children with at least one result above the 99th percentile and 3 measurements made.  145
Figure 4.2.11  Long-term biological variation in healthy children.  147
Figure 4.2.12  TnI biological variation by gender in 8, 10 and 12 year old children.  147

CHAPTER 4.3
Figure 4.3.1  Distribution of cTnI concentration in a population of 450 healthy 12 year old children.  160
Figure 4.3.2  Distribution of cTnI concentration in the central 95% of a population of 213 healthy 12 year old males.  160
Figure 4.3.3  Normality plot of data in Figure 4.3.2, showing no significant difference to a Gaussian distribution.  161
Figure 4.3.4  Non Gaussian distribution of cTnI concentration in the central 95% of a population of 237 healthy 12 year old females.  162
Figure 4.3.5  Gaussian distribution of cTnI concentration in the central 95% of a population after 2 highest 12 year old female results excluded.  162

CHAPTER 4.4
Figure 4.4.1  The troponin concentration in the blood of the same cohort of children, at ages 8, 10 and 12 years  173
Figure 4.4.2  Troponin positive results by school over different years. (2005,2007,2009)  175

CHAPTER 4.5
Figure 4.5.1  Study data analysis algorithm  189
Figure 4.5.2A  All subjects <75 years old with Dixon outlier exclusion (15) only.  193
Figure 4.5.2B  All subjects <75 years old with full exclusion criteria applied.  194
Figure 4.5.2C  Male subjects <75 years old with Dixon outlier exclusion (15) only.  194
Figure 4.5.2D  Male subjects <75 years old with full exclusion criteria applied.  195
Figure 4.5.2E  Female subjects <75 years old with Dixon outlier exclusion (15) only.  195
Figure 4.5.2F  Female subjects <75 years old with full exclusion criteria applied.  196
CHAPTER 5.2
Figure 5.2.1 Cartoon of hepatocyte bleb formation during ischemia and reperfusion.

Figure 5.2.2 Microbleb formation of adult cultured cardiac myocytes. A baseline. B 30 min of anoxia.

Figure 5.2.3 Mechanism of troponin release.

CHAPTER 5.3
Figure 5.3.1 Survival predicted by the old cTnT assay adjusted for all the covariates entered into the Cox regression model.

Figure 5.3.2 ROC curve for standard cTnT and hs-cTnT (ng/L) for predicting all-cause mortality.

Figure 5.3.3 Kaplan–Meier curve for hs-cTnT cut-off point of 24.15 ng/L.

CHAPTER 5.4
Figure 5.4.1 Raw cardiac intervals from a subject’s electrocardiograph recordings during the pre-exercise and recovery periods

Figure 5.4.2 Time-domain and non-linear parameters of cardiac intervals before exercise (Pre) and during recovery (15-, 30-, 45-, 60-min & 24h).

Figure 5.4.3 The QT interval corrected for heart rate (QTc, a), QT interval variability index (QTvi, b) and ratio of the QT/RR interval approximate entropy (QT/RR ApEn, c) before exercise (Pre) and during recovery (15-, 30-, 45-, 60-min & 24h).

Figure 5.4.4 Participant hs-cTnT response to exercise.

Figure 5.4.5 Participant hs-cTnl response to exercise

Figure 5.4.6 High sensitivity cardiac troponin T (hs-cTnT) concentrations before exercise (Pre-exercise) and during recovery (60 min and 24 hours).

Figure 5.4.7 High sensitivity cardiac troponin I (hs-cTnl) concentrations before exercise (Pre-exercise) and during recovery (60 min and 24 hours).

Figure 5.4.8 Average heart rate during a 2 hour constant load cycling test and relative increase in high sensitivity cardiac troponin T (hs-cTnT) concentration 60-min after the cycling test.

CHAPTER 5.6
Figure 5.6.1 Random Forest analysis of variable importance.

Figure 5.6.2 Decision tree: cTnl at presentation

Figure 5.6.3 Decision tree: delta cTnl (from presentation to 2 hours post presentation)

Figure 5.6.4 Median and inter quartile range for patients with observed MACE, stable cardiac conditions and no cardiac events.

Figure 5.6.5 ROC curve using PCA determined troponin algorithm

Figure 5.6.6 Abbreviated random forest analysis of stable cardiac patient cohort

Figure 5.6.7 SVM classification plot
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>American College of Cardiology</td>
</tr>
<tr>
<td>ACS</td>
<td>Acute coronary syndrome</td>
</tr>
<tr>
<td>ADAPT</td>
<td>Accelerated Diagnostic protocol to Assess Patients with chest pain</td>
</tr>
<tr>
<td>ADP</td>
<td>Accelerated diagnostic protocol</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine transaminase</td>
</tr>
<tr>
<td>AMI</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>APACE</td>
<td>Advantageous Predictors of Acute Coronary Syndromes Evaluation</td>
</tr>
<tr>
<td>ApEn</td>
<td>Approximate entropy</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>BNP</td>
<td>B-type natriuretic peptide</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine Kinase</td>
</tr>
<tr>
<td>CKD-EPI</td>
<td>Chronic Kidney Disease Epidemiological Collaboration</td>
</tr>
<tr>
<td>CK-MB</td>
<td>Creatine Kinase MB isoenzyme</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standards International</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>cTn</td>
<td>Cardiac troponin</td>
</tr>
<tr>
<td>cTnI</td>
<td>Cardiac troponin I</td>
</tr>
<tr>
<td>cTnT</td>
<td>Cardiac troponin T</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CV&lt;sub&gt;a&lt;/sub&gt;</td>
<td>Analytical variation (imprecision)</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CV&lt;sub&gt;g&lt;/sub&gt;</td>
<td>Between individual variation</td>
</tr>
<tr>
<td>CV&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Within individual variation</td>
</tr>
<tr>
<td>CV&lt;sub&gt;t&lt;/sub&gt;</td>
<td>Total variation</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimate of the glomerular filtration rate</td>
</tr>
<tr>
<td>EQA</td>
<td>External quality assessment</td>
</tr>
<tr>
<td>ESC</td>
<td>European Society of Cardiology</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GET</td>
<td>Gas exchange threshold</td>
</tr>
<tr>
<td>GGT</td>
<td>Gamma glutamyl transpeptidase</td>
</tr>
<tr>
<td>HA</td>
<td>Heterophile antibody</td>
</tr>
<tr>
<td>HAMA</td>
<td>Heterophilic anti mouse antibody</td>
</tr>
<tr>
<td>HbA1C</td>
<td>Haemoglobin A1C</td>
</tr>
<tr>
<td>HRV</td>
<td>Heart rate variability</td>
</tr>
<tr>
<td>hs-cTnI</td>
<td>High sensitivity troponin I</td>
</tr>
<tr>
<td>hs-cTnT</td>
<td>High sensitivity troponin T</td>
</tr>
<tr>
<td>IFCC</td>
<td>International Federation of Clinical Chemistry</td>
</tr>
<tr>
<td>II</td>
<td>Index of individuality</td>
</tr>
<tr>
<td>IQC</td>
<td>Internal Quality control</td>
</tr>
<tr>
<td>ISO</td>
<td>International organisation of standards</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>Ln</td>
<td>Natural log</td>
</tr>
<tr>
<td>LoB</td>
<td>Limit of blank</td>
</tr>
<tr>
<td>LoD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOOK</td>
<td>Lifestyle Of Our Kids</td>
</tr>
<tr>
<td>LoQ</td>
<td>Limit of quantitation</td>
</tr>
<tr>
<td>LVEF</td>
<td>Left ventricular ejection fraction</td>
</tr>
<tr>
<td>m</td>
<td>Metre</td>
</tr>
<tr>
<td>MACE</td>
<td>Major adverse cardiac event</td>
</tr>
<tr>
<td>MDRD</td>
<td>Modification of Diet in Renal Diseased</td>
</tr>
<tr>
<td>MDS</td>
<td>Classical multidimensional scaling</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative predictive value</td>
</tr>
<tr>
<td>NSTEMI</td>
<td>non ST-elevation myocardial infarction</td>
</tr>
<tr>
<td>NTproBNP</td>
<td>N-terminal pro B type natriuretic peptide</td>
</tr>
<tr>
<td>p</td>
<td>Probability</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal components analysis</td>
</tr>
<tr>
<td>PoCT</td>
<td>Point of Care Testing</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive predictive value</td>
</tr>
<tr>
<td>PSE</td>
<td>Prolonged strenuous exercise</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>RCV</td>
<td>Reference Change Value</td>
</tr>
<tr>
<td>RMSSD</td>
<td>Root mean squares of successive differences</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operator curve</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error mean</td>
</tr>
<tr>
<td>STEMI</td>
<td>ST-segment-elevation myocardial infarction</td>
</tr>
<tr>
<td>SVM</td>
<td>Support Vector Machine</td>
</tr>
<tr>
<td>TIMI</td>
<td>Thrombolysis in Myocardial Infarction</td>
</tr>
<tr>
<td>Tn</td>
<td>Troponin</td>
</tr>
<tr>
<td>TnC</td>
<td>Troponin C</td>
</tr>
<tr>
<td>TnI</td>
<td>Troponin I</td>
</tr>
<tr>
<td>TnT</td>
<td>Troponin T</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>ug</td>
<td>Micrograms</td>
</tr>
<tr>
<td>URL</td>
<td>Upper reference limit</td>
</tr>
<tr>
<td>VO$_2$</td>
<td>Oxygen uptake</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Troponins are regulatory proteins and part of the contractile apparatus that is integral to muscle contraction in skeletal and cardiac muscle but not smooth muscle [1].

Cardiac and skeletal muscle isoforms of troponin T and troponin I can be readily immunologically distinguished from each other as the amino acid sequences of the myocardial troponins differ [2] by approximately 40% from their skeletal muscle counterparts.

Cardiac troponins (cTn) are important clinically because they are very sensitive indicators of myocyte injury and they have become integral to the definition of myocardial infarction [3].

There continues to be uncertainty about several aspects of the significance of troponin [4] and how it should be used, both for the assessment of cardiac disease and in settings of non-cardiac illness. This thesis examines a number of these areas of uncertainty.

TROPONIN RELEASE ONLY OCCURS IN THE PRESENCE OF NECROSIS

At the time this thesis commenced, before the advent of the high-sensitivity assays for both cardiac troponin T (cTnT) and cardiac troponin I (cTnI), there was the belief that any detectable troponin indicated myocardial necrosis [5-8]. This thesis examines this belief that the presence of troponin always indicates that cardiac necrosis has occurred.
HOW SHOULD WE USE TROPOIN IN THE INVESTIGATION OF THE ACUTE CORONARY SYNDROME (ACS)?

With the first definition of acute myocardial infarction, the use of the 99th population percentile as a cut point when investigating persons with possible ACS was proposed [3,9]. This was in an attempt to reduce the number of false positives presenting to cardiologists. In this thesis I examine the validity of the 99th percentile and consider possible alternatives to its use.

WHAT IS THE SIGNIFICANCE OF TROPOIN IN THE PATHOLOGICAL NON-ACS SETTING?

It has been observed that there are elevations in troponin concentration in the peripheral circulation in settings other than the acute coronary syndrome. This is particularly evident in end stage renal disease, with patients receiving dialysis [10] and in athletes with vigorous training routines [11,12]. It will be demonstrated in this thesis, that the measurement of troponin concentrations, using assays with improved sensitivity, show superior performance to conventional analysis of troponin and other markers of cardiac function in identifying end stage renal disease (ESRD) patients undertaking dialysis at increases risk of all cause mortality. The recognition of transient seasonal changes in troponin concentrations in a cohort of healthy school children will be presented. This thesis will also describe the use of both clinical and biochemical parameters to determine cardio-health in adults when undertaking study population selection.
THESIS OUTLINE

ARE THE HIGH SENSITIVITY TROPONIN ASSAYS FIT FOR PURPOSE?

The first part of this thesis, Chapter 3, focuses on the validation of high sensitivity troponin assays, investigating the analytical characteristics of the high sensitivity assays in contrast to the conventional assays, particularly at concentrations that allow the detection of troponin in a cardio-healthy population. This chapter offers guidelines for a standardised approach to undertaking the verification of these analytical characteristics. Using these guidelines, two studies to determine the characteristics of high sensitivity assays are described.

The second part of this thesis, chapters 4, focuses on troponin physiology in the normal population.

Most of the time troponin is released by cellular necrosis. Necrosis is caused by factors external to the cell such as trauma that results in the unregulated digestion of cell components. This is where the plasma membrane is breached. There are relatively rare occurrences, where troponin is released without necrosis. A hypothesis is proposed how troponin may be released into the circulation if not due to cell death.

Cytoplasmic blebs have been described in liver. These blebs develop during cellular ischemia. If the ischemia is limited and re-oxygenation occurs, these blebs (which do contain mitochondria and hence have no energy source and rapidly disintegrate) may be released into the circulation without rupture of the plasma membrane, resulting in a one-off release of cytoplasmic contents including macromolecules. Evidence supporting the presence of these membranous blebs in cardiac myocytes, is presented.
WHAT IS THE DISTRIBUTION OF TROPONIN IN HEALTHY PEOPLE AND HOW DO WE DETERMINE APPROPRIATE DECISION POINTS?

I describe a series of studies with a population of healthy children. These studies determine the distribution of cardiac troponin I, including a longitudinal study over 4 years and investigate the significance of transient elevations in troponin concentrations. A discussion into the effect of coning or subject selection, in an adult population, using laboratory and clinical markers, on the 99th percentile for troponin is also included.

WHAT INFORMATION DOES THE TROPONIN CONCENTRATION PROVIDE TO US IN THE ACS AND NON-ACS SETTING?

The third part of this thesis, chapters 5, focuses on pathology and troponin. In this chapter I investigate the significance of cTn in the ACS and non-ACS settings. In the non-ACS setting, this chapter offers a discussion demonstrating that although both cTnI and cTnT are good prognostic indicators of all cause mortality for patients with end stage renal disease, cTnT offers slightly better performance. Also described is the biomarker release during recovery from prolonged strenuous exercise in trained male cyclists and a hospital and community survey of troponin concentrations over a 24 hour period.

In the ACS setting this chapter investigates the usefulness and applicability of a mathematical model for predicting a major adverse cardiac event (MACE) in a cohort of patients presenting to a hospital emergency room and compares that with the efficacy of the currently applied and recommended decision points.

The final chapter, chapter 6, contains a general discussion of the results described in this thesis and offers some general directions towards future research.
REFERENCES


CHAPTER 2

LITERATURE REVIEW

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the western world [1]. In 2008, about 3.5 million Australians had a long term cardiovascular disorder with about 50,000 deaths attributed to CVD which equates to about 34% of all death [2] with coronary heart disease (CHD), the most prominent CVD, accounting for approximately 17% of all deaths [3]. The acute coronary syndrome (ACS) is a subset of CHD and is defined across a range of medical conditions which result in the acute reduction of blood flow to the heart. ACS encompasses both chest pain at rest (unstable angina) and heart attack (acute myocardial infarction – AMI). Of those deaths attributed to CHD about half were attributed to AMI [2]. The number of Australians dying from repeat AMI is rising and expected to increase by over 40% by 2020 [2] which will have a significant impact on overall national healthcare costs [4].

When there is evidence of myocardial necrosis consistent with acute myocardial ischemia the term AMI is used. The detection of a rise and/or fall of cardiac troponin (cTn) in the peripheral circulation, with at least one concentration above the 99th percentile of the upper reference limit is used to aid in the diagnosis of AMI [5].

NORMAL PHYSIOLOGY OF TROPONIN AND ITS APPLICATION TO ACS

Myocardial injury is detected when blood levels of troponin (cTn) are increased [5,6]. As cardiac troponins are components of the contractile apparatus of myocytes, elevations of
troponin in the blood reflect injury that may lead to necrosis of myocardial cells. These elevations do not indicate the underlying mechanism [7].

When troponin analysis was introduced into the clinical laboratory, the initial response was to identify and report to the concentration that approximated to the old definition of myocardial infarction [8], using that concentration to differentiate myocardial infarction and unstable angina [9-11]. It has been stated unequivocally that troponin release only occurs in the presence of necrosis [12]. The belief or concept that the heart is a terminally differentiated organ and unable to regenerate working myocytes and that myocytes cannot divide originated in the 1920s. If this were true, as is the accepted dogma, the heart can only respond to increases in workload by hypertrophy of existing myocytes then when this hypertrophy reaches a maximum or is exhausted, cell death and heart failure should occur[13].

Approximately 3 -8% of troponin I and 6-8% of troponin T is unbound in the cytosol [9,14] or as part of an early releasable pool [15]. Regardless of the cause of the type of myocyte injury this unbound pool of troponin is released first. It would be expected that if there is release only from this pool that the troponin concentration in the blood would increase quickly and would also fall with rapidly. The half-life of troponin T and troponin I in the blood is about 2-4 hours [10], however longer half-lives have also been suggested. A rapid rise and fall in troponin concentrations in the blood over a 24 hour period would be consistent with release of this pool and reversible myocyte damage rather than myocyte necrosis where the fall in concentration would be over a longer period, up to 10 days, because of gradual degradation of myofibrils and release of the troponin complex [9]. The prolonged half-life seen in ACS may be due to continued breakdown of these contractile proteins.

A number of studies have suggested that troponins may be released from cardiac myocytes in situations other than myocyte necrosis without the cells becoming necrotic [5,16-19].
Over the past 5 years cTn has been demonstrated in the blood of healthy adults and patients without cardiac necrosis [20-23]. Various possibilities have been suggested for release of structural proteins from the myocardium, including normal turnover of myocardial cells, apoptosis, cellular release of troponin degradation products and increased cellular wall permeability [14], formation and release of membranous blebs and myocyte necrosis [16]

**CARDIAC TROPONIN COMPLEX**

The cardiac troponin complex belongs to the proteins of the contractile apparatus, is immobilized on the thin filament of the contractile apparatus of striated muscle and has a unique primary structure for cardiac muscle [9].

In striated muscle, the troponins consist of a complex the three proteins, Troponin I (TnI), Troponin T (TnT) and Troponin C (TnC), which reside on the thin filament of the myofibril in the myocyte (Figure 2.1). Together they regulate the force and velocity of striated muscle contraction in the heart. The nomenclature of these proteins derives from their respective function in muscle contraction [24,25]:

- Troponin C - ‘C’ for calcium binding,
- Troponin I - ‘I’ for inhibitory
- Troponin T, - ‘T’ for tropomyosin binding

Cardiac troponin I (MW ~21 kDa) is a key regulatory protein in cardiac muscle contraction linking Ca^{2+}TnC binding with the activation of the reaction between the thin and thick filaments. TnI inhibits actomyosin Mg^{2+}ATPase and leads to muscle relaxation by interrupting the actin–myosin linkage. cTnT (MW ~37 kDa) interacts with both cTnI and cTnC as well as tropomyosin to attach the cTn complex to the myofibrillar thin
filament cTnC; (MW ~ 18 kDa) binds Ca\(^{2+}\) ions, which induces conformational changes that are transmitted by cTnT and cTnI phosphorylation to modulate cTnI inhibition [24-26].

Figure 2.1: Schematic of troponin complex

Cardiac and skeletal muscle isoforms of TnT and TnI can be readily immunologically distinguished from each other as the amino acid sequences of the myocardial troponins differ [27] by approximately 40% from their skeletal muscle counterparts. Cardiac isoforms possess about 30 more amino acids on the N terminal than the skeletal muscle troponins. In contrast, Troponin C from cardiac and skeletal muscle is identical [28].

**NORMAL MYOCYTE CELL TURNOVER.**

The development of the concept of cardiac remodelling coincided with the development of the concept of apoptosis and the recognition that this phenomenon represents an important mode of cell death in physiological cell turnover and pathological processes [29]. A study by Bergmann [30] demonstrated that cardiac myocytes regenerate with a decrease from 1% annual turnover at the age of 25 to 0.45% at the age of 75 years with approximately 50% of cells exchanged during a normal life span.
DEFINITION OF THE ACUTE CORONARY SYNDROME (ACS)

The Acute coronary syndrome (ACS), a sub-group of CHD, is a continuum of diseases that range from health to angina without evidence of necrotic damage to the heart muscle, through ST-segment-elevation myocardial infarction (STEMI) to acute myocardial infarction (AMI) and death [31,32].

Atherosclerosis is the underlying cause of the ACS in the majority of cases [2-4]. The slow development of cholesterol plaque on the inside of the coronary arteries will ultimately restrict the lumen and cause a reduction in blood flow. If the plaque ruptures and the resulting unstable cap of fibrin and platelets dislodges producing emboli, significant blood flow reduction or complete blocking of blood flow to the heart can occur. The resultant ischaemia can give rise to the sudden onset of angina (unstable angina) leading to severe chest pain and potential myocyte necrosis, myocardial infarction (MI) [5]. Death can occur if blood flow is not restored. Under normal conditions blood flow may still be adequate but may be insufficient when an elevated blood flow is required such as in exercise. This is known as stable angina and is not considered part of ACS.

DEVELOPMENT OF ACS

Each condition of ACS, unstable angina, non (NSTEMI) and ST-elevation myocardial infarction (STEMI), share a common pathophysiological origin, which typically involves instability and fracture in the protective fibrous cap of an atheromatous plaque [5,33]. When these plaques rupture and core constituents such as lipid, smooth muscle and foam cells are exposed; it leads to the local generation of thrombin and deposition of fibrin [34]. Formation of an intracoronary thrombus occurs due to platelet aggregation and adhesion [35].
Embolisation from friable coronary thrombus may occur which can lead to cell necrosis and the release of cardiac troponins [36]

STEMI usually occurs when thrombus forms on a ruptured atheromatous plaque and blocks an epicardial coronary artery. Patient survival depends on several factors, the most important being restoration of blood flow, the time taken to achieve this, and the sustained patency of the affected artery [35].

Figure 2.2 provides a diagram of the development an atheromatous plaque. Figure 2.3 shows the preclinical and clinical phases of atheromatous plaque development

![Atherosclerotic Plaque Diagram](image)

Figure 2.2. The 7 stages of development of an atherosclerotic plaque. First LDL moves into the sub endothelium and is oxidized by macrophage and smooth muscle cells (1 & 2). Release of growth factors and cytokines attracts additional monocytes (3 & 4). Foam cell accumulation and smooth muscle cell proliferation result in growth of the plaque (6, 7 & 8)

Adapted from [37]
Patients with NSTEMI differ from patients with STEMI only through the absence of ST elevation on the presenting ECG. Although there is no universally accepted definition of unstable angina, it has been described as a clinical syndrome between stable angina and AMI [34]. Unstable angina can be recognised by ischemic-type chest pain that is more frequent, severe or prolonged than the patient’s usual angina symptoms, occurs at rest or minimal exertion, or is difficult to control with drugs. Recent onset angina is also classified as unstable [39].

Elevation in troponin and related biomarker concentrations provide robust evidence for the diagnosis and prognosis of ACS [5]. Figure 2.4 describes ACS as a continuum of disease.
### ACUTE MYOCARDIAL INFARCTION (AMI)

The term acute myocardial infarction is used when there is evidence of myocardial necrosis in a clinical setting consistent with acute myocardial ischemia.

Myocardial infarction (MI) can be recognized by clinical features, including electrocardiographic (ECG) findings, elevated values of biochemical markers of myocardial necrosis, by imaging, or defined histologically. It is a major cause of death and disability worldwide. MI may be the first manifestation of coronary artery disease (CAD) or it may occur, repeatedly, in patients with established disease [40].

MI is defined as myocardial cell death due to prolonged ischemia. After the onset of myocardial ischemia, histological cell death is not immediate, but takes a finite amount of time to develop but may be as little as 20 minutes [41]. It takes several hours after histological cell death before myocardial necrosis can be identified by macroscopic or
microscopic post-mortem examination. Complete necrosis of at risk myocytes requires 2–4 hours or longer, depending on the presence of collateral circulation to the ischemic zone, persistent or intermittent coronary arterial occlusion, the sensitivity of the myocytes to ischaemia, preconditioning and their individual demand for oxygen and nutrients [5].

PATHOPHYSIOLOGY OF MYOCARDIAL INFARCTION

The most common cause of MI is atheromatous plaque rupture with subsequent exposure of the basement membrane resulting in platelet aggregation, thrombus formation, fibrin accumulation, haemorrhage into the plaque, and varying degrees of vasospasm. This can result in partial or complete occlusion of the vessel and subsequent myocardial ischemia. Total occlusion of the vessel for more than 4-6 hours results in irreversible myocardial necrosis, but reperfusion within this period can salvage the myocardium and reduce morbidity and mortality.

The development of atherosclerotic plaque occurs over a period of years to decades [5]. The two primary characteristics of the clinically symptomatic atherosclerotic plaque are a fibromuscular cap and an underlying lipid-rich core [5,34-36]. Plaque erosion can occur because of the actions of matrix metalloproteases [14] and the release of other collagenases and proteases in the plaque, which result in thinning of the overlying fibromuscular cap. The action of proteases, in addition to hemodynamic forces applied to the arterial segment, can lead to a disruption of the endothelium and fissuring or rupture of the fibromuscular cap. The loss of structural stability of a plaque often occurs at the juncture of the fibromuscular cap and the vessel wall. Disruption of the endothelial surface can cause the formation of thrombus via platelet-mediated activation of the coagulation cascade. If a thrombus is large enough to occlude coronary blood flow, an MI can result [5,34,35].
Myocyte death first occurs in the area of myocardium most distal to the arterial blood supply: the endocardium. As the duration of the occlusion increases, the area of cell death enlarges, extending from the endocardium to the myocardium and ultimately to the epicardium. This cell death then spreads laterally. The extent of myocardial cell death defines the magnitude of the MI [8,35].

The severity of an MI depends on three factors [8]

- the level of the occlusion in the coronary artery
- the length of time of the occlusion
- the presence or absence of collateral circulation

The primary risk factors associated with the development of atherosclerotic coronary artery disease and MI are hyperlipidemia, diabetes mellitus, hypertension, tobacco use, male gender, and family history [2-4].

**CLINICAL FEATURES OF MYOCARDIAL ISCHEMIA AND INFARCTION**

The development of myocardial ischemia is the first step in the development of MI. The ischaemia results from an imbalance between oxygen supply and demand. Symptoms of ischaemia include chest, and upper extremity discomfort or dyspnoea or fatigue. The discomfort associated with AMI usually lasts for more than 20 minutes and may be diffuse. However, these symptoms are not specific for myocardial ischemia. Myocardial infarction may occur with atypical symptoms such as palpitations or cardiac arrest. MI may occur without symptoms such as seen sometimes in the elderly or postoperative and critically ill patients [6].
MI is classified into various types, based on histological, clinical and prognostic differences, along with different treatment strategies. International guidelines have been developed for the definition of myocardial infarction. The first guideline was released in 2000 and implied that any necrosis in the setting of myocardial ischemia should be labelled as MI [42]. A further refinement was released in 2007 and emphasized the different conditions which might lead to an MI [6]. The current Universal guidelines released in 2012 recognizes that very small amounts of myocardial injury or necrosis can be detected by with the introduction of more sensitive biochemical markers and/or imaging [5].

SPONTANEOUS MYOCARDIAL INFARCTION (MI TYPE 1)

Type 1 myocardial infarction is an event related to atherosclerotic plaque rupture, ulceration, fissuring, erosion, or dissection with resulting intraluminal thrombus in one or more of the coronary arteries. This event leads to decreased myocardial blood flow or distal platelet emboli and myocyte necrosis. Spontaneous MI is responsible for the majority of all cases of MI. In up to 20% of patients, particularly women there may be underlying severe CAD, but, no obstructive or coronary artery disease may be found with angiography. [5,43]

MYOCARDIAL INFARCTION SECONDARY TO AN ISCHEMIC IMBALANCE (MI TYPE 2)

In instances of myocardial injury with necrosis, where a condition other than CAD, such as coronary embolism, anaemia or arrhythmias, contributes to an imbalance between myocardial oxygen supply and/or demand, the term ‘myocardial infarction type 2’ is used [5]. Figure 2.5 shows the differentiation between types 1 and type 2 MI according to the condition of the coronary arteries. And the MI classifications are highlighted in table 2.1.
Figure 2.5. Differentiation between MI types 1 and 2 according to the condition of the coronary arteries. From [5]

Table 2.1: Classifications of Myocardial Infarction. Adapted from [5].

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1: spontaneous myocardial infarction</td>
<td>Spontaneous myocardial infarction related to atherosclerotic plaque rupture, ulceration, fissuring, erosion, or dissection with resulting intraluminal thrombus in one or more of the coronary arteries leading to decreased myocardial blood flow or distal platelet emboli with ensuing myocyte necrosis.</td>
</tr>
<tr>
<td>Type 2: MI secondary to an ischemic imbalance</td>
<td>Myocardial injury with necrosis where a condition other than coronary artery disease contributes to an imbalance between myocardial oxygen supply and/or demand.</td>
</tr>
<tr>
<td>Type 3: MI resulting in death when biomarker values are unavailable</td>
<td>Cardiac death with symptoms suggestive of myocardial ischemia and presumed new ischemic ECG changes or new left bundle branch block.</td>
</tr>
<tr>
<td>Type 4a: MI related to percutaneous coronary intervention</td>
<td>Myocardial injury or infarction associated with mechanical revascularization procedures such as percutaneous coronary intervention or coronary artery bypass grafting (CABG) surgery or myocardial infarction associated with stent thrombosis detected by coronary angiography or autopsy in the setting of myocardial ischemia. Elevation of cardiac troponin values may be detected following these procedures, since various insults may occur that can lead to myocardial injury with necrosis.</td>
</tr>
<tr>
<td>Type 4b: MI related to stent thrombosis</td>
<td></td>
</tr>
<tr>
<td>Type 5: MI related to coronary artery bypass grafting</td>
<td></td>
</tr>
</tbody>
</table>
BIOMARKER USE IN THE DETECTION OF MYOCARDIAL INJURY WITH NECROSIS

Myocardial injury is indicated when concentrations of biomarkers such as cardiac troponin (cTn) are elevated in the patients’ peripheral circulation. Whilst the elevations of cardiac troponin concentrations seen in the peripheral circulation are reflective of injury leading to necrosis of the myocyte, they do not indicate the underlying mechanism [44].

Where there is evidence of myocardial necrosis in a clinical setting consistent with acute myocardial ischemia, the Third Universal Definition of Myocardial Infarction [5] states that any one of the following criteria meets the diagnosis for myocardial infarction:

- “Detection of a rise and/or fall of cardiac biomarker values (preferably cardiac troponin) with at least one value above the 99th percentile URL and with at least one of the following: (i) symptoms of ischemia, or (ii) new or presumed new significant ST-segment–T wave (ST–T) changes or new left bundle branch block, or (iii) development of pathological Q waves in the electrocardiogram, or (iv) imaging evidence of new loss of viable myocardium or new regional wall motion abnormality, or (v) identification of an intracoronary thrombus by angiography or autopsy.”

- “Cardiac death with symptoms suggestive of myocardial ischemia and presumed new ischemic electrocardiographic changes or new left bundle branch block, but death occurred before cardiac biomarkers were obtained, or before cardiac biomarker values would be increased.”

- “Percutaneous coronary intervention related myocardial infarction is arbitrarily defined by elevation of cardiac troponin values (>5 × 99th percentile URL) in patients with normal baseline values (≤99th percentile URL) or a rise of cardiac troponin values >20% if the baseline values are elevated and are stable or falling. In addition,
either (i) symptoms suggestive of myocardial ischemia, or (ii) new ischemic electrocardiographic changes, or (iii) angiographic findings consistent with a procedural complication, or (iv) imaging demonstration of new loss of viable myocardium or new regional wall motion abnormality are required.”

- “Coronary angiography or autopsy in the setting of myocardial ischemia and with a rise and/or fall of cardiac biomarker values with at least one value above the 99th percentile URL.”

- “Coronary artery bypass grafting related myocardial infarction is arbitrarily defined by elevation of cardiac biomarker values (>10 × 99th percentile URL) in patients with normal baseline cardiac troponin values (≤99th percentile URL). In addition, either (i) new pathological Q waves or new left bundle branch block, or (ii) angiographic documented new graft or new native coronary artery occlusion, or (iii) imaging evidence of new loss of viable myocardium or new regional wall motion abnormality”.

THE CARDIOSPECIFICITY OF TROPONIN: EVIDENCE OF SKELETAL MUSCLE RELEASE OF cTnT

During ontogenetic development of cardiac and skeletal muscles the synthesis of multiple isoforms of troponin subunits occurs. Expression of these isoforms and the extent of phosphorylation of both cTnT and cTnI, under different pathological situations such as ischaemia, can affect the contraction function and the myofibrillar activity of the heart [45]. It has been demonstrated that foetal hearts showed different troponin I and T isoform expression and function compared with adult hearts.
It is generally thought that the myocardium expresses no more than two isoforms of cTnT. However, Anderson [46] resolved five isoforms: TnT1, TnT2, TnT3, TnT4, and TnT5, then demonstrated that the amount of TnT2 relative to the total amount of cTnT decreased significantly with age. In contrast, the relative amount of TnT4 increased with age. The relative amounts of the other two isoforms change biphasically with development. This suggests that developmental changes in cTnT expression may account for some of the maturational changes observed in the physiological and biochemical properties of the myocardium.

Western blots analysis has demonstrated that TnT1, the cardiac isoform with the slowest electrophoretic mobility, is expressed prominently in the immature hearts, in addition to TnT2, TnT3, and TnT4, but TnT1 was not evident in the 3-month and 6-month postnatal hearts. This group then examined the ontogenic expression of cTnT in human striated muscle. Western blots of normal and failing adult heart proteins demonstrated that the two isoforms, TnT1 and TnT2, are expressed in different amounts, with TnT2 being significantly greater in failing hearts [47]. In the foetal heart, four cTnT isoforms were found, two of which had the same electrophoretic mobilities as the adult cardiac isoforms TnT1 and TnT2. Foetal skeletal muscle expressed two of the four foetal cardiac TnT isoforms, one of which co-migrated with adult cardiac TnT1. These cardiac isoforms were expressed in low abundance in foetal skeletal muscle relative to seven fast skeletal muscle TnT isoforms [47,48].

**TROPONIN AND NON ACUTE CORONARY SYNDROME (ACS)**

Elevated cTn concentrations are seen in non-coronary-related myocardial conditions such as pulmonary embolism or myocarditis and should be considered when patients present with
chest pain. As assay sensitivity has increased, the list of non-ACS causes of an abnormally elevated cTn concentration (transient or prolonged) has expanded [49].

Table 2.2 identifies some non coronary causes of elevated troponin concentrations in the peripheral circulation.

Table 2.2: Non-coronary conditions that may cause elevated troponin. Adapted from [7]

<table>
<thead>
<tr>
<th>NON-CORONARY CAUSES OF INCREASE IN TROPONIN CONCENTRATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal dysfunction (chronic or acute)</td>
</tr>
<tr>
<td>Heart failure (chronic or acute)</td>
</tr>
<tr>
<td>Aortic valvular disease (aneurysm)</td>
</tr>
<tr>
<td>Severe burns</td>
</tr>
<tr>
<td>Rhabdomyolysis</td>
</tr>
<tr>
<td>Infiltrative disease (Haemochromatosis, amyloidosis)</td>
</tr>
<tr>
<td>Sepsis</td>
</tr>
<tr>
<td>Stroke</td>
</tr>
<tr>
<td>Strenuous exercise</td>
</tr>
</tbody>
</table>

Because sensitive cTn assays can measure troponin concentration in almost all individuals, we need to be able to discriminate slight increases in concentration, associated with acute myocardial damage as a result of ischemia, from other causes of myocardial necrosis [50] that lead to chronic increases in concentration.

These increases in troponin concentration reflect the sensitivity of the marker for myocardial cell injury and should not be labelled as a “false positive” observation.

TROPONIN AND RENAL DISEASE

Elevations in troponin concentration are frequently seen in patients with elevated serum creatinine concentrations (i.e. >221 umol/L) in the absence of proven ACS, and this is associated with an adverse prognosis [39,51].
There is a high morbidity and mortality among patients on renal dialysis. Death for patients on haemo- and peritoneal dialysis are as high as 15.4% [52]. Cardiovascular (CV) disease accounts for 43% of all deaths in patients with end-stage renal disease [53]. In particular, the mortality rate of patients on maintenance dialysis following myocardial infarction (MI) is extremely high [54]. CV disease continues to be the leading cause of mortality and morbidity following renal transplantation [55]. Measurement of the cardiac troponins to aid in the assessment of myocardial injury has been available since the mid-1990s and studies have reported that increased cTnT concentration in the blood of asymptomatic patients on dialysis is a predictor of adverse outcome in this population [16]. Similar associations have been shown for other biomarkers including cTnI [52,55]. Patients with renal failure have been also been shown to have a high baseline prevalence of abnormal cTnI levels, albeit lower than the prevalence of abnormal cTnT levels [53]. It has been suggested that cTnI is cleared by a predominantly non-renal mechanism and that cTnT is cleared through the kidney [50]. The underlying mechanism behind these elevations in troponin has not been fully elucidated. The high incidence of CAD in ESRD patients [53] along with the relationship between troponin concentrations and severity of the CAD [56,57] suggests that subclinical ischaemic cardiac damage may be present and be the cause of these elevations. A study by deFillipi [57] demonstrated that using cardiovascular magnetic resonance (CMR) 23% of ESRD patients with a cTnT concentration >70 ng/L had little to no evidence of myocardial ischaemia. This and the findings of Jacobs [58], where 15/32 ESRD patients had at least one elevated TnT in a 6 month period without evidence of ischaemia, may suggest that the half life of cTn and dialysis itself may have a direct effect on the troponin concentration [59].

Whatever the mechanism, elevations in cTn concentrations are highly predictive of adverse events [60-62].
Sepsis is a major source of morbidity and mortality. Elevated concentrations of cTnI and cTnT are frequently observed in patients with severe sepsis and septic shock even in the absence of an ACS [63].

The mechanisms underlying TnI release in patients with sepsis are still unknown. A number of hypotheses have been formulated [64]. One mechanism involves the release of TNF alpha, which increases permeability of endothelial monolayers to macromolecules. It is hypothesized that this may also occur at the myocyte cell membrane level causing the release of troponins [65]. In septic patients, elevated troponin concentrations have been shown to correlate with longer duration of hypotension, incidence of mechanical ventilation, the need for vasoactive medication, and longer bed stay in intensive care units [64].

Pre-existing CAD and demand ischemia may also account for TnI release in sepsis. However, it has been shown that cTnI release can also occur in patients in whom significant CAD has been excluded [65].

Altmann [66] found no differences in coagulation parameters analysed with rotational thromboelastometry between TnI positive and negative patients with systemic inflammatory response syndrome (SIRS), severe sepsis, and septic shock. This suggests that pathophysiological mechanisms other than thrombus-associated myocardial damage might play a major role, including reversible myocardial membrane leakage and/or cytokine mediated apoptosis in these patients.

**IRREVERSIBLE DAMAGE**

Cardiac troponins (cTnT and cTnI) are predominantly bound (94%–97%) via tropomyosin to actin filaments of sarcomeres, with only a small proportion of cTnT (6%–8%) and cTnI (3%–
8%) found in the soluble cytoplasmic pool [9,14] or as part of an early releasable pool [15,67]. Intracellular compartmentalization of cardiac troponins has significant impact on their rate of release following myocardial damage.

When a cardiac cell is irreversibly injured, loss of membrane integrity occurs [68] and the free cytoplasmic pool is immediately released followed by a slow continual release of the myofibril-bound proteins. A prolonged increase in troponin is seen. In patients with AMI, cardiac troponins appear in the serum relatively early after the onset of chest pain (from 2 h to 10 h), peak at 12–24 h and may remain elevated for 4–14 days (i.e., cTnI from 4 days to 7 days and cTnT from 10 days to 14 days) [12].

A study by Hessel et al.[69] using rat myocytes in culture demonstrated that during the first 12 hours of metabolic inhibition the myocyte viability was unchanged, resulting in no release of intact cTnI and cTnT. However, after treatment with azide the cardiomyocytes showed progressive cell death accompanied by release of intact cTnI and cTnT along with 4 cTnI and 3 cTnT degradation products over 12 to 30 hours. This suggests that metabolic inhibition of myocytes induces a parallel release of intact cTnI and cTnT along with their degradation products. This only occurs after the onset of irreversible damage.

**REVERSIBLE DAMAGE**

It has been formally stated that cardiac troponin is only released when cardiac myocytes undergo necrosis [12,70]. However, the possibility of troponin release due to ischemia without necrosis has been raised [68,71]. A number of papers in the literature over the past 10 years have demonstrated the presence of troponin in the circulation, without any subsequent evidence of coronary artery disease or cardiac myocyte necrosis.
Whilst many of these conditions have been associated with major systemic disease [72], there have been those that have specifically related to conditions affecting the myocardium causing transient myocardial ischemia such as supraventricular tachycardia (SVT), where patients presenting with SVT and no evidence of coronary artery disease on angiography had detectable troponin, which after treatment quickly became undetectable [73,74].

It is now accepted that there are a number of clinical situations where there is no apparent cardiac injury but troponin is present in variable concentrations in the circulation [5,7,22]. In these cases the half-life of the circulation troponin is usually substantially shorter than that seen when troponin is released where there is evidence of necrosis.

Apple et al. [75] found a significantly shorter troponin half-life of disappearance in patients with non-Q wave infarcts versus those with Q wave infarcts. It may be assumed that STEMI infarcts would have predominant necrotic release of troponin, whereas many patients with NSTEMI may have a significant ischemic release of troponin.

Katus [9] reported that if patients with MI were reperfused early, the release kinetics of troponin T showed a sharp peak and sharp initial fall in troponin concentration and the troponin released was cytoplasmic in origin. Troponin released upon later reperfusion was cleared more slowly and had the characteristics of structurally bound troponin. These studies all show that troponin can be released with markedly shorter half-lives of clearance from that characteristically found in association with acute myocardial infarction.

There are a number of potential major pathobiological mechanisms for this troponin release in the absence of necrosis.
FORMATION AND RELEASE OF MEMBRANOUS BLEBS.

It has been hypothesised by Hickman [16] that the presence of membranous blebs in cardiac myocyte may enable troponin to be released from cardiac cells due to ischemia alone, without necrosis.

Membranous blebs are bubbles in the plasma membrane which develop in response to ischemia. As the ischemia is prolonged these blebs grow and will eventually rupture and cell necrosis occurs. If however, re-oxygenation occurs before blebs rupture, then they are either resorbed or shed into the circulation releasing their cytoplasmic contents, with the cells plasma membrane remaining intact. This mechanism has been described in liver cells, where large molecules can pass from intracellular to extracellular spaces without necrosis occurring [76,77]. Because the blebs are released into the circulation only at the time of re-oxygenation they represent a one-off release of cytoplasmic contents which is then cleared at the half-life of that substance.

Cultured cardiac myocytes have been shown to develop blebs during anoxia and to release cytosolic enzymes without undergoing necrosis [78-81]. Remppis, [82] demonstrated that short duration ischemia followed by re-oxygenation led to a very short burst of cTnT release, but if the period of ischemia was extended before re-oxygenation, troponin release was greater and more prolonged. Hamm [83] and Sabatine [84] have demonstrated that in unstable angina or patients experiencing transient ischaemia that the short lived troponin detected in the patient’s blood may reflect bleb formation but there is little evidence supporting this concept in man. Figures 2.6 illustrate mechanisms of troponin release.
Figure 2.6. Mechanism of troponin release. A: Release of cytosolic troponin followed by structural troponin into blood following irreversible injury with permanent myocyte membrane damage. B: Release of cytosolic troponin only into blood following reversible injury and bleb formation. From [16]

**TROPONIN AND EXERCISE**

Whilst the cardiac troponins appear to be specific for myocardial tissue, troponin release has been documented in a variety of conditions other than the acute coronary syndrome [72]. The most common of these is the association with vigorous exercise. This has been demonstrated in several studies where exercise has been shown to induce the release of cTnT or cTnI in obviously healthy athletes [85,86]. Most exercise studies have utilized single point
measurements of troponin, some have looked at two or more measures and in nearly all cases, troponin half-life has been short [87-89].

Significant elevations of cTnT and cTnI have been reported after prolonged and competitive endurance exercise bouts such as marathon and ultra-marathon running, ultra-triathlon, road cycling, long-distance mountain bike races, or cross-country skiing in adult athletes [89-92]. Elevations in cTnT and cTnI concentrations have been reported in 60% of young males after a 21-km run [92]. Besides the structurally bound cTnT and cTnI, approximately 6–8% of cTnT and 2–8% of cTnI are in the cytosolic pool as unbound cTnT and cTnI [93].

In patients with MI, at first the cytoplasmic unbound cardiac troponin is released, followed by the structurally bound troponin of the troponin complex [94] and elevations of cTnT and cTnI appear in the blood after 2–4 h and can persist for up to 21 days [94]. In contrast, with athletes, exercise-associated elevations of cardiac troponins typically decrease significantly within 24 hours after exercise and usually reach normal values within this period [78,88,91-97]. A meta-analysis looking at exercise-associated troponin release [98,99] found that running by non-elite individuals was also associated with the highest incidence of troponin release. A relationship between exercise-associated elevations of cardiac troponins and cardiovascular risk factors seems not to exist [97]. A relationship associated with the amount of prerace endurance training undertaken before a marathon has demonstrated greater increases in cTnT concentrations in non-elite runners with <56km per week training, when compared with non-elite runners with higher training distances [97100]. One study has reported that less marathon experience and younger age were associated with troponin increases after the Boston marathon [101].

In athletes, these increases in cardiac troponin concentrations tend to be relatively small and of short duration and therefore are more likely to reflect a reversible membrane leakage of
cardiomyocytes with troponin release from the free cytosolic pool, not bound in the contractile apparatus [14].

**PROTEOLYTIC TROPONIN DEGRADATION PRODUCTS AND INCREASED CELLULAR WALL PERMEABILITY.**

Proteolysis creating small fragments could allow troponin to pass through a cell membrane with normal membrane integrity. Fifteen minutes of mild ischemia has been shown to cause development of troponin I degradation products [102].

Reversible injury to the cardiac myocyte due to myocardial stretch, or ischemia caused by pressure or volume overload may activate some intracellular proteases, such as metalloproteinase 2 and 14. These proteases are able to intracellularly degrade troponin [14].

Overload-induced stretch at the cardiomyocyte level is sensed by integrins. Simulation of stretch responsive integrins has been shown to result in release of intact troponin from cultured viable cardiomyocytes without lactate production suggesting that the troponin release may occur without ischemia or necrosis [103]. The integrins link the extracellular matrix to the intracellular and this mechanism may also be involved in the stretch-induced release of troponin and its degradation products. The integrins have been associated with sepsis and septic shock [104]. Figure 2.7 illustrates the release mechanisms of cTnI and cTnT from cardiomyocytes following reversible or irreversible damage.
Figure 2.7: Schematic model illustrating the release mechanisms of cTnI and cTnT from cardiomyocytes following reversible or irreversible damage. The release of cardiac troponins may be the result of leakage from reversibly damaged myocardial membranes as intact non-degraded protein chain (left side corner of the lower panel) or by release of proteolytic troponin degradation products through the intact myocyte membrane (right side corner of the lower panel). In the latter case, troponins are presumed to be degraded by matrix metalloproteinases (MMP) activated by integrin mediated myocardial stretch. The biggest fragments of asymmetrical troponin degradation are unlikely to directly traverse the intact myocyte membrane and may be released only in cases of membrane leakage. From [14]

TROPONIN ASSAYS

Troponin assays consist of a range of research, pre-commercial and commercial cTnI and cTnT methods that differ from each other in assay format by the types of antibody used. For cTnI, the antibodies may be combinations of mouse monoclonal and/or polyclonal antibodies or the epitopes to which they bind, and by the type of indicator molecule that is used.
The detection method for these assays is by spectrophotometry, fluorescence, chemiluminescence and electrochemistry [105]. The latest generation of troponin assays providing improved sensitivity are able to measure cTn concentrations up to 100-fold lower than previous generation assays. These higher sensitivity assays incorporate longer incubation times and/or signal amplification techniques to achieve this improvement.

cTnI assays should recognise complexed, free and modified forms of cTnI equally. This allows monitoring of total cTnI concentrations present in samples over the course of a patient’s infarct as there is progressive modification of cTnI of these patients after tissue release resulting in a heterogeneous mixture of forms present in plasma including predominantly the binary cTnI-C complex with smaller amounts of the ternary cTnI-T-C complex, binary cTnI-T complex and free cTnI [106]. After tissue release, the main form of cTnT antigen found in blood is the free cTnT form from the breakdown of the cTnI-T-C complex into binary cTnI-C and cTnT forms.

The limit of blank (LoB) and the limit of detection (LoD) are important characteristics of a troponin assay and are used to discriminate between the presence and absence of troponin. LoB is the highest apparent troponin concentration expected to be found when replicates of a blank sample containing no troponin are tested. LoD is the lowest troponin concentration likely to be reliably distinguished from the LoB and at which detection is feasible. The limit of quantitation (LoQ) is the lowest concentration at which troponin cannot only be reliably detected but at which predefined goals for bias and imprecision are met [107]. This may be a predetermined consensus % CV, such as an imprecision of 10% for troponin that is recommended for the diagnosis of MI [5] or a desirable total error goal of ±22.5% CV based on the biological variability of cTnI for example [108,109].

A manufacturer establishing the LoB, LoD, or LoQ typically would perform studies using more than one analyser and one lot of reagent to encompass the variability that users can
expect to encounter in the routine laboratory [107]. Clinical laboratories can validate these characteristics using a smaller number of samples and generally will use only one analyser and one lot of reagent.

The imprecision performance of a new troponin assay should be verified to confirm the imprecision of the manufacturer’s performance claims. Ideally both patient samples and manufacturer’s quality controls (QC) are tested at the time of assay evaluation. The assessment of longer-term imprecision using quality control materials to monitor assay transferability and bias across different reagent and calibrator lots is required.

Troponin imprecision is ideally assessed using a patient pooled material with concentrations that approximate the decision limit for that assay in line with the CLSI EP15-A2 guidelines [110]. This decision limit is recommended to be the 99th percentile concentration of the value distribution of a cardio-healthy reference population [5]. Troponin assays should have an imprecision of ≤10% CV at the 99th percentile limit of the value distribution in a reference population [110]. Of the 24 commercially available cTn assays currently listed on the IFCC website by the Working Group on Standardization of Troponin I [111], only seven appear to fulfill the guideline recommendations based on manufacturers’ declared performance.

The Study Group on Biomarkers in Cardiology of the ESC Working Group on Acute Cardiac Care endorses the recommendation of the ESC/AACF/AHA/WHF Task Force that troponin imprecision of 10% CV or less at the 99th percentile is desirable [112]. The group also recommend not utilising troponin assays with an imprecision at the 99th percentile of above 20% CV because of the lack of data on the potential risk of misclassification of patients. The 99th percentile and imprecision limits for commercial and pre-commercial/research troponin assays are provided in table 2.3. A scorecard developed by Apple [113] to define the generation and type of assay is presented in table 2.4.
Table 2.3: 99th percentile and imprecision levels for troponin assays as at December 2012.

NA indicates not available. Adapted from [114].

<table>
<thead>
<tr>
<th>COMMERCIALY AVAILABLE ASSAYS - COMPANY/PLATFORM</th>
<th>99th % (µg/L)</th>
<th>CV at 99th %</th>
<th>10% CV (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbott AxSYM ADV</td>
<td>0.04</td>
<td>14.0</td>
<td>0.16</td>
</tr>
<tr>
<td>Abbott ARCHITECT</td>
<td>0.028</td>
<td>14.0</td>
<td>0.032</td>
</tr>
<tr>
<td>Abbott i-STAT</td>
<td>0.08</td>
<td>16.5</td>
<td>0.10</td>
</tr>
<tr>
<td>Alere Triage SOB</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Alere Triage Cardio 3</td>
<td>NA</td>
<td>17.0 (at 0.02)</td>
<td>NA</td>
</tr>
<tr>
<td>Beckman Coulter Access Accu</td>
<td>0.04</td>
<td>14.0</td>
<td>0.06</td>
</tr>
<tr>
<td>bioMerieux Vidas Ultra</td>
<td>0.01</td>
<td>27.7</td>
<td>0.11</td>
</tr>
<tr>
<td>Mitsubishi Chemical PATHFAST</td>
<td>0.029</td>
<td>5.0</td>
<td>0.014</td>
</tr>
<tr>
<td>Ortho VITROS Troponin I ES</td>
<td>0.034</td>
<td>10.0</td>
<td>0.034</td>
</tr>
<tr>
<td>Radiometer AQT90 FLEX TnI</td>
<td>0.023</td>
<td>17.7</td>
<td>0.039</td>
</tr>
<tr>
<td>Radiometer AQT90 FLEX TnT</td>
<td>0.017</td>
<td>15.2</td>
<td>0.025</td>
</tr>
<tr>
<td>Response Biomedical RAMP</td>
<td>NA</td>
<td>18.5 (at 0.05)</td>
<td>0.21</td>
</tr>
<tr>
<td>Roche Cardiac Reader cTnT</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Roche E 2010 /cobas e 411 / E 170 / cobas e 601 / 602 TnI (4th gen)</td>
<td>NA</td>
<td>NA</td>
<td>0.03</td>
</tr>
<tr>
<td>Roche E 2010 /cobas e 411 / E 170 / cobas e 601 / 602 hs-TnT</td>
<td>0.014</td>
<td>10.0</td>
<td>0.013</td>
</tr>
<tr>
<td>Roche E 2010 /cobas e 411 / Roche E 170 / cobas e 601 / 602 cTnl</td>
<td>0.16</td>
<td>NA</td>
<td>0.3</td>
</tr>
<tr>
<td>Siemens Centaur Ultra</td>
<td>0.04</td>
<td>8.8</td>
<td>0.03</td>
</tr>
<tr>
<td>Siemens Dimension RxL</td>
<td>0.07</td>
<td>20.0</td>
<td>0.14</td>
</tr>
<tr>
<td>Siemens Dimension EXL</td>
<td>0.056</td>
<td>10.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Siemens Immulite 2500 STAT</td>
<td>0.2</td>
<td>NA</td>
<td>0.42</td>
</tr>
<tr>
<td>Siemens Immulite 1000 Turbo</td>
<td>NA</td>
<td>NA</td>
<td>0.64</td>
</tr>
<tr>
<td>Siemens Stratus CS</td>
<td>0.07</td>
<td>10.0</td>
<td>0.06</td>
</tr>
<tr>
<td>Siemens VISTA</td>
<td>0.045</td>
<td>10.0</td>
<td>0.04</td>
</tr>
<tr>
<td>Tosoh ST AIA-PACK</td>
<td>0.06</td>
<td>8.5</td>
<td>NA</td>
</tr>
<tr>
<td>RESEARCH ASSAYS</td>
<td>99th % (ng/L)</td>
<td>CV at 99th%</td>
<td>10% CV (ng/L)</td>
</tr>
<tr>
<td>Beckman Coulter Access hs-cTnl</td>
<td>8.6</td>
<td>10.0</td>
<td>8.6</td>
</tr>
<tr>
<td>Nanosphere VeriSens hs-cTnl</td>
<td>2.8</td>
<td>9.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Singulex hs-cTnI</td>
<td>10.1</td>
<td>9.0</td>
<td>0.88</td>
</tr>
<tr>
<td>Abbott hsTnl</td>
<td>13.6</td>
<td>4.8</td>
<td>3.9</td>
</tr>
</tbody>
</table>
Table 2.4 Scorecard designation of troponin assays. Adapted from [113]

<table>
<thead>
<tr>
<th>Acceptance designation</th>
<th>Total imprecision at the 99th percentile, CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guideline acceptable</td>
<td>≤10</td>
</tr>
<tr>
<td>Clinically usable</td>
<td>≤20</td>
</tr>
<tr>
<td>Not acceptable</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Assay designation</th>
<th>Measurable normal values below the 99th percentile, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 4 (third generation, hs)</td>
<td>≥95</td>
</tr>
<tr>
<td>Level 3 (second generation, hs)</td>
<td>75 to &lt;95</td>
</tr>
<tr>
<td>Level 2 (first generation, hs)</td>
<td>50 to &lt;75</td>
</tr>
<tr>
<td>Level 1 (contemporary)</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>

**TROPONIN ASSAY ISSUES - STANDARDISATION**

With existing on-market TnI assays marketed by different in vitro diagnostic companies and the use of various standard materials for calibration and antibodies with different epitope specificities, results that are unique to a certain method or instrument are obtained.

Immunoassays using anti-cTnI monoclonal antibodies are dependent on the epitope regions recognized by the antibodies incorporated into each assay. Protein regions within the cTnI molecule are susceptible to the modifications as well as the variable nature of epitope-dependent binding to heterophile and autoantibodies are likely to exhibit variable immune reactivity and even loss of reactivity in some cases. This variability can lead to altered signal generation within sandwich-type immunoassays that use capture and detection antibodies directed against these modified regions [115].

With the acute ongoing release of cTnI during MI, the ongoing cTnI modifications in myocardial tissue and blood, and with different clearance mechanisms and rates of clearance for the multiple isoforms of cTnI that accumulate over the first 24 h after an MI, it is unlikely that individual patients blood will contain the same cTnI isoform composition [116].
High-sensitivity assays have begun to improve our understanding of the clinical implications of measurable cTnI concentrations in healthy individuals [23] but whether the distributions of cTnI isoforms are the same from individual to individual or whether they remain the same within healthy individuals is unclear. This is highlighted where some assays show gender differences but where others do not. Because of these factors, cTnI values obtained with different assays cannot currently be interchanged. cTnT assays are provided by only one manufacturer and as such do not suffer from this lack of standardisation.

**TROPOIN ASSAY ISSUES - INTERFERENCE**

Haemolysis, icterus, lipaemia, anticoagulant(s) and sample storage may interfere with the measurement of troponin in some assays resulting in either false-positive or false-negative values. Florkowski [117] and Bais [118] have reported on haemolysis in cTnI and cTnT assays. These studies showed haemolysis is likely to be more clinically significant at low troponin concentration close to the decision limit and haemoglobin concentration as low as 1.9 g/L can cause >20% change in cTn concentration [118].

Interference is method-dependent and may vary for each assay. Endogenous antibody interferences which are sources of interference for the sandwich-type immunometric troponin assays due to endogenous antibodies directed against proteins of non-human species. These heterophile antibodies (HA) commonly include natural or autoimmune rheumatoid factors. Human anti-animal antibodies such as human anti-mouse antibody (HAMA), are high affinity, specific polyclonal antibodies produced against a specific animal whole immunoglobulin may also interfere. These compete with the troponin antigen by cross-reacting with reagent antibodies of the same species to produce a false signal. The incidence of HA in cTn immunoassays is uncertain although an incidence of 1 in 5000 (0.02%) by Architect cTnI assay has been reported [119]. Since this report the frequency of interference
from HA has reduced, probably because of the addition of heterophile blocking antibodies to the assay format and the humanisation of the Ab’s used.

Troponin autoantibodies (cTnAAb) may cause false negative troponin values by preventing troponin from binding to the reagent antibodies [120,121]. Autoantibodies to both cTnI and cTnT have been reported to be prevalent in blood donors [122,124]. They may also cause false positive values when present as circulating immunoglobulin-troponin complexes or macrotroponin [123-127].

Interference can be either positive or negative; positive when the Ab links the capture and signal antibodies, and negative when the Ab blocks the binding site for either capture or signal Ab. Strictly, both HA and cTnAAb could cause both, however we are only aware of HA causing positive interference and cTnAAb causing negative interference. Patient samples that show constant but stable troponin elevation over a period of days or weeks should be investigated to determine if a HA, HAMA, a troponin autoantibody or a macrotroponin is present.

No routine procedures have been proposed for the investigation of possible troponin assay interferences. Possible laboratory procedures to evaluate the presence of HAMA and HA include the use of a larger dilution of the sample with reagent containing non-immune mouse IgG, use of heterophile blocking tubes or performing the analysis on a differently configured assay [128].

Macrotroponins that are immunoglobulin-troponin complexes have been reported to give persistently elevated troponin due to their slow turnover in blood [127,128]. They result in persistently elevated troponin concentrations. Isolation of the immunoglobulin-troponin complex can be obtained by using protein G affinity chromatography [128].
False-negative cTnI concentrations due to the presence of a circulating autoantibody (probably IgG), have been reported [120]. The autoantibodies show high affinity for cTnI preventing its recognition by the two-site immunoassays.

**TROPONIN ASSAY ISSUES - REFERENCE VALUES**

Reference values for cTnI and derived decision limits need to be determined separately for each assay and platform and must not be extrapolated to other assays until there is adequate cTnI standardisation.

The standard material for calibration with the cTnT assay is recombinant human cTnT. The new high sensitivity cTnT assay has been standardised to the earlier 4th generation cTnT assay at higher concentrations but, due to the different assay detection limits and other analytical differences, there is limited comparability observed at low cTnT concentrations <100 ng/L [129].

With the improved sensitivity of the latest generation of troponin assays, clinical validation in the low concentration range is required. Small troponin elevations above the recommended decision limit, 99th percentile URL [5], can occur either acutely or chronically in both ACS and non-ACS conditions, where there is myocardial injury, as well as in the aging community population who are at increased cardiovascular risk, and, asymptomatic persons with cTn above the 99th percentile are at an increased risk of major adverse cardiac events including death [130-133]. Depending on an assay’s performance characteristics, some assays will recognise different at-risk patient groups [131-133].

Defining a healthy population to determine the 99th percentile is challenging. Different studies have published very different numbers even for the same cTn assay. For example, for the high sensitivity (hs)-cTnT assay, 99th percentiles have ranged from 12 ng/L to 20 ng/L.
For hs-cTnI assays, 99th percentiles have ranged from <34 ng/L to 80 ng/L for the Beckman assay [23], from 7 ng/L to 36 ng/L for the Singulex assay [21], and from 13 ng/L to 32 ng/L for the Abbott assay [133]. These studies are variable in the total number of subjects enrolled, the age of subjects, and by differences in gender composition. Most but not all have reported separate male and female 99th percentiles [133]. No definitive number of individuals that should be included in a reference population has been defined on the basis of evidence nor has the criteria on what constitutes cardio-health.

**TROPONIN ISSUES – CHANGES IN TESTING PROTOCOLS**

Higher sensitivity troponin assays have shown excellent diagnostic performance in the evaluation of patients with possible AMI; however, clinicians are concerned that because of lower specificity for the diagnosis of AMI that many patients may require unnecessary investigations because of elevated troponin values [134].

When a patient presents to the Emergency Department (ED) and a diagnosis of ACS is obvious from the history and ECG, the patient is rapidly transferred to Cardiology for active treatment. When the diagnosis is less clear-cut, an important part of the decision making process is measurement of troponin at presentation and 6-8h from the time of onset of pain [135]. An increase in troponin concentration would be suggestive of ACS and the patient would be transferred to Cardiology. The up to 8 hour prolonged assessment of these patients contribute to overcrowding, increased costs and possible adverse patient outcomes including increased mortality.

Guidelines have been released which stated that in persons with a diagnosis of possible ACS, an extra sample should be collected at 3h from the onset of pain [136]. Further clinical
studies are required to assess this strategy in conjunction with the high sensitivity cTnI assays.

USE OF THE TROPONIN 99th PERCENTILE URL FOR THE DIAGNOSIS OF ACS AND IN THE ASSESSMENT OF CARDIAC RISK?

The concept and use of the 99th percentile was introduced with the redefinition of myocardial infarction in 2000, at a time when few assays could detect troponin at this concentration let alone none could meet the criteria of an assay CV at that concentration of 10%. Most healthy persons have low concentrations of troponin present in their peripheral circulation and troponin may be released in response to minor non-cardiac illness, thus need to accept the concept that troponin may be released by physiological as well as pathological mechanisms.

There is evidence that biological variation of troponin is small and significant changes could occur within the 99th percentile[137] Transient non-cardiac illness, age, male gender and subclinical cardiovascular disease may all push up the 99th percentile artifactually. Also there is variation between assays [138,139] and problems with population selection [140], making the 99th percentile an unreliable boundary to use in assessing patients with presumed acute coronary syndrome. What are the alternative? As studies have shown a Gaussian or near Gaussian distribution of troponin in healthy adults [141] and children [142], the assessment of health or otherwise in a population should assess how closely that population lies to that Gaussian distribution. If the distribution is Gaussian, then the standard procedure for setting reference intervals, the central 95% of the reference population, could be used. With our understanding that there is a “physiological” troponin concentration, consideration should be given to going back to the original proposal from the National Academy of Clinical Biochemistry (NACB) reporting troponin concentrations to the 97.5th percentile [143]. This will result in 2.5% of the population having an abnormally high troponin and will cause some anxiety. Troponin has for many years troponin has been used inappropriately as an absolute
diagnostic test, rather than an aid to diagnosis. Troponin concentrations and changes in troponin concentration are only meaningful when taken in a clinical context [144].

OTHER BIOCHEMICAL CARDIAC MARKERS

While cardiac troponins are the key biomarkers for initial risk stratification, multiple other biomarkers have been evaluated for incremental prognostic information. Whilst this thesis is about troponin in health and disease, and in disease it is especially important in considerations of the ACS, there are other biomarkers which are also informative. Although not directly relevant to this thesis, I provide a short summary of several of these markers, to provide a more complete overview.

These biomarkers can be grouped according to their pathophysiological mechanisms [145] associated with the acute coronary syndrome such as B type natriuretic peptide (BNP) and LV dysfunction and C-reactive protein (CRP) and inflammation.

B TYPE NATRIURETIC PEPTIDE (BNP) AND C-REACTIVE PROTEIN (CRP)

Natriuretic peptides such as BNP or its N-terminal pro-hormone fragment (NT-proBNP) are highly sensitive and fairly specific markers for the detection of left ventricular dysfunction. Robust retrospective data in NSTE-ACS show that patients with elevated BNP or NT-proBNP levels have a three- to five-fold increased mortality rate when compared with those with lower levels independent of troponin and hsCRP measurements [146]. The level is strongly associated with the risk of death even when adjusted for age, Killip class, and left ventricular ejection fraction (LVEF) [20]. Values taken a few days after onset of symptoms seem to have superior predictive value when compared with measurements on admission. Natriuretic peptides are useful markers in the emergency room in evaluating chest pain or dyspnoea and were shown to be helpful in differentiating cardiac and non-cardiac causes of
dyspnoea. However, as markers of long-term prognosis, they have limited value for initial risk stratification and hence for selecting the initial therapeutic strategy in NSTE-ACS [146].

Natriuretic peptides (BNP and NTproBNP) have been shown to be useful in the prediction of heart failure [147,148] however there is only limited support for the measurement of these peptides to guide clinical decision making for early invasive strategies for patients with acute coronary syndrome [148].

Of the numerous inflammatory markers investigated over the past decade, CRP measured by high-sensitivity assays is the most widely studied and is linked to adverse events. There is solid evidence that even among patients with troponin-negative NSTE-ACS, elevated levels of hsCRP (>10 mg/L) are predictive of long-term mortality (6 months to 4 years) [21 149,150]. Kavsak has demonstrated (table 2.5) that for early serious cardiac outcome in patients presenting to an emergency department that NTproBNP is a useful biomarker [150].

Table 2.5: Association between biomarker and serious cardiac outcome after 72 hours.

Adapted from [150]. *Beckman Coulter AccuTnI, *Roche Elecsys 2010

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>n</th>
<th>Serious 72 h cardiac outcome (n=24)</th>
<th>Non serious 72 cardiac outcome (n=162)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Y)</td>
<td>186</td>
<td>65 (56-73)</td>
<td>59 (49-73)</td>
<td>NS</td>
</tr>
<tr>
<td>hs-cTnI (ng/L)*</td>
<td>159</td>
<td>39 (19-367)</td>
<td>6.8 (4.7-12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hs-cTnT (ng/L)*</td>
<td>178</td>
<td>43 (14-94)</td>
<td>4.6 (2.9-12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NT-proBNP (ng/L)*</td>
<td>172</td>
<td>506 (139–3137)</td>
<td>123 (38–419)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
HEART TYPE FATTY ACID BINDING PROTEIN AND COPEPTIN

Heart-type fatty acid binding protein (H-FABP) is a low molecular weight protein involved in myocardial fatty-acid metabolism, transporting fatty acids from the cell membrane to mitochondria for oxidation [151]. Because of its small molecular size and predominant cytoplasmic localization, it displays a very early rise and detectability in the peripheral circulation after MI [152]. Although H-FABP has elicited interest as an early diagnostic tool for MI it has been suggested that it cannot be used as the sole biomarker for diagnosis. [153]

Copeptin, a glycopeptide, is the c-terminal part of the vasopressin prohormone which has important effects on osmoregulation and indirectly cardiovascular homeostasis. Copeptin, as a biomarker, has been studied in early rule-out of MI in patients with acute chest pain, diagnosis of heart failure in patients with acute dyspnea and determining the prognosis of destabilized or chronic stable heart failure [154]. The release pattern of copeptin in MI patients is an immediate rise after the onset of chest pain followed by a return to physiological levels within 5 days [155]. An AUC of 0.97 has been cited using copeptin in combination with cTnT in ruling out AMI. This is in contrast to an AUC of 0.86 using TnT alone [156]. A recent report by Eggers and co-workers [157] investigated H-FABP and copeptin combined with hs-cTnT in the early evaluation of patients with acute chest pain, showed that hs-cTnT provides excellent early diagnostic accuracy for non-ST segment elevation myocardial infarction (NSTEMI), and that neither H-FABP nor copeptin performed better or provided diagnostic increment to hs-cTnT data. Even though some reports document the potential of each of them in improving the sensitivity of MI diagnosis in combination with cTn [158] other evidence suggests that neither-FABP or copeptin adds diagnostic information to conventional or hs-cTn data [157]. Further data are needed to determine whether a multi-marker approach using H-FABP and/or copeptin in addition to cTn can improve the accuracy in diagnosing MI.
GROWTH-DIFFERENTIATION FACTOR-15

Growth-differentiation factor-15 (GDF-15) is a distant member of the transforming growth factor-β-cytokine super family that is induced in the myocardium following pathological stress associated with inflammation or tissue injury [145]. The expression of GDF-15 in virtually all tissues suggests its importance in general and basic cellular functions. Although the exact biological functions of GDF-15 are still poorly understood, it has been shown to be involved in regulating inflammatory and apoptotic pathways and its expression is upregulated in many different pathological conditions, including inflammation, cardiovascular disease, and renal disease. GDF-15 has been shown to be a strong and independent predictor of mortality and disease progression in patients with a number of established disease, such as ACS, heart failure, stroke, chronic kidney disease, and different types of cancer [159,160]. Because of its lack of tissue specificity compared with markers such as cTnT, cTnI, and BNP, GDF-15 has no useful role as a diagnostic marker. Its characteristic as a prognostic marker for a wide range of outcomes could in fact be its major weakness as a clinically useful marker of risk. [161]

MicroRNAs

Most of the currently available biomarkers of ACS used in clinical applications are proteins and polypeptides. Novel biomarkers such MicroRNAs (miRNAs) have recently been studied looking at the opportunity to improve diagnosis of MI.

MicroRNAs are short non-coding RNA molecules involved in post-transcriptional gene regulation by binding to the 3’ untranslated region of a messenger RNA (mRNA); they can therefore inhibit translation or induce mRNA destabilization [162].
Studies are showing that cardiac miRNAs are markedly dysregulated in MI. MI induces dramatic changes in the structure and composition of heart tissue, triggering a dynamic process characterized by time changes in gene expression. However, current knowledge of which miRNAs are dysregulated in MI is largely incomplete and a meta analysis by Lippi and co-workers [163] found that the diagnostic performance of circulating miRNAs used as stand-alone tests was at best comparable to that of commercial hs-cTn assays.

There are limitations with the introduction into routine clinical use of miRNA’s. Fast, standardised and automated complex technologies such as real-time PCR are required and there is subsequent high cost and slow time to results.

The detection of miRNAs is also hampered by the characteristics of miRNA’s being small in size, sequence similarities, lack of common features facilitating selective amplification, low abundance, and tissue-specific or development stage-specific expression [163].
REFERENCES

3. Australian Institute of Health and Welfare, 2010, Cardiovascular disease mortality: trends at different ages, Cardiovascular series no. 31, Cat. No.47, Canberra
4. Access Economics, 2005 The Shifting Burden of Cardiovascular Disease in Australia, Report the Heart Foundation
33. Libby P. Current concepts of the pathogenesis of the acute coronary syndromes, Circulation 2001;104:365-72
38. Robbins and Cotran pathological basis of Disease 8th edition 2009 Elsevier
41. Jennings RB, Ganote CE. Structural changes in myocardium during acute ischemia. Circ. Res 1974;35:156–72


114. Tate JR, Panteghini M, Koerbin G, Hickman PE, Schneider HG, Jaffe A. Verification of the analytical characteristics of troponin assays in the laboratory – a how to guide Clin Biochem Reviews Troponin Monograph 2012 69-85
141. Venge P, Johnston N, Lindahl B, James S. Normal plasma levels of cardiac troponin I measured by the high-sensitivity cardiac troponin I Access prototype assay and the impact on the diagnosis of myocardial ischemia. J Am Coll Cardiol 2009; 54: 1165-72


CHAPTER 3.1

THE DETERMINATION OF THE PERFORMANCE CHARACTERISTICS OF HIGHLY SENSITIVE TROPONIN ASSAYS AND VALIDATION OF THEIR FITNESS FOR PURPOSE IN THE CLINICAL LABORATORY

The basic principle underlying any diagnostic test should be that it provides a valid and reliable basis to distinguish between a result that is most likely to lie within a “healthy” category and a result that is most likely to lie within a “disease” category. We now have acceptance that an evidence-based culture underpins the practice of laboratory medicine and this is now perceived as the scientific foundation of medicine [1].

In line with this evidence-based requirement, performance characteristics for each procedure used in a laboratory should relate to the intended use of that procedure. Evaluation of these characteristics is part of good laboratory practice and a requirement of laboratory accreditation [2]. Laboratories should validate the performance characteristics that are claimed by the manufacturer of all new measurement procedures and analytic systems before reporting patient test results.

Early troponin assays were unable to detect low concentrations in the peripheral circulation. The newer high sensitivity assays now appear to be able to measure physiological concentrations which may or may not improve diagnostic accuracy.

There are a large number of commercially available troponin assays utilized in laboratories and at point of care worldwide, all of which have different performance characteristics. It is important that analysts performing troponin measurement understand the performance characteristics of the assay used in their laboratory. It is important that the analyst is able to
relay that knowledge to the clinician to enable appropriate interpretation of the results generated to optimise patient care.

The terminology used to describe the evolving generations of troponin assays may not always be representative of the assay’s performance characteristics, rather, may have association only with marketing considerations. Terms such as “ultra sensitive” or “highly sensitive” do not necessarily represent superior performance [3]. A proposed means of classifying assays as to their analytical capabilities will be discussed.

This chapter focuses on investigating and validating the performance characteristics of these high sensitivity assays in contrast to the conventional assays. Particular emphasis will be on the ability of these assays to detect troponin concentrations in a cardio-healthy population. Without this ability, we are unable to address the belief that the presence of cTn indicates that necrosis has occurred, adequately determine the 99th percentile troponin concentration of a healthy population or the implications of detectable cTn in the non-ACS setting.

Chapter 3.2 offers guidelines that we developed for a standardised approach in undertaking the verification of the analytical characteristics of a troponin assay. The development of these guidelines, provide to Australian laboratories a template to determine and/or confirm the analytical characteristics of an assay including limits of blank and detection, possible interference from endogenous and exogenous sources and the sensitivity of the assay and its ability to detect troponin concentrations in the blood of cardio healthy subjects and not necessarily only where there is myocyte necrosis.

Chapter 3.3 we report on the analytical characteristics of the Roche high sensitivity troponin T (hs-cTnT) assay and demonstrated its application to a cardio-healthy adult population.
In chapter 3.4 we characterised the Abbott high sensitivity troponin I (hs-cTnI) assay in its pre-commercial form and demonstrated its application to a cardio-healthy adult Australian population.

REFERENCES

CHAPTER 3.2

VERIFICATION OF THE ANALYTICAL CHARACTERISTICS OF TRO Ponin ASSA YS IN THE LABORATORY – A HOW TO GUIDE

Part of this work was published in:

Tate JR, Panteghini M, Koerbin G, Hickman PE, Schneider HG, Jaffé A.

Clin Biochem Reviews Troponin Monograph 2012; 69-85
ABSTRACT: It is important that both laboratory staff performing troponin measurement and clinicians who interpret troponin results should ‘know their routine laboratory’s troponin assay’ and its performance characteristics. International quality systems established by ISO and accepted by clinical laboratories worldwide require the verification of a new method. As a minimum a troponin method must be verified as showing ‘fitness for purpose’ before being used to produce analytical data for patient care.

The analytical performance characteristics of troponin assays that require an initial verification include imprecision and limit of quantitation. Validation of the upper reference limit that has been established by the manufacturer is also highly desirable, if possible. If the assay is replacing a previous assay, a method comparison and if possible an audit of performance should also be done. Investigation of other characteristics such as limit of blank, limit of detection, interferences and linearity is optional and depends on a laboratory’s situation and the availability of resources to do this work.

In addition, the introduction of newer more sensitive troponin assays may require clinical validation in collaboration with local cardiology units and emergency medicine departments, and auditing of the impact of implementation of these more analytically sensitive assays.
INTRODUCTION

The determination of cardiac troponin (cTn) is widely used to diagnose acute myocardial infarction (AMI) and for risk prognostication of acute coronary syndrome (ACS). Over the last 20 years there has been an evolution of troponin assays from the initial research cTnI and cTnT assays to the commercial assays produced by diagnostic manufacturers and used by clinical laboratories and at Point of Care Testing (PoCT). As troponin assays have developed there have been improvements in their analytical performance, e.g. less imprecision at lower concentrations and less heterophile antibody interference. At the same time the clinical knowledge about the physiology and pathology affecting troponin concentrations in blood has increased. The advent of the latest generation of highly-sensitive (hs) troponin assays has heralded a new wave of information about very low concentrations of troponin in blood. Whereas previous generations of cTnI and cTnT assays were unable to detect these low concentrations, the more highly sensitive assays appear to be able to measure physiological concentrations of troponin. This property may or may not improve diagnostic accuracy.

Due to the large number of different cTnI assay systems in use, utilisation of 4th- and 5th-generation cTnT assays and use of different Point of Care assays, it is important that both laboratory staff performing troponin measurement and clinicians who interpret troponin results should ‘know their routine laboratory’s troponin assay’ and its performance characteristics [1]. Laboratory personnel should know troponin assay performance characteristics including imprecision, limit of detection and quantitation, interference and the potential for false-positive results and false-negative results together with pre-analytical pre-requisites and post-analytical reporting and interpretive commenting.
VERIFICATION AND VALIDATION STUDIES

In daily clinical laboratory practice the performance of troponin assays can be different from that quoted in the manufacturer’s package insert [2]. Various 10% coefficient of variation (CV) concentrations and 99th percentile upper reference limits (URL) have been reported for some 2nd generation cTnI assays [3-5]. Often assays perform better in a highly quality-controlled setting such as research and development laboratories than in clinical laboratories and reference populations may differ in size and makeup [2,6].

Verification requires the confirmation that performance claims by diagnostic manufacturers are achieved whereas validation is a more extensive process of gathering independent data to compare directly with the manufacturer’s performance claims. The extent to which a laboratory can undertake the verification or validation of a new method depends on the existing status of the method and the resources of the laboratory. As a minimum, a troponin assay must be verified as showing ‘fitness for purpose’ before being used to produce analytical data for patient care.

According to the ISO standard 15189 for quality and competence of medical laboratories [7], verification confirmation requires, through provision of objective evidence (performance characteristics), that the performance claims for the examination method have been met. The analytical performance characteristics of troponin assays that require mandatory verification are imprecision, especially at the clinically relevant decision cut-off as recommended by the European Society of Cardiology (ESC) Study Group on Biomarkers in Cardiology [6], and limit of quantitation. If the assay is replacing a previous assay, a comparison of performance should also be performed. Verification of the limit of blank, limit of detection, interferences and linearity are optional but desirable if the resources are available. Validation of the upper reference limit that has been previously established by the manufacturer is also highly desirable, if possible, as recommended by the practice guidelines of the National Academy of
Clinical Biochemistry [8]. The pre-analytical requisites for robustness and the long-term monitoring of imprecision across different reagent lots should also be determined.

Several evaluations [9] show that local laboratory troponin assay performance may differ from the manufacturer’s stated claims. The data provided for marketed assays often portrays better precision than can be obtained in clinical practice. Studies that include multiple variables, such as the number of days of testing, the total number of replicates, the number of calibrations, the number of reagent batches and multisite evaluations provide the most robust data for local laboratories; hence, the importance of each laboratory ‘knowing its troponin assay’s performance’. It should also be noted however that individual patient evaluations occur over a constrained period minimising longer term variation. All of these considerations should be taken into account.

In the specific case of clinical trials where the aggregation of data across multiple institutions is used to provide statistical power to clinical outcomes, the performance characteristics of cTn assays to be used, including optimal precision and decision cut-off concentration, should be determined prior to the analysis of patient specimens.

**TROPONIN ASSAYS**

**ASSAY PRINCIPLES:** Assays for troponin consist of a range of research and commercial cTnI and cTnT immunoassay methods that differ from each other in assay format, by the types of antibody used, which for cTnI may be combinations of mouse monoclonal and/or polyclonal antibodies, by the epitopes to which they bind, and by the type of indicator molecule that is used. Detection is by spectrophotometry, fluorescence, chemiluminescence and electrochemistry (Table 3.2.1) [9,10]. The troponin assays providing higher sensitivity may include longer incubation times and/or signal amplification techniques and measure
troponin concentrations approximately 10- to 100-fold lower than previous generation assays [11].

**ANTIBODY SPECIFICITY:** Current assays for cTnT and cTnI are two- or three-site immunoassays. All the assays are of the capture type where an immobilised antibody specifically binds the troponin present in the serum or plasma. The captured troponin is then reacted with a second antibody and in some assays a third antibody that is coupled to an indicator molecule. Immunoassays for cTnI are influenced by the antibody species and the specificity of the polyclonal and/or monoclonal anti-cTnI antibodies. Due to proteolytic susceptibility of N- and C-terminal parts of cTnI, most currently available cTnI assays use antibodies that are directed to the mid-fragment epitopes, i.e. amino acids 30-110 which is the more stable part of the molecule (Table 3.2.1) [12].
Table 3.2.1: Analytical characteristics of commercial cardiac troponin I and T assays declared by the manufacturer as at December 2012 [9]

<table>
<thead>
<tr>
<th>Commercially available assays - Company/platform(s)/ assay</th>
<th>LoB* (ng/L)</th>
<th>LoD* (ng/L)</th>
<th>99th %</th>
<th>10% CV at 99th %</th>
<th>Reference population N: age range (y)</th>
<th>Epitopes recognised by Antibodies</th>
<th>Detection Antibody Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbott AsSYM ADV</td>
<td>20</td>
<td>40</td>
<td>14.0</td>
<td>170</td>
<td>C: 87-91, 41-49; D: 24-40</td>
<td>ALP</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Abbott Architect</td>
<td>&lt;10</td>
<td>28</td>
<td>14.0</td>
<td>32</td>
<td>C: 87-91, 24-40; D: 41-49</td>
<td>Fluorophor</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Abbott STA</td>
<td>20</td>
<td>50</td>
<td>16.5</td>
<td>100</td>
<td>C: 41-49, 85-91; E: 28-39, 62-78</td>
<td>ALP</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Alere Triage SOb</td>
<td>50</td>
<td>NA</td>
<td>16.5</td>
<td>100</td>
<td>C: NA; D: 27-40</td>
<td>Fluorophor</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Alere Triage Cardio 3</td>
<td>2</td>
<td>10</td>
<td>17.0</td>
<td>37</td>
<td>C: 27-39; D: 83-93, 196-196</td>
<td>Fluorophor</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Beckman Coulter Access Accu</td>
<td>10</td>
<td>20</td>
<td>14.0</td>
<td>60</td>
<td>C: 41-49, 24-40</td>
<td>ALP</td>
<td>Acidinium</td>
</tr>
<tr>
<td>bioMérieux Vidas Ultra</td>
<td>10</td>
<td>20</td>
<td>17.7</td>
<td>90</td>
<td>C: 24-40, 41-49; D: 87-91</td>
<td>ALP</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Chibro VIROS (Troponin I)</td>
<td>7</td>
<td>12</td>
<td>15.2</td>
<td>26</td>
<td>C: 125-131, D: 136-147</td>
<td>Europium</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Roche Centaur XPT</td>
<td>3</td>
<td>17</td>
<td>15.2</td>
<td>26</td>
<td>C: 125-131, D: 136-147</td>
<td>Europium</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Response Biomedical RAMB</td>
<td>30</td>
<td>NA</td>
<td>13.5</td>
<td>110</td>
<td>C: 85-92, D: 26-38</td>
<td>Fluorophor</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Roche Cardio Reader cTnT</td>
<td>30</td>
<td>NA</td>
<td>13.5</td>
<td>110</td>
<td>C: 125-131, D: 136-147</td>
<td>Fluorophor</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Roche cobas 600 cTnT</td>
<td>50</td>
<td>NA</td>
<td>13.5</td>
<td>110</td>
<td>C: 125-131, D: 136-147</td>
<td>Fluorophor</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Roche Elecsys 1010/411 / E 170 / cobas 600/682 cTnT (2nd gen)</td>
<td>10</td>
<td>NA</td>
<td>13.5</td>
<td>110</td>
<td>C: 125-131, D: 136-147</td>
<td>Fluorophor</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Roche Elecsys 1010/411 / E 170 / cobas 600/682 hs.cTnT</td>
<td>5</td>
<td>14</td>
<td>16.0</td>
<td>13</td>
<td>C: 125-131, D: 136-147</td>
<td>Fluorophor</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Roche Elecsys 1010/411 / E 170 / cobas 600/682 hs.cTnT</td>
<td>160</td>
<td>160*</td>
<td>NA</td>
<td>300</td>
<td>C: 87-91, 190-196; D: 23-29, 27-43</td>
<td>Fluorophor</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Siemens ADVIA Centaur TNI Ultra™</td>
<td>40</td>
<td>70</td>
<td>8.8</td>
<td>30</td>
<td>C: 41-49, 85-91; D: 27-40</td>
<td>ALP</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Siemens Dimension XLI™ CTNI</td>
<td>40*</td>
<td>70</td>
<td>8.8</td>
<td>30</td>
<td>C: 41-49, 85-91; D: 27-40</td>
<td>ALP</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Siemens IMMULITE® 1000 Turbo²</td>
<td>100</td>
<td>190</td>
<td>10.0</td>
<td>50</td>
<td>C: 87-91, 24-40</td>
<td>ALP</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Siemens IMMULITE® 2000 XPI²</td>
<td>100</td>
<td>190</td>
<td>10.0</td>
<td>50</td>
<td>C: 87-91, 24-40</td>
<td>ALP</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Siemens IMMULITE® 2500 STAT™</td>
<td>100</td>
<td>190</td>
<td>10.0</td>
<td>50</td>
<td>C: 87-91, 24-40</td>
<td>ALP</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Siemens IMMULITE® 2500 Turbo²</td>
<td>100</td>
<td>190</td>
<td>10.0</td>
<td>50</td>
<td>C: 87-91, 24-40</td>
<td>ALP</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Siemens IMMULITE® 2500 Turbo²</td>
<td>100</td>
<td>190</td>
<td>10.0</td>
<td>50</td>
<td>C: 87-91, 24-40</td>
<td>ALP</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Siemens IMMULITE® 2500 Turbo²</td>
<td>100</td>
<td>190</td>
<td>10.0</td>
<td>50</td>
<td>C: 87-91, 24-40</td>
<td>ALP</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Siemens IMMULITE® 2500 Turbo²</td>
<td>100</td>
<td>190</td>
<td>10.0</td>
<td>50</td>
<td>C: 87-91, 24-40</td>
<td>ALP</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Siemens IMMULITE® 2500 Turbo²</td>
<td>100</td>
<td>190</td>
<td>10.0</td>
<td>50</td>
<td>C: 87-91, 24-40</td>
<td>ALP</td>
<td>Acidinium</td>
</tr>
<tr>
<td>Tosoh ST AIA-PACK</td>
<td>60</td>
<td>60</td>
<td>8.5</td>
<td>NA</td>
<td>C: 41-49, 87-91</td>
<td>FLAP</td>
<td>Acidinium</td>
</tr>
</tbody>
</table>

Low B, limit of blank; LoD, limit of detection; NAD = indeterminate; NA, data are not available; 99th %: 99th percentile concentration 10% CV, lowest concentration that has been shown to have a 10% CV (total imprecision); risk stratification claim per US Food and Drug Administration (FDA); epitopes (amino acid residues) recognised by antibodies were supplied by manufacturers; C, capture antibody(s); D, detection antibody(s); ALP, alkaline phosphatase; hs, high sensitivity designation per manufacturers; HRP, horseradish peroxidase.

NB – assays cannot be compared by the stated values in the table since they are derived with different metrics for the various assays.
TROPONIN PLASMA FORMS AND DEFINITION OF THE MEASURAND

TROPONIN I: The progressive modification of cTnI found in blood of AMI patients after tissue release results in a heterogeneous mixture of forms present in plasma including predominantly the binary cTnI-C complex with smaller amounts of the ternary cTnI-T-C complex, binary cTnI-T complex and free cTnI [13]. The different plasma forms may also undergo oxidisation, reduction, phosphorylation, dephosphorylation and degradation.

One way to address the definition of cTnI that exists in blood in multiple forms is to define the measurand as a unique, invariant part of the molecule that is common to all cTnI forms present in blood (e.g., a specific amino acid sequence) and is the clinically relevant form(s) [14]. Ideally, cTnI assays should recognise complexed, free and modified forms of cTnI equally, to allow monitoring of total cTnI concentrations present in samples over the course of a patient’s AMI.

TROPONIN T: Monoclonal antibodies are directed to amino acid residues 125-131 and 136-147 on the cTnT molecule. The main form of cTnT antigen found in blood of AMI patients after tissue release is the free cTnT form from the breakdown of the cTnI-T-C complex into binary cTnI-C and cTnT forms. Degraded cTnT is also detected by the cTnT assay [15].

STANDARDISATION

TROPONIN I: Current cTnI assays are marketed by different companies and use various standard materials for calibration and antibodies with different epitope specificities. Hence, assays may give results that are unique to a certain method or instrument and different results may be obtained for the same patient sample depending on the assay and platform used. As a consequence, cTnI values obtained with different assays cannot be interchanged; hence reference values for cTnI and derived
decision limits need to be determined separately for each assay and platform and must not be extrapolated to other assays until there is adequate cTnI standardisation.

**TROPONIN T:** Only one company, Roche Diagnostics, manufactures troponin T. Radiometer, under license, also market this assay for their AQT point of care device. They use recombinant human cTnT as standard material for calibration. The new high sensitivity cTnT assay has been standardised to the earlier 4th generation cTnT assay at higher concentrations but, due to the different assay detection limits and other analytical differences, there is limited comparability observed at low cTnT concentrations (<100 ng/L).

**LIMIT OF BLANK, LIMIT OF DETECTION, AND LIMIT OF QUANTITATION**

The limit of blank (LoB) and the limit of detection (LoD) are important characteristics of a troponin assay and are used to discriminate between the presence and absence of troponin. LoB is the highest apparent troponin concentration expected to be found when replicates of a blank sample containing no troponin are tested. The manufacturer’s zero calibrator or assay diluent, or human serum without troponin are used for determining LoB. LoD is the lowest troponin concentration likely to be reliably distinguished from the LoB and at which detection is feasible. It is determined by measuring replicates of a sample known to contain a low concentration of troponin. Samples for LoD estimation are recommended to have concentrations in the range from LoB to 4×LoB (CLSI EP17-A) [16].

The limit of quantitation (LoQ) is the lowest concentration at which troponin cannot only be reliably detected but at which some predefined goals for bias and imprecision are met. This may be a predetermined consensus % CV, e.g. imprecision of 10% CV for troponin that is recommended...
for clinical diagnosis of AMI [17], or a desirable total error goal of ±22.5% CV based on the biological variability of cTnI for example [2,18].

A manufacturer establishing the LoB, LoD, or LoQ typically would perform studies using more than one analyser and one lot of reagent to encompass the variability that users can expect to encounter in the routine laboratory. Clinical laboratories are able to validate these characteristics using a smaller number of samples and generally will use only one analyser and one lot of reagent. The estimation of LoD for commercially available cTnI assays is described by the CLSI guideline EP17-A [16] with a more rigorous estimation described by Arrebola [19].

IMPRECISION AND LIMIT OF QUANTITATION

The imprecision performance of a new troponin assay should be verified to confirm the imprecision of the manufacturer’s performance claims. Ideally both patient samples and manufacturer’s quality controls (QC) are tested at the time of assay evaluation (and note that third-party-manufactured QC may be subject to matrix effects). The assessment of longer-term assay alignment uses manufacturer’s quality control (QC) materials to monitor assay transferability and bias across different reagent and calibrator lots. Lot to lot variation in QC material must be taken into account when undertaking monitoring.

DERIVING BASIC INFORMATION ON IMPRECISION

Troponin imprecision is optimally assessed by using a patient pool with concentrations close to the decision limit for troponin, which should be the 99th percentile concentration of the value distribution of a reference population. An initial evaluation should include a number of pooled patient samples (at least 4) (refer to imprecision profiling). Minimum imprecision testing for troponin to verify a manufacturer’s claims requires that runs are performed twice a day, for five
days, with a minimum time separating each run (CLSI EP15-A2) [20]. A more comprehensive imprecision evaluation over 20 days (CLSI EP05-A2) [21] is used to determine assay repeatability and reproducibility. Arrebola [19] and Kelley [22] provide examples of laboratory based imprecision studies.

**IMPRECISION PROFILING**

Imprecision profiles express imprecision characteristics of an assay over a range of concentration values and provide a total imprecision picture. Profiling is a useful tool but is not mandatory. Variance function estimation can be readily applied to the duplicates accumulated in a CLSI experiment to obtain a repeatability imprecision profile provided that four (minimum number) or more (widely spaced) specimens have been used in the study [23]. A troponin imprecision profile and the assessment of LoQ require an adequate number of replicate measurements at the low-end of the measuring range. A worked example is shown using direct variance function estimation and plotting [24] is seen in Figure 3.2.1
Figure 3.2.1: Imprecision profiles. Coefficient of variation (CV%) and standard deviation (SD) versus troponin concentration (logarithmic scale) are shown for Vitros ECi cTnI (100 observations in 10 data sets) and E-170 hs-TnT (220 observations in 13 data sets) assays. The imprecision profile curve and 95% confidence interval (shaded area) were determined by a variance function program [24]. Troponin concentrations at 10% CV and 20% CV indicated by dashed and solid horizontal lines respectively, for Vitros ECi cTnI were 0.034 µg/L and 0.019 µg/L respectively, and for E-170 cTnT were 11.86 ng/L and 6.80 ng/L respectively [24].

**CONTROVERSIAL ISSUES ABOUT IMPRECISION**

The 2007 recommendations for diagnosis of MI and risk stratification of ACS recommend troponin assays have an imprecision of ≤10% CV at the 99th percentile limit of the value distribution in a reference population [17]. Of the 28 commercially available cTn assays listed on the IFCC website by the Working Group on Standardization of Troponin I [9] as at December 2012, only nine appear to fulfill the guideline recommendations if the reliability of manufacturers’ declared performance is assumed to be correct. Nine others are clinically acceptable. All 4 pre commercial assays fulfill the recommended guidelines. The imprecision obtained at the 99th percentile concentration by diagnostic manufacturers varies from 5-28% CV for currently available troponin assays. More
sensitive cTnI research assays show an imprecision of 10% CV or lower at the 99th percentile and
detect physiological concentrations of troponin. Their improved imprecision at low-level troponin
concentrations means that there should be better analytical performance at physiological
concentrations.

The Study Group on Biomarkers in Cardiology of the ESC Working Group on Acute Cardiac Care
endorses the recommendation of the ESC/AACF/AHA/WHF Task Force that troponin imprecision
of 10% CV or less at the 99th percentile is desirable [6,25]. The group recommends not using the
10% CV value as clinical cut off, but rather the 99th percentile of a reference population. They
recommend not utilising troponin assays with an imprecision at the 99th percentile of above 20% CV
because of the lack of data on the potential risk of misclassification of patients.

INTERFERENCES

Haemolysis, icterus, lipaemia, anticoagulant(s) and sample storage may interfere with the
measurement of troponin in some assays resulting in either false-positive or false-negative values

HAEMOLYSIS TESTING: Haemolysis can interfere with some troponin immunoassays.

Florkowski [26] and Bais [27] have recently reported on haemolysis in cTnI and cTnT assays.

These studies showed haemolysis is likely to be more clinically significant at low troponin
concentration close to the decision limit, where troponin values may be falsely low (cTnT) or high
(most cTnI assays) compared with at higher troponin concentrations where the values remain
positive. Figure 3.2.2 demonstrates the effect of increasing haemolysis on commercial troponin
assays.
Some analysers use automated haemolysis index (HI) to estimate the haemoglobin concentration that may be present in the patient sample (serum or plasma). Haemoglobin concentration as low as 1.9 g/L can cause >20% change in cTn concentration and may be of significance [27].

Interference is method-dependent and may vary for each assay. Diagnostic manufacturers should specify in their package inserts upper limits above which interference due to haemolysis may occur. Laboratories are recommended at a minimum to apply this information by using the HI if available and, if possible, to validate the level of haemoglobin that may interfere with the troponin assay. General testing of interference by haemolysis, bilirubin and lipaemia has been described by Dimeski [28] and the CLSI protocol EP07-A2 [29].

Figure 3.2.2: Effect of haemolysis on cTn assays. Increasing hemolysis added to samples analysed on the Ortho Clinical Diagnostics TnI ES assay (open symbols) and the Roche TnT hs assay (closed symbols). A 20% change was considered clinically significant. The 3 cTnI concentrations were 24 ng/L (◊), 36 ng/L (□), and 49 ng/L (△), and the 3 cTnT concentrations were 6 ng/L (●), 12 ng/L (■), and 23 ng/L (▲). (Note that the negative and positive scales are not equal.) From Bais [27]
Endogenous antibody interferences refer to sources of interference for the sandwich-type immunometric troponin assays due to endogenous antibodies directed against proteins of non-human species, i.e. heterophile antibodies (HA). Circulating heterophile and human anti-animal antibodies may be acquired from iatrogenic and non-iatrogenic causes that may include the use of mouse monoclonal antibodies for therapeutic and imaging purposes, blood transfusions, vaccination against infectious diseases, exposure to microbial antigens, animal husbandry or the keeping of animals as pets, transfer of dietary antigens across the gut wall in celiac diseases, and autoimmune diseases that may give rise to autoantibodies such as autoimmune rheumatoid factors. Human anti-animal antibodies, which are high affinity, specific polyclonal antibodies produced against a specific animal whole immunoglobulin, e.g. human anti-mouse antibody (HAMA), may also interfere. Immunoassays for cTn often employ 2-site (sandwich) or non-competitive reactions which contain 2 antibodies specific at 2 sites for the measured analyte. The first or "capture" antibody, initially binds to any cTnI in the sample. The second or "label" antibody is then added after a wash phase and binds any "captured" cTn, providing a detectable signal that can be measured to quantify the cTn concentration. Human anti-animal and heterophile antibodies, which are specific for the Fc portion of the assay species immunoglobulin, may crosslink the capture or label antibodies in the absence of the intended analyte, which may cause a positive assay response. Because of this potential interference, manufacturers generally add non-specific blocking antibodies of the assay species, which are intended to limit the effect of any heterophile antibodies present in the sample. In some cases when sufficient quantities of interfering antibodies are present, analytical errors may occur.

The incidence of HA in cTn immunoassays is uncertain although Lam et al. reported an incidence of 1 in 5000 (0.02%) by Architect cTnI assay [3]. Since the original report the frequency of interference from HA has reduced, probably because of the addition of heterophile blocking antibodies to the assay format.
Troponin autoantibodies (cTnAAb) are formed after exposure of troponins to circulation. They could be observed in very low quantities in normal individuals and detected more frequently in patients after a myocardial infarction and dilated cardiomyopathy [30-33]. These may cause false negative troponin values by preventing troponin from binding to the reagent antibodies [30,31]. Autoantibodies to both cTnI and cTnT are reported to be prevalent in blood donors [32,33]. The prevalence of IgG autoantibodies reactive with cTnI has been reported to be as high as 12.7% in the normal blood donor population [32]. Alternatively, they may present as false positive values when present as circulating immunoglobulin-troponin complexes or macrotroponin [34-38]. An association with anti-cTnI antibodies and cardiomyopathy has been demonstrated, but anti-cTnT antibodies appear incapable of inducing myocardial damage, possibly due to sarcoplasmic location of cTnT [33].

Constant troponin elevations over days to weeks that show little change in concentration should be investigated to determine if a HA, HAMA, a troponin autoantibody or possibly a macrotroponin is present [37]. Grossly abnormal cTn values that are inconsistent with the patient’s clinical status may alert the laboratory or clinician to a falsely elevated result. Negative interference is harder to detect.

**HETEROPHILE ANTIBODIES AND HAMA:** Laboratory procedures to evaluate the presence of HAMA and HA include the use of a larger dilution of the sample with reagent containing non-immune mouse IgG, use of heterophile blocking tubes or performing the analysis with a differently configured assay [37]. Current and new generation assays may be affected by HA interference and has been reported in the literature [39].

**MACROTROPONIN:** Cases of macrotroponin that are immunoglobulin-troponin complexes have been reported to give persistently elevated troponin due to their slow turnover in blood [36,37,38].
They result in persistently elevated troponin concentrations. Protein G affinity chromatography is used to isolate the immunoglobulin-troponin complex [38].

**LINEARITY**

Linearity could be assessed to verify the stated linearity range on dilution and to detect any potential for bias, but testing is optional [40]. Select a sample with a high cTn concentration and mix it in linearly related proportions with one containing undetectable concentrations (<LoD) or use the manufacturer’s sample diluent. Sequential dilutions of the cTn-positive sample down to the lowest concentration where troponin is measurable are recommended. Suitable mixtures may contain 0, 5, 10, 20, …up to 100 % of the high sample. All dilutions and high and low sample should be assayed in duplicate. Plot measured values versus their expected values and analyse the data by least squares linear regression. An upper limit should be no higher than the highest troponin concentration that falls on the apparently linear segment by eye. An alternative means of data analysis is by residual plot.

**METHOD COMPARISON STUDIES**

Generally method comparison studies are performed using from 20 samples as a minimum to a more thorough evaluation requiring 40 samples or more (CLSI EP09-A2) [41]. Duplicate determinations are required and multiple batches with the two methods run at the same time over several days are preferred to single runs to accommodate between-day variation [42]. To allow for variability in both compared methods, the study results can be compared using Deming model least squares regression analysis. Undue influence of outliers can be handled with Passing-Bablok regression. Difference plots (absolute and logarithmic) can be used to assess method agreement. Testing should be done at concentrations throughout the measuring range, especially at the low end
(Figures 3.2.3A and 3.2.3B). In this example, the difference plots shows cTnT concentrations above a cut-off point of 93 ng/L for the hs-cTnT assay were lower than those obtained using the 4th generation cTnT assay. Below this cut-off point hs-TnT concentration was higher than for values obtained by the 4th generation assay. Difference (%) is calculated as (hs-TnT – cTnT)/cTnT *100. The new high sensitivity cTnT assay has been standardised to the earlier 4th generation cTnT assay at higher concentrations but, due to the different assay detection limits and other analytical differences, there is limited comparability observed at low cTnT concentrations (<100 ng/L) [46].

**CLINICAL VALIDATION**

Clinical validation in the low concentration range is important when assessing the performance of latest generation assays. Small troponin elevations above the 99th percentile URL can occur either acutely or chronically in both ACS and non-ACS conditions, where there is myocardial injury, as well as in the aging community population who are at increased cardiovascular risk. Depending on an assay’s performance characteristics, some assays will recognise different at-risk patient groups [43,44]. Ideally split-patient sample studies that use panels of archived samples from subjects with known clinical outcomes for a range of clinical conditions involving ACS and non-ACS aetiologies are desirable when validating an assay’s clinical performance. However, this is usually beyond the capability of clinical laboratories and involves research or clinical trial laboratories.
Figure 3.2.3A and B: Bland Altman analysis of hs-cTnT and 4th generation cTnT assays. Adapted from [46]
THE 99th PERCENTILE UPPER REFERENCE LIMIT (URL) OF A REFERENCE POPULATION

One reason why the 99th percentile URL of the value distribution in cardio-healthy people was selected as the cut-off value for AMI was to minimise the number of false positive values at a time when it was unclear how low normal values were [47]. However, there are issues with the 99th percentile URL as cut-off, including the analytical performance of the assays and how to define a ‘cardio-healthy’ reference population [48]. With regard to the first aspect, two critical questions can be raised [49]:

- If troponin is (partially or totally) undetectable in a healthy population, how can a reference distribution be accurately defined
- If the measurement error at the decision limit (99th percentile cut-off) is high, how can it be relied on to define true positives and negatives? Different studies have also shown variability of the 99th percentile URL depending on the reference population used, the skewness of the distribution and the number of outliers.

REFERENCE POPULATION

The rationale for determining the 99th percentile in a cardio-healthy reference population as opposed to a general community population is the presence of cardiac muscle damage in otherwise healthy people. For example, in a general population study of Swedish elderly men with no cardiac symptoms at the time of blood collection, a proportion went on to develop cardiac disease or had increased mortality at follow-up several years later [50]. Slightly elevated cTnI concentrations above a 99th percentile of 0.021 µg/L (AccuTnI) were associated with signs of ongoing subclinical processes in the myocardium in this group of older subjects.
The ESC Study Group on Biomarkers in Cardiology recommends that the ideal reference population should have negative exercise stress tests and physiological cardiac function as assessed by imaging [6]. Any effects of age, gender and ethnicity need to be evaluated, and to avoid the effect of outliers and to achieve a 95% probability that at least 99% of the population will have normal troponin concentration, a sample size of at least 300 individuals per sex- and age-matched healthy reference population is the minimum number required [7,51]. The 99th percentile URL of the manufacturers’ troponin assays seen in Table 1 (Analytical characteristics of commercial cardiac troponin I and T assays declared by the manufacturer as at December 2012 [9]) has been determined using a range of different reference population samples. The number of subjects, their cardiovascular status, ethnicity, gender and age are different for each manufacturer’s reference value distribution.

DETERMINATION AND VERIFICATION OF THE 99TH PERCENTILE URL

The National Academy of Clinical Biochemistry laboratory practice guidelines recommend either that the 99th percentile URL of a troponin assay should be determined in each laboratory by internal studies or that a reference interval based on findings in the literature should be validated [8]. In practical terms, few laboratories can determine their own troponin reference intervals as this is generally beyond their capability and resources. On the contrary, it is relatively easier to validate previously established reference limits for the population that laboratories service. The validation can be done according to the CLSI document C28-A3, by examining 20 reference individuals from a laboratory’s own subject population [52]. Note that some highly sensitive cTn assays have shown gender-related differences in the 99th percentile URL [45,46,53]. Hence, validation studies may require both male and female subjects to be tested.

An example of determining cardio-healthy cTn ranges is given by Koerbin et al. for hs-cTnT [46]. The removal of subjects with pathology is important so as to avoid markedly skewing the 99th
percentile URL. The authors note that contemporary cTn assays, which lack analytical sensitivity and accuracy close to the assay LoD, give undetectable cTn concentration for a large percentage of reference subjects. This study also observed a significant difference (P <0.01) between the 99th percentile of the hs-cTnT distribution for both men (12.9 ng/L) and women (11.0 ng/L). (Figure 3.2.4)

As a minimum, laboratories should select a 99th URL that is supported by findings in the peer-reviewed literature relating specifically to the assay they are using. This should be done after consultation with clinicians using the test. Routine laboratories can verify that their assay measures ‘normal’ troponin concentrations that are below the 99th percentile URL by using blood samples from young males and females, e.g. medical students. cTnI decision limits for one manufacturer’s assay should not be extrapolated to other assays until there is adequate cTnI standardisation and results are shown to be comparable.

Figure 3.2.4: hs-cTnT population distribution. In an Australian study the 99th percentile upper reference limit (URL) of a cardio-healthy reference population by hs-cTnT was 12.5 ng/L [46],
which is similar to the manufacturer’s URL [45]. The study also observed a significant difference (P <0.01) between the 99th percentile of both men (12.9 ng/L) and women (11.0 ng/L).

**AUDITING THE IMPACT OF IMPLEMENTATION INTO CLINICAL PRACTICE OF A NEXT GENERATION ASSAY**

New generations of cardiac troponin assays have improved analytical performance at low-range troponin concentrations, thus enabling safe clinical application of international recommendations for AMI (i.e. the 99th percentile limit concept). Whereas on one hand this results in markedly improved assay sensitivity for detecting troponin elevations, on the other hand it may increase the number of positive results not related to ACS, hence challenging physician decision-making. As the new generation assays are cleared for use, the corresponding previous generation assay manufactured by the same company will be replaced. Laboratories and institutions using specific analytical platforms are often ‘obliged’ to adopt the more sensitive assay into clinical practice. However, ignoring the 99th percentile and keeping higher cut-off values based on historical conventions can lead to preventable deaths in patients not reported and not acted upon [54]. Consequently, laboratories need to be prepared and plan for more sensitive assays and their implementation by not only using available scientific knowledge but by auditing the impact of the use of a more sensitive assay in their specific setting soon after introduction. This should be done by comparing current data with those from the same period of the year when the previous generation of troponin assay was used. If no important changes in the hospital organisation have occurred and, consequently no variations in the case-mix between the two periods are expected, this approach represents the best way to obtain the desired information.

There are few recent examples in the literature where health institutions that replaced conventional with new highly sensitive troponin assays have performed a comprehensive audit on the impact of this change on test volumes, troponin positivity rates, and interpretation of results [54-56]. As expected, the number of positive cardiac troponin results have markedly increased, due exclusively
to the results falling into the low-positive range that could not be quantitatively determined with acceptable precision by conventional assays. What is different among studies is the magnitude of the increase of positive results after introduction of new assays into routine practice. This is dependent on the types of assay used, their selected cut-offs, as well as on the type of healthcare institution and the patient case-mix.

In a recent study performed in a 600-bed university hospital in Milan, Italy, replacement of the 4th-generation cTnT assay with the new highly sensitive hs-cTnT assay, both from Roche Diagnostics, increased the number of positive examinations from approximately 25% to more than half of the total without markedly changing test volume [56]. Surprisingly though, when cTnT and hs-cTnT were compared, there was no difference in the percentage of patients with the ‘typical’ troponin release (defined as a rising and/or falling pattern showing a troponin variation between two consecutive samples exceeding +46% for increasing and -32% for decreasing troponin results) [18]. This shows that the use of an interpretative approach based on the demonstration of an acute pathophysiological release of troponin in blood results in the same test performance in terms of biological specificity when using different generations of cTnT assays. After hs-cTnT implementation, the absolute number of hospitalised patients in the Emergency Department (ED) population with positive troponin significantly increased, although the rate of admission to intensive and non intensive care wards was unchanged [56]. On the other hand, about 25% of hs-cTnT positive patients were discharged and, importantly, in the follow-up their rate of readmission did not increase when compared with the cTnT period [56]. The preliminary results show that the implementation of a more sensitive troponin assay can be associated with major reductions in morbidity and mortality [54].

**SETTING GOALS FOR ANALYTICAL PERFORMANCE OF TROPOIN MEASUREMENTS**
Clinical laboratories must verify the consistency of the performance declared by the manufacturer of their troponin assay during daily routine operations performed in accordance to the manufacturer’s instructions. This requires that the internal quality control materials (IQC) used to monitor the analytical performance of the employed analytical system should be organised into two independent components:

- To confirm that current measurements are acceptable (‘unbiased’) according to the manufacturer’s established parameters (i.e., the acceptable range of control materials); and
- Using a different control material designed to evaluate the system imprecision.

Theoretically, External Quality Assessment (EQA) schemes for cardiac troponins should meet requirements reported shown in table 3.2.2.

As there are no high-order reference methods available for troponins (I and T), system-dependent target values should be used to evaluate the performance of participating laboratories. The values assigned to the EQA materials should preferably be determined by reference institutions (possibly including the manufacturer of that specific analytical system), working under strictly controlled conditions in order to maintain measurement uncertainty as low as possible, and group method means are not recommended.

Table 3.2.2. Requirements for the applicability of EQA results to evaluation of the performance of individual laboratories in the measurement of cTn. Adapted from Panteghini [57]
Once the estimate of the analytical performance of troponin measurement has been obtained in appropriately structured IQC and EQA programmes, acceptability limits for such performance must be used to properly identify analytical systems (and laboratories) that require corrective action. As we are faced with the clinical usefulness of laboratory measurements, simple statistical criteria are not enough; rather, measurement performance (i.e., bias, imprecision, total error) should fall within limits based on medical relevance so that results are reliable for patient management. The definition of acceptable performance for troponin measurement is still under discussion [25], but the biological variability data can help in objectively defining the allowable goals (Table 3.2.3) [2].

Table 3.2.3. Analytical performance goals for cTnI measurements using routine methods based on data of biological variability obtained by Wu [18].

<table>
<thead>
<tr>
<th>Quality Level</th>
<th>Imprecision goal</th>
<th>Bias goal</th>
<th>Total error goal*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>≤7.3%</td>
<td>≤±21.6%</td>
<td>≤33.6%</td>
</tr>
<tr>
<td>Desirable</td>
<td>≤4.9%</td>
<td>≤±14.4%</td>
<td>≤22.5%</td>
</tr>
<tr>
<td>Optimum</td>
<td>≤2.4%</td>
<td>≤±7.2%</td>
<td>≤11.2%</td>
</tr>
</tbody>
</table>

CONCLUSIONS

Both laboratory staff performing troponin measurement and clinicians who interpret troponin results should ‘know their routine laboratory’s troponin assay’ and its performance characteristics.
Verification of the following characteristics by the clinical laboratory is necessary to determine whether a new troponin assay is fit-for-purpose:

- Total imprecision, including biological samples with troponin concentrations around the established clinical decision limit (mandatory);
- Limit of blank and limit of detection (optional);
- Linearity of analytical response (optional);
- Method comparison and analytical concordance if the assay is replacing a previous assay; and, if possible,
- Validation of the previously established 99th percentile URL.

In the case of latest generation, high sensitivity troponin assays, clinical validation of these assays is required in collaboration with local cardiology units and emergency medicine departments, together with auditing of the impact of their implementation on troponin positivity rates and interpretation of results. Monitoring of the long-term assay performance by use of appropriately organised IQC and participation in EQA schemes using performance goals based on medical relevance provide unique information that permit verification of the suitability of laboratory measurements for their clinical use.
REFERENCES


CHAPTER 3.3

THE ANALYTICAL CHARACTERISTICS OF THE ROCHE hs-cTnT ASSAY AND ITS APPLICATION TO A CARDIO-HEALTHY POPULATION

Part of this work has been published in:

Koerbin G, Tate J, Hickman PE.

Ann Clin Biochem. 2010;47:524-8
ABSTRACT: It is desirable that current assays for cardiac troponin (cTn) be able to meet the recommended criterion that the diagnosis and risk assessment of patients who present with symptoms of myocardial infarction requires a rise and fall in cTn with at least one point above the 99th percentile of a reference population. We have evaluated the analytical characteristics of the new high sensitivity troponin T (hs-cTnT) assay to see if it meets this criterion and applied it to a carefully defined, cardio-healthy Australian reference population.

An imprecision profile was determined for the Roche hs-cTnT assay using multiple samples analysed on 9 separate occasions. Distribution of troponin T was determined using 104 samples from a cardio-healthy population.

The new hs-cTnT assay meets the specifications of a coefficient of variation (CV) of 10% at the 99th percentile of our cardio-healthy reference population. Of the 104 samples analysed 44 showed troponin T concentrations above the manufacturer quoted limit of detection. Age and gender differences in the median and 99th percentile troponin T concentrations were observed.

The new high sensitivity troponin T (hs-cTnT) assay shows improved precision and sensitivity at very low troponin concentrations. We have shown that a significant number of individuals in this cardio-healthy population had detectable circulating troponin concentrations. With many apparently healthy people having detectable troponin, clinical judgment will become more important in interpreting troponin results.
INTRODUCTION

Myocyte necrosis can be recognised by the appearance of biochemical markers in the blood. The recommended biochemical marker for the diagnosis and risk assessment of patients presenting with myocardial infarction by the European Society of Cardiology and the American College of Cardiology (ESC/ACC) is a rise and fall of cardiac troponin (cTn) with at least one value above the 99th percentile of a reference population the upper reference limit (URL) and the assays used should have an imprecision, coefficient of variation (CV) of ≤10% at this cut point [1]. Use of the 99th percentile of a reference population as a decision point (URL) has some limitations in that many of the currently used assays lack the sensitivity to measure troponin in healthy individual [2].

The criteria for defining a reference population are still undefined [2,3]. The National Academy of Clinical Biochemistry suggests that reference values and decision limits should be based on a healthy population without a history of known cardiac disease [4]. Tate [5] has suggested that troponin reference intervals need to be established that allow differentiation of people at cardiovascular risk using populations that are cardio-healthy.

The new generation of highly sensitive assays with greater analytical sensitivity and precision at low concentration have demonstrated the presence of troponin in the majority of apparently healthy persons [6,7,8]. There have however been only a limited number of reference interval studies where the population used has been screened and deemed to be cardio-healthy. As these new highly sensitive assays become common place in clinical practice more individuals will present to emergency rooms with detectable troponin. It is particularly important that we review troponin levels in a cardio-healthy reference population to address this issue.

Tate et al [9] performed a study using 111 apparently healthy Australian community individuals in whom questionnaire, blood tests and stress echocardiography were used to
determine cardiovascular health. Of these 111 individuals, three were rejected based on cardiovascular assessment. We have revisited this population using the 5th generation high sensitivity cTnT (hs-cTnT) assay from Roche Diagnostics and report our findings in this paper.

MATERIALS AND METHODS

ETHICS

Ethics approval was received from the Princess Alexandra Hospital Ethics Committee and written consent was obtained from the reference subjects.

REFERENCE POPULATION SUBJECTS

Community volunteers from Brisbane, Australia whose ages ranged between 25 and 74 years completed a questionnaire to determine eligibility. The questionnaire was used to exclude those volunteers with diabetes mellitus, hypertension, known cardiac disease, known or treated hyperlipidaemia and those taking cardio-active medications such as beta blockers, calcium channel blockers and ACE inhibitors. The final population selected for the study were 62 men and 49 women. The subjects underwent stress echocardiography and routine chemistry testing on blood and urine including electrolytes, urea, creatinine, calcium, phosphate, liver enzymes, glucose, iron, ferritin, transferrin, cholesterol, triglycerides, HDL-cholesterol, HbA1c and urine total protein and albumin. Three individuals were identified as not meeting the criteria to be considered cardio-healthy and were excluded. These individuals showed cardiovascular abnormalities by stress angiography such as angina and ST depression with apial/anterior abnormal wall motion, left bundle branch block and apial hypokinesia at
rest or apial hypokinesia with probable segmental scar. Sufficient sample was available for further testing on 104 (57 male, 47 female) of these 108 subjects.

REFERENCE POPULATION SAMPLES

Blood samples were collected into Greiner SST (Serum) tubes, centrifuged at 3000 g for 10 min within 2 h of collection and then stored at -80°C within 8 h prior to analysis. These samples were later transported to the Canberra Laboratory on dry ice where they were stored at -80°C prior to analysis. The samples were thawed at room temperature, mixed and re-centrifuged at 3000 g for 10 min prior to analysis.

IMPRECISION PROFILE SAMPLES

Blood samples were collected from four patients known to have had a myocardial infarct and with detectable levels of troponin into Becton Dickinson PST gel (lithium heparin) collection tubes. The plasma from these samples was pooled after centrifugation at 3000g for 10 minutes and cTnT determined by the hs-cTnT assay. This patient pool was linearly diluted in pooled plasma (lithium heparin) collected from 2 donor samples with cTnT concentrations below the manufacturer quoted limit of detection (<5 ng/L). The dilutions range between cTnT concentrations of 5 – 340 ng/L and were stored in 0.2 – 0.3 mL aliquots at -80°C. These samples were thawed at room temperature, mixed and re-centrifuged at 3,000g for 10 min prior to analysis in duplicate on 9 separate occasions for hs-cTnT. Analysis was according to modified CLSI guidelines [10], with imprecision profiling according to Sadler [11].

METHOD COMPARISON SAMPLES
Blood samples were collected into Vacuette tubes with separator gel (Greiner SST (serum) and PST (lithium heparin)) from 96 patients undergoing either hemo or peritoneal dialysis and having routine cTn testing performed. This population was selected because a much higher proportion of patients have detectable troponin in their blood [12]. All blood samples were collected immediately pre-dialysis, and processed within 2 h of collection. Samples were centrifuged at 3000g for 10 min and 0.2 – 0.3 ml aliquots prepared and stored at -80°C. Prior to assay each aliquot was thawed and re-centrifuged at 3000g for 10 min. The 96 lithium heparin plasma samples were used in the method comparison of hs-cTnT and the cTnT 4th generation assays. Forty-two paired (serum and heparin) samples with cTnT concentrations above the manufacturer quoted limit of quantitation, LoQ, cut point (13 ng/L) were used to assess any difference in sample type for the hs-cTnT assay.

CARDIAC TnT ASSAYS

Reference population, imprecision and method comparison samples were analysed by hs-cTnT (single reagent lot number) and the 4th generation cTnT assays on the Roche E411 analyser (Roche Diagnostics, Sydney, Australia). The hs-cTnT assay was calibrated three times during this study.

DATA ANALYSIS

Troponin concentrations are reported as ng/L. Between-run standard deviation of replicate hs-cTnT measurements (N=18 for each hs-cTnT concentration) was used in the variance function program (VFP Version 8.1, W.A. Sadler, Christchurch, New Zealand) to determine the hs-cTnT imprecision profile. Troponin concentrations at 10% and 20% CVs were determined from the profile. Method comparison was by Passing-Bablok regression analysis.
For statistical analysis to determine the 99th percentile URL, all results below the manufacturer quoted limit of blank (LoB; 3 ng/L) were assigned a concentration of 3 ng/L. Statistical analysis was undertaken using Analyse-it for excel.

RESULTS.

IMPRECISION

Between-run imprecision for the hs-cTnT assay using commercial quality control material (PreciControl Troponin, Roche Diagnostics, Sydney, Australia) during the study period was 8.4% at 28.5 ng/L and 5.1% at 2,313 ng/L cTnT. Linearly related dilutions in the hs-cTnT range 5-340 ng/L were used for the precision study. From the imprecision profile (Figure 3.3.1) the 10% CV corresponded to a cTnT concentration of 11.9 ng/L and the 20% CV corresponded to a TnT concentration of 6.8 ng/L. Between-run imprecision and recovery of TnT on sample dilution are seen in Table 3.3.1.

METHOD AND SAMPLE TYPE COMPARISONS

96 paired samples in the range 27-330 ng/L by hs-cTnT assay and 11 – 500 ng/L by cTnT 4th generation assay were compared. Passing Bablok regression analysis gave a slope of 0.77 [95%CI 0.73 – 0.81], intercept 20.4 [95%CI 18.7 - 23.9] and r² of 0.96 (Figure 3.3.2). Bland Altman analysis, seen in figure 3.3.3, shows concentrations above a cutpoint of 93 ng/L for the hs-cTnT assay were lower than those obtained by the 4th generation cTnT assay. Below this cutpoint, hs-cTnT concentrations were higher than those obtained by the 4th generation assay (Figure 3.3.4). No significant difference was seen between serum and plasma by hs-cTnT assay in the cTnT range 13-188 ng/L (slope 0.99 [95%CI 0.96-1.02], intercept 0.52 [95%CI -1.92-2.95], r² 0.99). (Figure 3.3.5
Table 3.3.1: Measured mean cardiac troponin (cTn) concentrations, recovery and imprecision for nine daily measurements of thirteen linearly related plasma samples. * Sample 1 represents the MI patient pool; # Sample 13 represents the healthy donor pool.

<table>
<thead>
<tr>
<th>SAMPLE NUMBER</th>
<th>1*</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution</td>
<td>Nil</td>
<td>40%</td>
<td>20%</td>
<td>12%</td>
<td>10%</td>
<td>8%</td>
<td>6%</td>
<td>4%</td>
<td>3%</td>
<td>2.5%</td>
<td>2.0%</td>
<td>1.5%</td>
<td>Nil</td>
</tr>
<tr>
<td>Expected TnT (ng/L)</td>
<td>338</td>
<td>137</td>
<td>69</td>
<td>41</td>
<td>34</td>
<td>27</td>
<td>21</td>
<td>13.7</td>
<td>10.3</td>
<td>8.6</td>
<td>6.8</td>
<td>5.1</td>
<td>4.6</td>
</tr>
<tr>
<td>Measured TnT (ng/L)</td>
<td>338</td>
<td>141</td>
<td>66</td>
<td>43</td>
<td>34</td>
<td>27</td>
<td>21</td>
<td>13.7</td>
<td>12.4</td>
<td>8.1</td>
<td>6.0</td>
<td>5.0</td>
<td>4.6</td>
</tr>
<tr>
<td>SD (ng/L)</td>
<td>9.5</td>
<td>5.1</td>
<td>2.5</td>
<td>1.7</td>
<td>1.3</td>
<td>1.2</td>
<td>0.9</td>
<td>1.2</td>
<td>1.4</td>
<td>1.2</td>
<td>1.5</td>
<td>1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>CV (%)</td>
<td>2.8</td>
<td>3.7</td>
<td>3.8</td>
<td>3.8</td>
<td>3.9</td>
<td>4.4</td>
<td>4.6</td>
<td>8.7</td>
<td>10.9</td>
<td>15.0</td>
<td>25.5</td>
<td>38.5</td>
<td>21.9</td>
</tr>
</tbody>
</table>
Figure 3.3.1: Imprecision profile of hs-cTnT. % coefficient of variation (CV) and standard deviation (SD) versus troponin concentration (logarithmic scale) determined by measurement of low-level cardiac troponin (Roche hs-cTnT) on 9 separate occasions. Troponin concentrations at 10%CV and 20% CV are shown by the dotted and solid horizontal lines respectively.
Figure 3.3.2: Passing-Bablok regression analysis plot of cardiac troponin T for 96 plasma samples. The values for the regression slope and y-intercept are shown with the 95% confidence intervals given in brackets and as dotted lines on the figure.
Figure 3.3.3: Bland Altman analysis of cardiac troponin T for 96 plasma samples. The 95% confidence intervals are shown by the dotted line. Polynomial regression analysis shows an x-intercept at 93 ng/L.

Figure 3.3.4: Expanded Bland Altman analysis of cardiac troponin T for 96 plasma samples.
Figure 3.3.5: Regression analysis, serum vs lithium heparin plasma

REFERENCE POPULATION

One hundred and four cardio-healthy subjects had samples available for analysis (57 male and 47 female). All samples tested were below the manufacturer quoted LoD for the cTnT 4th generation assay (<10 ng/L), which is consistent with the findings of Tate et al [9]. The concentration distribution for the hs-cTnT assay is seen in figure 3.3.6, and shows a non-Gaussian distribution being skewed towards the manufacturer quoted LoB; (3 ng/L).
Sixty samples gave hs-cTnT concentrations below the manufacturer quoted LoD (5 ng/L). Twenty-seven of these were below the quoted LoB with 33 between LoB and the LoD, 43 were between this LoD and the manufacturer quoted 10% CV concentration of 13 ng/L, with one sample greater than the manufacturer quoted 99th percentile upper reference limit (URL). For this study cohort the 99th percentile URL was determined to be 12.5 ng/L. It is acknowledged that without bootstrapping the cohort of samples is too small to provide absolute confidence in this concentration.

A significant difference in median hs-cTnT concentrations (p < 0.01, Mann-Whitney U test) was observed between men and women (figure 3.3.7). A difference was also seen between median troponin concentrations and the age ranges of <60 and 60+ years for both males and females. These relationships are seen in Table 3.3.2
Figure 3.3.7: Differences in cardiac troponin T concentrations in men and women younger and older than 60 years. The diamond indicates the median concentration and the dotted vertical line the 99th percentile.

Table 3.3.2: Median cardiac troponin T concentrations and 99th percentile values in men and women younger and older than 60 years.

<table>
<thead>
<tr>
<th></th>
<th>Male (n = 57)</th>
<th>Female (n = 47)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (range)</td>
<td>99th Percentile</td>
</tr>
<tr>
<td>All</td>
<td>5.7 (&lt;3 - 13.3)</td>
<td>12.9</td>
</tr>
<tr>
<td>≤ 60 years</td>
<td>5.0 (&lt;3 - 10.7)</td>
<td>10.5</td>
</tr>
<tr>
<td>&gt;60 years</td>
<td>6.9 (&lt;3 - 13.3)</td>
<td>13.1</td>
</tr>
<tr>
<td>p (≤60 and &gt;60 y)</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

The new hs-cTnT assay has significantly improved analytical performance at very low concentrations of troponin. In this cardio-healthy population 42% had cTnT greater than the manufacturer quoted LoD (5 ng/L), whereas the 4th generation cTnT assay did not detect troponin in any of these 104 subjects. Jacobs et al [13], using precommercial hs-cTnT reagent and a LoD of 1 ng/L found 92% of a healthy population had troponin concentrations above the LoD. In a recent study by Reichlin et al [14] a LoD for the hs-cTnT assay of 2 ng/L was used. If the cTnT concentrations of the subjects in our study are compared with these detection limits then at least 74% had detectable troponin. The method comparison of the 4th generation cTnT with the hs-cTnT assay showed a good correlation with $r^2 >0.96$. Regression analysis was different to that of the manufacturer ($y^{hs-cTnT} = 0.77x^{4th-gen cTnT} + 20.4 \text{ ng/L}$) compared with manufacturer ($y^{hs-cTnT} = 0.99x^{4th-gen cTnT} + 0.47 \text{ ng/L}$). This difference may reflect variation in the analytical ranges used (manufacturer 3 - 8,100 ng/L; our study 5 – 338 ng/L) or the subject groups (ie. healthy versus dialysis patients). Degraded forms of cTnT have been reported in renal dialysis patients [15] and may react differently in the two cTnT assays. Analysis of paired serum and plasma samples for the hs-TnT assay indicated that there is no significant difference between serum and plasma down to a concentration of 13 ng/L.

International guidelines [1] have recommended that the 99th percentile and the 10%CV need to be validated for each troponin assay by routine testing laboratories. Contemporary troponin assays, which lack analytical sensitivity and accuracy close to the assay detection limit, give undetectable troponin concentration for a large percentage of reference subjects. As a consequence the 99th percentile URL could be
markedly skewed by including subjects with pathology. We found and excluded three such persons from our study. If these subjects had been included our conclusions regarding the 99th percentile may have been markedly different. Our study has demonstrated that even using this hs-cTnT assay, 60/104 samples were below the manufacturer quoted LoD with 27/104 below the manufacturer quoted LoB contributing to the skewness of this reference population.

There is still the requirement to define what is a normal apparently cardio-healthy reference subject [2,3] and for manufacturers to use the same set of reference subjects to determine the URL for same-generation troponin assays. Eggers et al. [16] have demonstrated that the reference population selected has an effect on the determination of the 99th percentile. Recent reports [9,17,18] have demonstrated that the measurement of cTnI in sample sets from different groups of apparently healthy subjects can produce different 99th percentile values for the same assay. Using the ADVIA Centaur TnI-Ultra\textsuperscript{TM} 2nd-generation assay (Siemens, US), in three different reference populations the 99th percentile URLs were 0.021, 0.039, and 0.040 µg/L with the cardio-healthy population who were negative by stress echocardiography giving the lowest cTnI URL [9,17,18].

In our study, using the same reference population, the 99th percentile URL by hs-cTnT was 12.5 ng/L which is similar to the manufacturer’s limit. Giannitsis et al [19] in their study of 616 individuals demonstrated a 10%CV of 13.0 ng/L with the 99th percentile for the entire population of 13.5 ng/L. This study also observed a significant difference (p<0.01) between the 99th percentile of both males (14.5 ng/L) and females (10.0 ng/L). Mingels et al [7] using a pre-commercial version of the hs-TnT assay in a reference population consisting of 546 apparently healthy persons from a health-check hospital program obtained a 99th percentile URL of 16 ng/L. This
study also observed significant differences in age and gender. Although small in number, our study supports both Mingels’ [7] finding of gender and age differences and Giannitsis’ [19] findings of analytical performance.

The object of our study has been to define the analytical characteristics of the new Roche hs-cTnT assay and to apply it to a carefully defined, cardio-healthy reference population. Of great interest is that the majority of our cardio-healthy subjects had detectable cTnT in their blood. Clerico [18] has suggested that detectable troponin in the healthy population may be due to myocyte turnover and the physiological remodelling of the myocardium. This myocyte turnover may reflect the physiological as well as the pathological process [20,21]. If troponin is present in the blood of many healthy persons it will require greater reliance on clinical judgment reminiscent of when CK-MB was the cardiac marker used. The Universal Definition of Myocardial Infarction requires a rise and fall in troponin concentration with at least one value greater than the 99th percentile of the reference value distribution. With this in mind we need to understand the biological variation of cardiac troponin T in the cardio-healthy population and the physician may need to consider serial sampling to determine the acute or chronic nature of an elevated cTnT.

A lot to lot variation in hs-cTnT assay performance was identified in 2012 [22]. This decrease or drift in low end sensitivity caused up to 88% of the samples from a healthy cohort to be measured with values below the limit of the blank (3 ng/L), rather than the ≤50% observed in most studies [23]. This shift was not due to a change in reagent formulation but to the standardization of assay lots against the master lot. Whilst it has been observed that this did not affect concentrations above the 99th percentile URL [23,24] it does highlight the need for laboratories to monitor low end performance of their assays.
REFERENCES

CHAPTER 3.4

CHARACTERISATION OF A HIGHLY SENSITIVE cTnI ASSAY
AND ITS APPLICATION TO A CARDIO-HEALTHY
POPULATION

This work was published in:

Koerbin G, Tate J, Potter JM, Cavanaugh J, Glasgow N, Hickman PE.

**ABSTRACT:** Abbott Diagnostics have developed a new highly sensitivity troponin I assay. We have assessed its analytical characteristics and applied the assay to a population of apparently cardio-healthy persons.

We assessed imprecision, bias compared to the previous generation assay, matrix effects, and interferences and applied the assay to an apparently healthy population, deriving the 99th percentile limit of the distribution of values in a reference population for men and women separately.

The dynamic range of the assay was from 0.5-50,000 ng/L (pg/mL). The 10% CV was at a concentration of 3.9 ng/L, and the 20% CV was at a concentration of 1.8 ng/L. The new and current version of the cTnI assay were highly correlated (Slope: 0.98 (95%CI: 0.88 – 1.07), y- intercept: 1.20 (95%CI:-2.35 – 4.75) r² = 0.99). The 99th percentile limit of the distribution of values in a reference population was different for males and females: males 14.0 ng/L and females 11.1 ng/L. At these concentrations the assay CV was 5.0%. cTnI was detectable in nearly all patient samples from the healthy reference population (98.6%).

This new hs-cTnI assay is able to measure to an order of magnitude lower than the current generation TnI assay from the same manufacturer. With TnI being detectable in nearly all apparently healthy subject samples this suggests that cTnI presence does not always indicate cardiomyocyte necrosis.
INTRODUCTION

Cardiac troponin is now clearly established as the superior biomarker for investigation of the acute coronary syndrome and troponin rise and fall is central to the diagnosis of this condition [1].

Recently, new highly sensitive assays for both troponin T (cTnT) and troponin I (cTnI) have been evaluated, both of which can measure to an order of magnitude lower than previously possible. Investigation of apparently healthy populations has shown both of these biomarkers detectable in the blood of the majority of subjects, and in an apparently Gaussian distribution, suggesting that low background concentrations of troponin may be physiological [2,3] and that the presence of troponin is not always a sign of cardiomyocyte necrosis. It is important now to characterise these new hs-cTn assays and apply them to distinct populations and assess the information obtained [4].

In this paper we report the characterisation of a research prototype in final configuration of a highly sensitive troponin I (hs-cTnI) assay from Abbott Diagnostics. We have applied this assay to an apparently healthy population of 497 persons.

MATERIALS and METHODS

All studies using human blood samples were approved by the ACT Health Human Research Ethics Committee, the Australian National University Human Research Ethics Committee or the Princess Alexandra Hospital Ethics Committee, Woolloongabba, Queensland.
The new hs-cTnI assay from Abbott Diagnostics was performed on the Abbott ARCHITECT ci16200 analyser. This assay uses 1 capture antibody (epitope aa 24-40) and 1 detection antibody (epitope aa 41-49) and the detection antibody is a chimeric human antibody. The assay uses 150 μL of sample and assay run time is <16 minutes.

SAMPLES

For both the imprecision profile and dilution linearity testing, blood samples were collected from three known myocardial infarction patients with detectable concentrations of troponin in their blood. These samples were collected into Becton Dickinson SST gel (no additive) collection tubes. The serum from these samples was pooled after centrifugation at 3000g for 10 minutes and cTnI concentration by the hs-cTnI assay determined. This patient pool was linearly diluted in pooled serum collected from 2 donor samples with cTnI concentrations below the manufacturer quoted limit of detection (1.0 ng/L). The cTnI concentration of the dilutions ranged between 1.4 and 354 ng/L. These dilutions were stored at -80°C in 0.4 mL aliquots for the imprecision profile and 0.25 mL aliquots for the linearity testing. Prior to analysis, all samples were thawed to room temperature, mixed and re-centrifuged at 10,000g for 10 min prior to analysis in duplicate on 10 separate occasions. For the linearity testing samples were thawed to room temperature, mixed and re-centrifuged at 10,000g for 10 min prior to analysis in singlicate on 10 separate occasions on the same day.

For comparison between the current on market cTnI and hs-cTnI assays, blood samples were collected into Becton Dickinson SST gel (no additive) collection tubes from a cohort of 143 end stage renal disease (ESRD) patients [5] undergoing either
hemo- or peritoneal dialysis and 177 community volunteers. All blood samples had cTnI concentrations above the current on market assay LoD of 0.01µg/L (10 ng/L).

Blood samples from renal patients were collected immediately pre-dialysis. All samples were processed within 4 hours of collection. Samples were centrifuged at 3,000g for 10 min and aliquots for each assay stored at -80°C. Prior to assay each aliquot was thawed and re-centrifuged at 10,000g for 10 min.

To compare serum and plasma values with the new assay, we used 340 paired samples from community and hospital outpatients collected into Becton Dickinson SST gel (no additive) and PST (Lithium heparin) collection tubes and 197 matched serum / K2-EDTA (Becton Dickinson collection tubes) samples. All samples had TnI concentrations above the manufacturer quoted assay LoD of 1.0 ng/L, and covering the range of 1.0-5,400 ng/L and were assayed in singlicate.

We looked at 2 similar populations of healthy persons. One was a population of 103 cardio-healthy volunteers from Queensland (59 males, 31-74 years, 44 females, 26-72 years) [6], the other of 394 healthy persons from Canberra (162 males, 20-78 years, 222 females, 21-84 years). Both groups were community living, with no history of cardiac disease and provided detailed health histories (combined:116 males <50 years, 105 males 50+ years; 122 females <50 years, 154 females 50+ years). NT-proBNP (Roche Diagnostics) was performed as a surrogate marker of left ventricular dysfunction using FDA approved cut-points of 125 ng/L for subjects <75 years and 450 ng/L for subjects 75+ year. [7]. Creatinine concentrations < 110 µmol/L and eGFR (MDRD equation) with a cutoff of 60mL/min/1.73m² was used to demonstrate adequacy of renal function [8].

We have previously used samples from the Queensland population to assess the performance of the hs-cTnT assay from Roche Diagnostics [9]. The samples were
collected in Brisbane and transported to the Canberra Laboratory on dry ice where they were stored at -80°C prior to analysis. All samples were thawed at room temperature, mixed and re-centrifuged at 10,000g for 10 min prior to analysis. The samples used in this study had been stored for 5 years and had not previously undergone a freeze-thaw cycle.

SAMPLE INTEGRITY

We have demonstrated the long-term stability of cTnT when stored frozen at -80 °C [10]. Similarly, long term stability of cTnI has been demonstrated [2,11]. To confirm sample integrity for cTnI we reanalysed samples stored for up to 5 years at -80°C from a renal dialysis patient cohort [10] and samples stored for up to 5 years at -80°C from the community cohort [6] using the on market assay. All 67 samples had detectable TnI with this on market assay (>10 ng/L). To assess any possible interferences associated with use of a gel separator 20 blood samples with TnI concentrations between the LoD and 99th percentile were collected into Becton Dickinson no anticoagulant SST (gel) and non gel tubes and assayed using the research prototype cTnI method.

METHODS

Hs-cTnI analysis was performed using two separate reagent lots and two calibrator sets. Each reagent lot was calibrated twice using each calibrator set during the study.
LIMIT OF BLANK, LIMIT OF DETECTION, AND LIMIT OF QUANTITATION

The limit of blank (LoB) was determined using human troponin-free serum (Abbott Diagnostics), which was analysed 60 times over 6 runs on the Abbott ARCHITECT ci16200. The limit of detection was determined using 3 samples analysed 20 times each whose troponin concentration was not greater than 4 x LoB (2.0 ng/L). Mean and SD were calculated. Calculations of LoB and LoD were performed according to the CLSI EP17-A specifications [12]

LoB = μB + 1.645 σB, where μB and σB are the mean and standard deviation of the analyte free material.

LoD = LoB + cβ SDS, where cβ = 1.645/(1-1/(4 x f)), f = degrees of freedom, SDS = √(average of variances).

To determine the limit of quantitation (LoQ) the total error was assessed as bias + 1.96 X CVa with a desirable goal of ≤24.0% and a minimum goal of ≤35.9% [13,14]. Bias was assessed by comparison of the hs-TnI assay versus the on-market assay in the ranges of 10 – 950 and 10 – 40 ng/L.

IMPRECISION TESTING

Analysis was according to CLSI EPS-A2 guidelines [15] with 12 samples analysed in duplicate on 10 separate occasions. Imprecision profiling was according to Sadler [16]. Troponin concentrations at 10% and 20% were determined from the imprecision profile.
Within and between run imprecision was determined using the manufacturer’s tri-level quality control (QC) material. QC samples were analysed within 1 run (n=20) and between-runs (n=70).

LINEARITY TESTING

We assessed linearity in accordance with CLSI EP6-A [17] over the concentration range of 1.5ng/L to 180 ng/L by serial dilution of a pool of 3 human serum samples. The pool sample was serially diluted up to 1:2 with pooled human serum with hs-TnI concentration below the quoted manufacturer LoD (1.0 ng/L) in 10 consecutive steps enabling 11 final concentrations ranging from 180 ng/L to 1.5 ng/L. We measured all dilutions on 10 separate occasions in singlicate on the same day. Acceptable linearity was determined if there was no difference between simple linear regression and 2nd and 3rd order polynomial regression [18].

COMPARISON OF SERUM AND PLASMA

All samples were frozen at -80°C within 4 hours of collection and stored for between 2 weeks and 2 years. Prior to analysis each aliquot was thawed and re-centrifuged at 10,000g for 10 minutes. Troponin assays were performed in singlicate. The samples used in this study had not previously undergone a freeze-thaw cycle. Deming regression analysis was used for serum/plasma comparison and the Mann-Whitney non parametric test for group comparison was used for the evaluation of statistical difference between paired serum and plasma samples. Analysis was undertaken using Analyse-it for Excel.
HEMOLYSIS TESTING

Donor samples with cardiac troponin concentrations around the LoD, 99th percentile cutoff and above were collected into Becton Dickinson SST collection tubes. Hs-TnI analysis was undertaken in duplicate before and after preparation of these samples according to the method of Dimeski et al [19], over the free haemoglobin concentration range 0.1-5.5 g/L. The average pre-preparation hs-cTnI concentrations were 1.9ng/L, 3.8ng/L, 10.1ng/L, 24.1ng/L and 72.9 ng/L. Standard errors were calculated for troponin recovery at each Hb concentration.

ASSAY COMPARISON

We compared the on-market TnI assay with the research prototype hs-cTnI assay using 322 routine specimens from randomly selected patients with on market TnI concentrations between 10 and 950 ng/L. All samples were measured in singlicate. Regression data was calculated using the Deming method. Statistical analysis was performed using Analyse-it.

RESULTS

The comparison of 67 samples stored for up to 5 years at -80 °C with the on market TnI assay concentrations >10 ng/L found a very high correlation between the two assay measurements. The regression equation showed a slope of 1.03, y-intercept of 0 with $r^2=0.95$, confirming excellent sample integrity.

We found excellent correlation between no anticoagulant gel (SST) and non gel tubes using the research prototype cTnI assay showing a slope 0.97 (95% CI 0.84 – 1.08) and $r^2 = 0.99$ (n= 20; range <14 ng/L). Within run imprecision was calculated using 20
replicates of control materials and was 3.10% at 19.6 ng/L, 2.43% at 196 ng/L and 2.06% at 14,617 ng/L respectively. Between run imprecision was calculated using 70 determinations and was 4.22% at 20.0 ng/L, 2.81% at 198 ng/L and 2.70% at 14,905 ng/L.

From the imprecision profile (Figure 3.4.1) hs-cTnI concentration was 3.9 ng/L at 10% CV and 1.8 ng/L at 20% CV. Analyses were performed on 2 separate analysers, using 2 separate reagent lots each calibrated twice during the study. By comparison, the current on-market assay has a 10% CV at a concentration of 55 ng/L and a 20% CV at a concentration of 23 ng/L [6].

![Imprecision profile showing assay total CV versus log concentration for the Abbott hs-cTnI assay. Concentration at 10% CV (3.9 ng/L) is marked.](image-url)
Using the CLSI criteria, the LoB was 0.5 ng/L and the LoD was 1.0 ng/L. The LoQ, with a desirable total error of ≤24%, was determined to be <10.0 ng/L when bias was calculated according to regression analysis from 10-950 ng/L and <16 ng/L from 10-40 ng/L.

The 20% CV is becoming widely used as the appropriate reporting limit for troponin [20-22]. With the hs-cTnI assay, we found 490 of our population of 497 healthy subjects (98.6%) had concentrations above the LoD, and 84.5% had cTnI concentrations above the 20% CV of 1.8 ng/L. By comparison only 57 of these 497 subjects (11.5%) had detectable cTnI using the on market assay and 7.4% had cTnI concentrations above the 20% CV of 23 ng/L. Linearity testing showed recovery of 83 – 115% over the range of 1.5 – 180 ng/L and between 97 and 100% for concentrations above the 99th percentile limit of the distribution of values in a reference population. Linear, 2nd and 3rd order polynomial regression analysis (y = 1.00x + 0.35, y = 6*10^-6x^2 + 1.00x + 0.36, y = 3*10^-6x^3 + 6*10^-4x^2 +1.02x + 0.27, respectively with r^2 =0.997) showed no statistical differences.

The current on market cTnI assay and the new hs-cTnI assays produced very similar results for samples with values above the LoD of the older assay (0.01 μg/L – 10 μg/L) as shown in Figures 3.4.2A and 3.4.2B. Deming regression of troponin concentrations 10-950 ng/L gave: Slope: 0.98 (95%CI: 0.88 – 1.07), y- intercept: 1.20 (95%CI:-2.35 – 4.75) n = 322, r^2 =0.99, average difference 7.2% (95%CI: 4.7 – 9.8) and for concentrations 10-40 ng/L Slope: 0.88(95%CI: 0.80-0.96) y-intercept: 4.12 (95%CI: 2.27-5.97) n=135 r^2 =0.99, average difference 7.5% (95%CI: 4.4 – 10.7).
Figure 3.4.2A. Deming regression comparison between on market cTnI assay and research prototype hs-cTnI assay over the range 10-950 ng/L (n=322). Insert shows range 11-40 ng/L, (n=135)
Figure 3.4.2B. Difference plot showing comparison between on market cTnI assay and research prototype hs-cTnI assay over the range 10-950 ng/L. (Average bias and 95% confidence intervals are shown) Insert: Difference plot shows range 11-40 ng/L with average bias and 95% confidence interval shown.

Below 10 ng/L lithium heparin plasmas gave apparently lower concentrations than the corresponding serums. The average difference was 13.8% (95%CI: -17.4 to -10.2). However this was not statistically significant (Deming regression: Slope = 1.10 (95%CI:0.99-1.21), y-intercept = -0.69 (95%CI:-1.04 to -0.35), n = 180, r² =0.76, Mann-Whitney non parametric test: p = 0.80). As the concentration reached approximately 10 ng/L, serum and plasma gave very similar results. Between 10 and 5400 ng/L no significant differences were seen (Deming regression: Slope = 1.02 (95%CI:0.95-1.09), y-intercept = 2.88 (95%CI:-2.46-8.22), n = 160, r² =0.99, average difference 1.0% (95%CI -1.5 to 3.5) Mann-Whitney non parametric test: p = 0.86 (Figure 3.4.3A). Maximal deviations of >60% were seen in 15 samples whose cTnI
concentration was below 3 ng/L. The largest negative deviation (>40%) was seen in 2 samples whose cTn concentration was also below 3 ng/L.

There was good correlation between serum and EDTA plasma (Figure 3.4.3B) over the concentration range 1-5400 ng/L (Deming regression: Slope = 1.04 (95%CI:0.97-1.12), y-intercept = -2.84 (95%CI:-6.88-1.21), n = 197, $r^2 =0.99$, average difference -4.2% (95%CI: -7.2 to -1.2), Mann Whitney non parametric test $p = 0.58$) with no variance for samples below 10 ng/L (Deming regression: Slope = 1.04 (95%CI:0.93-1.15), y-intercept = -0.33 (95%CI:-0.91-0.26), n = 50, $r^2 =0.89$) A maximal positive difference of >45% was seen in 4 samples whose concentration was <3 ng/L with the largest negative deviation of >30% seen in 1 samples (8.9 ng/L).

Figure 3.4.3A. Difference plot showing comparison between serum and lithium heparin plasma over the range 1-5400 ng/L. (Average bias and 95% confidence intervals are shown) Insert: Difference plot shows range 11-40 ng/L with average bias and 95% confidence interval shown.
Figure 3.4.3B. Difference plot showing comparison between serum and EDTA plasma over the range 1-5400 ng/L. (Average bias and 95% confidence intervals are shown). Insert: Difference plot shows range 11-40 ng/L with average bias and 95% confidence interval shown.

Haemolysis up to a free haemoglobin concentration of 5.5 g/L had little effect on hs-cTnI concentration as shown in Figure 3.4.4. Recovery of hs-cTnI was from 91% to 108% at Hb concentrations up to 3 g/L, (i.e. at concentrations below 3 g/L, Hb did not interfere) when tested in 5 cTnI-positive samples. At a higher Hb concentration of 5.5 g/L hs-cTnI concentration was under-recovered by up to 14%. An apparent difference in results may be real or artifactual due to assay imprecision. To be confident at the
95% level that an apparent difference is real, the change needs to be ≥2.77 SD [23]. If we apply these objective criteria to our assessment of the effect of haemolysis on measured cTnI concentration, it is only at the highest level of haemolysis (5.5 g/L) that we find a significant underestimation in cTnI concentration by between 14% at 1.9 ng/L, 12% at 3.8 ng/L and 6% at 10.1 ng/L, 24.1 ng/L and 72.7 ng/L.

Figure 3.4.4: Effect of haemolysis (0 - 5.5 g/L Haemoglobin) on troponin concentrations 1.9 - 72.7 ng/L (♦ 1.9ng/L; ● 3.8 ng/L; ▲ 10.1 ng/L; □ 24.1 ng/L; ◊ 72.7 ng/L). Standard error is shown for each concentration.

We applied the hs-cTnI assay to a population of 497 healthy persons. All subjects had TnI concentration above the limit of blank, with a range for men (n=221) of 1.0 - 25.7 and for women (n=276) of 0.7 - 23.6 ng/L. The 99th percentile for men was determined to be 14.0 ng/L (90%CI: 13.2 - 14.6) and for women 11.1 ng/L (90%CI:
10.7 – 11.5). The all-subject 99th percentile was determined to be 13.6 ng/L (90%CI: 13.2 – 14.0). At the 99th percentile the total error was calculated as 28.2% (regression analysis from 10-40 ng/L hs-cTnI) and meets the guidelines for minimum analytical performance. The reference values between men and women were highly significantly different (t-test: p<0.01). Using a partition of 50 years, we found no age-related differences for women (t-test: p=0.90) or men (t-test: p=0.20). These data for men and women are presented in Figure 3.4.5A and 3.4.5B.

Figure 3.4.5A Distribution of serum hs-cTnI concentrations in cardio-healthy males
DISCUSSION

The new hs-cTnI assay is able to measure down to an order of magnitude lower than the currently available assay from this manufacturer. We found the 10% CV corresponded to a concentration of 3.9 ng/L, the 20% CV was at 1.8 ng/L and the 99th percentile limit of the distribution of values in a reference population for men was at a concentration of 14.0 ng/L and for women at 11.1 ng/L. Thus this assay easily meets the criteria recommended in Apple’s troponin scorecard [24]. The assay is robust with minimal interference from hemolysis and good relatedness at higher concentrations to the current on market cTnI assay. Serum and plasma samples gave essentially comparable results.

What is of particular interest with the new assay is that nearly all persons (98.6%) in this apparently healthy population had detectable troponin in their blood. Clerico has
suggested [25] that troponin may be present in blood as a physiological finding due to cardiomyocyte turnover. In addition, Bergman [26] has presented evidence for physiological turnover of cardiomyocytes. Venge has reported a similar population distribution to ours, using the Beckman hs-cTnI assay [27] and Apple [20] has reported that the Singulex TnI assay was able to detect cTn in 100% of subjects showing a near Gaussian distribution of results but with no gender differences.

The advent of these new highly sensitive assays for both cTnT and cTnI is exciting. The body of evidence is steadily growing that the presence of troponin in low concentration is a common finding in healthy subjects [2,3,9,28]. The new assay we describe in the current paper is a particularly sensitive assay with nearly all subjects in the reference population we have studied having detectable troponin. The current dogma that an increase in troponin always indicates cardiomyocyte necrosis needs to be reconsidered. Evidence has been presented which suggests that troponin may be released without necrosis occurring, whether by apoptosis or by some other undefined mechanism [29-31]. It appears that the presence of low concentrations of troponin is a common finding in healthy persons.

However, these new highly sensitive assays have been applied in several population studies and there continues to be evidence that detectable troponin in apparently healthy persons is associated with higher rates of mortality [32,33,34]. For example in the ARIC study looking at the value of cTnT in predicting future cardiovascular disease, there was a continuous increase in hazard ratio from cTnT concentrations well below the 99th population percentile limit [34] though mortality was substantially higher in persons with cTnT >14 ng/L which corresponds to the 99th percentile limit in several published studies. It will be of particular interest with the new hs-cTnI
assays, which can detect cTnI in a larger proportion of apparently healthy persons
(20) than can the hs-cTnT assay, to see whether this same predictive power is present.

The implications of this are substantial. The major use of cTn is and is likely to
continue to be for investigation of presumed acute coronary syndrome. Besides the
confusion associated with (nearly) everyone having detectable troponin in their blood,
assessing the cause of an increased troponin has the potential to be particularly
problematic. Simply documenting troponin rise and fall will be insufficient as non-
ACS conditions such as pulmonary embolism [35] may also display this pattern.
There is currently debate occurring as to whether absolute or delta changes in troponin
concentration will be the most useful when assessing persons with detectable troponin
in baseline samples [36,37]. This situation is further complicated as more information
will need to be collected on the biological variation in troponin concentration [38], to
enable us to assess whether apparent observed increases in troponin are real or
artifactual due to a combination of assay imprecision and biological variation. A
careful clinical assessment and assessing pre-test probability of ACS will be essential.
For those clinicians and laboratorians who gained their initial cardiac marker
experience with CK-MB, there will be a sense of “Back to the Future” as, similar to
CK-MB, the simple presence of the marker was insufficient for a diagnosis, but rather
the whole picture with an emphasis on the clinical presentation was required.
REFERENCES


CHAPTER 4.1

PHYSIOLOGY OF CARDIAC TROPONIN IN THE NORMAL POPULATION

Cardiac troponins are ternary structural protein components of the contractile apparatus of myocardial cells and are expressed almost exclusively in the heart [1]. Although elevations of these biomarkers in the blood reflect injury leading to necrosis of myocardial cells, they do not indicate the underlying mechanism [2]. The mechanism by which troponins are released into circulation has not been fully elucidated but possibilities suggested include normal turnover of myocardial cells, apoptosis, cellular release of cTn degradation products, increased cellular wall permeability, formation and release of membranous blebs, and myocyte necrosis [3]. This hypothesis of formation and release of membranous blebs will be discussed in chapter 5.

With the advent of troponin assays with greater analytical sensitivity, it has become possible to reliably measure troponin concentrations in a cardio-healthy population. This ability contrasts with conventional assays, in which reliable measurement of troponin was possible only at concentrations significantly higher than the 99th percentile of the upper reference limit of that healthy population.

The ability of sensitive assays to detect smaller quantities of troponin release provides earlier reliable detection of clinically important elevations.

There has been concern that sensitive assays with their lower detection limits and adopting existing knowledge, could confused the clinical picture in patients. The
quotes by Jesse [4] “when troponin was a lousy assay it was a great test, but now that it’s becoming a great assay, it’s getting to be a lousy test.” highlights the need for further study on the physiology of troponin.

The second part of this thesis focuses on the physiology of troponin in the normal population. This section will examine the validity of the currently accepted determination of the 99th percentile URL and consider possible alternatives to its determination.

In chapter 4.2 I describe a longitudinal study over 4 years on the cTnI concentrations measured with a high sensitivity assay in a cohort of healthy children. We further explored this cTnI distribution in the cohort of children in chapter 4.3. In chapter 4.4 we discuss the transient elevations in troponin concentrations that are sometimes observed. The cohort of healthy children is used as the model. I describe the effect of coning using laboratory and clinical measures on the determination of the 99th percentile of the upper reference limit for cTn in a normal healthy adult population in chapter 4.5.

REFERENCES

CHAPTER 4.2

LONGITUDINAL STUDIES OF CARDIAC TROPONIN I IN A
LARGE COHORT OF HEALTHY CHILDREN.

Part of this work was published in:

Koerbin G, Potter JM, Abhayaratna WP, Telford R, Badrick T, Apple FS, Jaffe A,
Hickman PE.

Clin Chem 2012;58 :1665-72
ABSTRACT: There is little information available on cardiac troponin (cTn) concentrations in healthy young children.

cTnI was measured in longitudinal blood samples collected at ages 8, 10 and 12 years from a cohort of 800 healthy, community-dwelling children using a pre-commercial high sensitivity assay from Abbott Diagnostics. Of this cohort 453 children had more than one collection. The 99th percentile values were calculated and estimates of the long-term biological variation made.

cTnI concentrations were above the limit of detection in 87%, 90% and 98% of the children at ages 8, 10 and 12 years. The 99th percentiles were lower in both male and female children at all ages studied, compared to a healthy adult population. At the 3 periods of study assessment, different children had cTnI concentrations above the 99th percentile. The calculated 99th percentile varied markedly depending upon whether the lowest or highest cTnI measurement for an individual child was included in the calculation. Biological variation varied markedly between 0% and 136%, the index of individuality was low at 0.36 and the RCV was an increase of 147% or a decrease of 59%.

In this longitudinal study of cTnI concentrations in healthy children, different children had concentrations of cTnI above the 99th percentile at the 3 episodes of assessment. This suggests that in children the 99th percentile may not be a reliable index of silent cardiac disease, but rather may be indicating low-grade intercurrent illness.
INTRODUCTION

The 99th percentile is well established as an essential component of the definition of myocardial infarction [1-3]. It has been demonstrated on innumerable occasions in adult populations, with both conventional and high sensitivity assays that persons with cTn concentrations above the 99th percentile are at a markedly increased risk of death from myocardial infarction [4-9]. Studies defining the 99th percentile are always cross-sectional in nature, with samples being taken at a single time point. To our knowledge there are no studies where the same population has had repeat sampling to look at the reproducibility of the 99th percentile.

Studies of cTn concentration in children are uncommon. We have had the opportunity to collect blood from the same cohort of 800 healthy children on 3 separate occasions at 2-yearly intervals and measure cTnI and cTnT [10] in these samples. All children have been closely monitored during the study period, had physical examinations and serial echocardiographic confirmation of normal cardiac structure and function and were free of any identified disease. In the current study we report our longitudinal studies on the cTnI concentrations measured with a high sensitivity assay in this cohort of healthy children.

MATERIALS AND METHODS

This study was approved by the ACT Health Human Research Ethics Committee. Informed consent was obtained from the parents or guardians of all subjects.
STUDY PARTICIPANTS

The LOOK (Lifestyle Of Our Kids) study is a multidisciplinary study examining the beneficial effects of exercise in healthy young children. In 2005 a cohort of healthy 8-year old children was enrolled from Canberra schools, assigned to intervention and control groups and followed for 4 years. The details of this study have been published elsewhere [11].

As part of this study, serum was collected in 2005, 2007 and 2009, when the children were aged 8, 10 and 12 years old. The children were requested to fast overnight and refrain from vigorous exercise on the morning of the collections. 800 children were included in this study of which 453 had multiple phlebotomies. Details of the sample handling procedures have been published elsewhere [12]. After the initial analyses were performed, serum was stored frozen at -80°C.

ECHOCARDIOGRAPHY

Echocardiography was performed on all children at ages 8, 10 and 12 years. Cardiac structure and function was assessed by one of two experienced sonographers using transthoracic echocardiography (Vivid 7, General Electric Healthcare, Wisconsin, USA) according to a standardized protocol. Measurements were made on-line and recorded digitally with study number as their only identification. A cardiologist, blinded to the participant’s clinical data, interpreted the echocardiogram after review off-line. Cardiac chamber sizes and left ventricular mass were quantified and indexed for body size according to current American Society of Echocardiography guidelines [13]. Left ventricular ejection fraction (LVEF) was quantified by the biplane disc summation method (Simpson’s rule) using the two-dimensional echocardiography
images from the apical four- and two-chamber views. A small number of children with abnormal cardiac structure, or function, including congenital or valvular heart disease, were excluded from this study.

**TROPONIN ANALYSES**

Samples used in this study were subjected to only one freeze-thaw cycle. The long-term stability of cTnI has been demonstrated under these conditions using both the Beckman Coulter hs-cTnI [14] and the Abbott ARCHITECT STAT hs-cTnI assays [15]. Assays were performed in singlicate. The 30 highest results from each year were repeated and no discrepancies were found.

Analytical studies of the ARCHITECT STAT hs-cTnI assay from Abbott Diagnostics, which was made available in a pre-commercial form by the manufacturer (Abbott USA) found that the limit of blank (LoB) was 0.5 ng/L, the limit of detection (LoD) was 1.0 ng/L, the concentration corresponding to the 20% CV was 1.8 ng/L, the concentration corresponding to the 10% CV was 3.9 ng/L, and the concentration corresponding to the 99th percentile values in adult males was 14.0 ng/L and in adult females 11.1 ng/L [15].

**STUDIES WITH hs-cTnI IN LOOK CHILDREN**

Not all children had blood collected on every occasion. Thus we have some children who had only one sample collected, some with 2 samples and some had 3 samples collected. We calculated the 99th percentile value for males and females separately at ages 8, 10 and 12 years, and looked to see what the effect would be on the calculated 99th percentile if we aggregated data for children who had more than one sample
collected, using either the highest or lowest concentration obtained. All subjects had baseline hs-cTnT measurements [10]. Where there was any discordance between results for cTnT and cTnI, samples were checked for possible heterophile antibody interference using Scantibody tubes (Scantibodies labs Inc. USA) and one sample was excluded as a result.

For children who had a result above the 99th percentile and had more than one sample collected, we looked at whether such increases were reproducible, i.e. if a person had a result above the 99th percentile on one occasion, we investigated the likelihood of it being above the 99th percentile on a subsequent occasion. To enable some statistical comparison of reproducibility of results, we partitioned results arbitrarily using 3 cutpoints – 99th, 95th and 75th percentiles (one tail). Concordance between the repeat measures was calculated as kappa values using MedCalc™ version 9.2 software. A kappa value of 0.21-0.40 is considered to be fair agreement, 0.41-0.60 moderate agreement and 0.61-0.80 good agreement.

As will be shown below, we were able to eliminate a peri-pubertal growth spurt as a source of variation. Hence the only contributions to the total variation were analytical variation and biological variation. As this was a large cohort with multiple measurements on many of the subjects, the total variation for each subject was calculated as the standard deviation of the 2 or 3 measurements taken over the course of the study and expressed as SD and CV_T for each individual. We fitted a power function to our precision profile for this hs-cTnI assay (15) \( y = 26.457x^{-0.6466}, \) \( r^2=0.92 \), and calculated the assay CV at the mean cTnI concentration for that child. This in turn allowed us to calculate the biological variation for each child as the difference between total variation and analytical variation: \( CV_i = \sqrt{(CV_T^2 - CV_A^2)}. \)
We independently verified these data by performing analysis of variance (ANOVA). We compared those subjects where there were two measurements against those where we had three measurements using a Mann-Whitney test, to see if these subjects were members of the same population. Analysis was performed using Anayse-it for Excel. We used our data from the multiple measurements on the children to calculate the Index of Individuality (II) and the Reference Change Value (RCV) as described by Fokkema et al [16].

**RESULTS**

In figures 4.2.1 to 4.2.6 we show the distribution of results for 8, 10 and 12 year old children, with the 99th percentile concentration marked. The hs-cTnI concentrations of all three child age group were statistically different (p<0.001, Mann Whitney non parametric analysis) and with 99th percentiles lower than found in healthy adults [15]. Males showed little change over the 3 sampling periods but the females at 10 years of age had a significantly higher 99th percentile (p<0.001) due to 3 children having markedly higher cTnI concentrations.

Figures 4.2.7 and 4.2.8 shows the distribution of cTnI for both within subject and between-subject results. The great majority of results lie below 4 ng/L, with occasional results that are higher.
Figure 4.2.1: hs-cTnI frequency distribution for 8 year old males. x-axis hs-cTnI (ng/L)

Figure 4.2.2: hs-cTnI frequency distribution for 10 year old males. x-axis hs-cTnI (ng/L)
Figure 4.2.3: hs-cTnI frequency distribution for 12 year old males. x-axis hs-cTnI (ng/L)

99th percentile: 10.4 ng/L

Figure 4.2.4: hs-cTnI frequency distribution for 8 year old females. x-axis hs-cTnI (ng/L)

99th percentile: 8.2 ng/L
Figure 4.2.5: hs-cTnI frequency distribution for 10 year old females. x-axis hs-cTnI (ng/L)

Figure 4.2.6: hs-cTnI frequency distribution for 12 year old females. x-axis hs-cTnI (ng/L)
Figure 4.2.7: Within- and between-child cTnI concentrations for 453 children who had more than 1 measurement made. The dots represent mean cTnI concentrations and the bars show the spread in cTnI concentrations. The children are ordered according to school starting with the school that was coded AR and finishing at the school coded WH. The results from 1 child with a peak cTnI concentration of 59 ng/L have been excluded from this figure.
Figure 4.2.8: Within- and between-child cTnI concentrations for 453 children who had more than 1 measurement made (lowest to highest). The dots represent mean cTnI concentrations and the bars show the spread in cTnI concentrations. The children are ordered according to maximal difference between samplings.

Table 4.2.1 shows the proportion of children who had cTnI concentrations above the LoB, LoD, 20% CV, 10% CV and adult 99th percentiles respectively. Over the 3 sampling periods, 87%, 90% and 98% of the children had results above the LoD. (Mann Whitney non-parametric analysis was used to compare all results by year of collection; p <0.001 2005 vs 2007, 2007 vs 2009 and p <0.05 2005 vs 2009). Between the ages of 8 and 12 years, median left ventricular mass had increased by 65% (data not shown).
Table 4.2.1: Number of LOOK children from each year of the study who had cTnI concentrations above the indicated cut-point.

*aThe number under 99th percentile refers to the number of children who were above the adult 99th percentile [15]. Male and female data have been combined.*

<table>
<thead>
<tr>
<th>hs-cTnI</th>
<th>n</th>
<th>Boys 99th percentile</th>
<th>Girls 99th percentile</th>
<th>LoB (0.5 ng/L)</th>
<th>LoD (1.0 ng/L)</th>
<th>20% CV (1.8 ng/L)</th>
<th>10% CV (3.9 ng/L)</th>
<th>Adult 99th percentile (13.6 ng/L)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005</td>
<td>386</td>
<td>9.4</td>
<td>8.2</td>
<td>376(97.4%)</td>
<td>337(87.3%)</td>
<td>265(69.4%)</td>
<td>45(11.7%)</td>
<td>3(0.7%)</td>
</tr>
<tr>
<td>2007</td>
<td>452</td>
<td>10.5</td>
<td>10.1</td>
<td>450(99.5%)</td>
<td>407(90.0%)</td>
<td>186(41.2%)</td>
<td>24(53.1%)</td>
<td>2(0.4%)</td>
</tr>
<tr>
<td>2009</td>
<td>451</td>
<td>10.2</td>
<td>7.9</td>
<td>450(99.8%)</td>
<td>444(98.4%)</td>
<td>348(85.1%)</td>
<td>32(7.1%)</td>
<td>2(0.4%)</td>
</tr>
</tbody>
</table>

In a post hoc examination we explored the relationships between cTnI and the inflammatory marker CRP, and found no evidence of any association (data not shown). Furthermore, an examination of the physical activity records of this cohort showed that none of the children were involved in training or physical activity of the intensity or volume likely to markedly raise inflammatory markers and no child had undertaken any form of strenuous exercise in the 12 hours prior to providing the blood sample.

We assessed the reproducibility of a high value in an individual child. We had a total of 11 children who were above the 99th percentile on at least one occasion and had at least 2 measurements made. We show these data for the individual children in Figures 4.2.9 and 4.2.10, divided on the basis as to whether they had 2 or 3 measurements made. There was no predictable pattern, with some having an initial high concentration which fell or vice-versa. The kappa statistic, a measure of concordance between measurements, was 0.00 indicating that if a child had one measurement
above the 99\textsuperscript{th} percentile, it offered no predictive value on any other measurement in this child.

Figure 4.2.9: Change in results for the 6 of 11 children with at least one result above the 99\textsuperscript{th} percentile and two measurements made.
Figure 4.2.10: Change in results for the 5 of 11 children with at least one result above the 99th percentile and 3 measurements made.

With our longitudinal data we investigated the effect of utilizing, in turn, the highest and lowest measurements where children had repeated values. These data are shown in Table 4.2.2. For both males and females, there was an approximately 2-fold increase in apparent 99th percentile when the highest value was used as opposed to the lowest (p < 0.001).

Table 4.2.2: Variable 99th percentiles based upon whether highest or lowest cTnI concentration used, where multiple blood samples collected from the one child.

<table>
<thead>
<tr>
<th>Age</th>
<th>cTnI (ng/L)</th>
<th>Gender</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>n (male)</td>
</tr>
<tr>
<td>8-12(high)</td>
<td>10.54</td>
<td>14.77</td>
<td>343</td>
</tr>
<tr>
<td>8-12 (Low)</td>
<td>5.71</td>
<td>6.47</td>
<td>343</td>
</tr>
<tr>
<td>p (High/Low)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Children with multiple samples and results changed</td>
<td></td>
<td></td>
<td>229</td>
</tr>
</tbody>
</table>

For children with more than 1 cTnI measurement, we were able to derive an estimate of total CV, and with our data for assay CV, estimate the biological CV for each child. These data are shown in Figure 4.2.11 and 4.2.12. The results ranged from 0% through to 136% with a median value of 33%.
Figure 4.2.11: Long-term biological variation in healthy children.

X-axis: average hs-cTnI concentration for children who had either 2 or 3 samples assayed. CV<sub>i</sub> was calculated from CV<sub>t</sub> and the CV<sub>a</sub> for the assay at the specified concentration. 11 children with mean cTnI < 1.0 ng/L (LoD) were excluded.

Figure 4.2.12: cTnI biological variation by gender in 8, 10 and 12 year old children.
Table 4.2.3 also shows the spread of both cTnI concentrations and the biological CVs. Because one possible reason for a large biological variation might be the peri-pubertal growth spurt, we also calculated biological variation for children who had 2 samples collected at ages 8 and 10, compared to children who had 2 samples collected at ages 10 and 12, that is we compared relatively younger with relatively older children. Using Mann-Whitney testing, we found no significant difference between these 2 groupings.

Table 4.2.3: Median, 2.5th and 97.5th percentiles for both cTnI concentration in the different groups, and the biological variation of cTnI in these groups. The numbers are less for the biological variation data as a small number of subjects who had average cTnI concentrations <1.0 ng/L (LoD for the assay) are excluded.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>cTnI (ng/L)</th>
<th>SD</th>
<th>n</th>
<th>CVi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>2.5th percentile</td>
<td>97.5th percentile</td>
<td>Median</td>
</tr>
<tr>
<td>3 measurement</td>
<td>153</td>
<td>2.1</td>
<td>1.2</td>
<td>5.5</td>
<td>0.7</td>
</tr>
<tr>
<td>2005 and 2007</td>
<td>66</td>
<td>1.9</td>
<td>0.8</td>
<td>5.7</td>
<td>0.6</td>
</tr>
<tr>
<td>2005 and 2009</td>
<td>78</td>
<td>1.9</td>
<td>1.0</td>
<td>5.7</td>
<td>0.6</td>
</tr>
<tr>
<td>2007 and 2009</td>
<td>156</td>
<td>2.0</td>
<td>1.1</td>
<td>4.8</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Mann-Whitney       p
3 vs 2 measurements | 0.14
2005/7 vs 2007/9   | 0.55

For this population CV\textsubscript{I} was 33%, CV\textsubscript{G} was 106%, the index of individuality was 0.36 and the RCV for a significant rise was 147% and for a significant fall 59%. These data are shown in table 4.2.4.
DISCUSSION

Our study is unique in two regards. Firstly we have had a unique opportunity to assess a cohort of truly healthy community-dwelling children, in whom cardiovascular health had been monitored during the study period and cardiac structure and function had been carefully evaluated by serial echocardiography. This is by comparison with other studies such as CALIPER which have used samples collected from hospital outpatients [17]. We have demonstrated that these latter data are probably showing subtle effects of ill-health [12]. The other unique element to our study was in our opportunity to collect longitudinal data, three times over a period of 4 years.

A high proportion of our cohort of children had cTnI above the LoD. At age 8 years this was 87%, at age 10 years it was 90% and at age 12 years it was 98%. This compares with 14.8%, 20.3% and 14.0% at ages 8 years, 10 years and 12 years respectively using hs-cTnT (10) and >98% of a population of healthy adults having a cTnI concentration above the LoD using this same assay [15]. This is a population with either no cardiac disease or an extremely low level of disease. The fact that nearly all members of this population have detectable troponin, strongly suggests that

---

Table 4.2.4: Index of Individuality and RCV data for hs-cTnI in healthy children.

<table>
<thead>
<tr>
<th></th>
<th>Long Term Variability (2 - 4 years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical Variation^a CV_A %</td>
<td>5.6</td>
</tr>
<tr>
<td>Biological Variation CV_I %</td>
<td>33</td>
</tr>
<tr>
<td>CV_G %</td>
<td>106</td>
</tr>
<tr>
<td>Index of individuality</td>
<td>0.36</td>
</tr>
<tr>
<td>RCV increase, %</td>
<td>147</td>
</tr>
<tr>
<td>RCV decrease, %</td>
<td>-59</td>
</tr>
</tbody>
</table>

^a CV_A at 13.6 ng/L (99th percentile)
cTn release is not always pathological, as was widely believed until very recently. Bergmann has shown [18] that there is a slow but significant turnover of cardiac myocytes each year and this can explain the very high prevalence of low concentrations of cTn in healthy persons. It is of interest that this increase in cTnI detectability was accompanied by a 65% increase in median left ventricular mass between the ages of 8 and 12 years, suggesting that physiological myocardial growth may have been an important contributor. Based on our findings, the performance of the cTnI assay used in this study meets the guideline acceptable and level 4 third generation hs-assay criteria according to the troponin scorecard [19], with imprecision \( \leq 10\% \) at the 99th percentile and with \( \geq 95\% \) of samples providing a measureable concentration above the assay’s limit of detection.

The 99th percentile values were lower at the three ages in both the preadolescent males and females than for adults. Male 99th percentiles changed little over the three sampling periods though the female 99th percentile was higher in the 10 year olds than for 8 or 12 year old females, due to 3 children having high cTnI concentrations. However, the 97.5 percentile was slightly lower in this group (7.4, 5.9 and 6.5 ng/L in 8, 10 and 12 year old females respectively) suggesting that the 99th percentile was distorted by an unidentified event, as will be considered below.

It is of great interest that it is not the same children repeatedly above the 99th percentile. There were 11 children who had at least one result above the 99th percentile and who had at least one more sample collected. None of these 11 children had a repeat result above the 99th percentile. Review of these data shows a wide scatter, with no consistent pattern of increased or decreased concentrations.

Why is there this wide scatter? One possibility is that preanalytical or analytical factors may be responsible. However, sample collection was meticulous with trained
phlebotomists taking blood from the children, and the serum being separated, centrifuged and frozen at -80°C within 4h. Assays were only performed after 1 freeze-thaw cycle with re-centrifugation before analysis and we have documented the stability of cTnI under these conditions. The assay shows excellent between run precision down to low concentrations [15]. All assays on the 3 separate sample collections of the study were performed over a short time frame, using the same lot numbers of reagents. For the reasons above we believe pre-analytical or analytical causes are very minor contributors to the total variation in results and that the scatter is a reflection of the children in their environment. Previous studies to determine the 99th percentile have all used cross-sectional data. Our study is unique in having longitudinal data available, and when longitudinal data are available it is a moot point as to which value should be used – highest, lowest or mean. Where we have multiple samples from the one child it is of interest to assess the 99th percentile depending upon whether the lowest or highest result is used (Table 4.2.2). This matter will require careful consideration.

We have been able to estimate the long term biological variability of cTnI in children who had more than one measurement made. The range is substantial, from 0% to 136% with a median of 33%. Our analysis suggests that the peri-pubertal growth spurt is not a major contributor to this biological variability. We have shown previously with hs-cTnT that there is a significant clustering of children with detectable cTnT within the same school and same year, suggestive of an infective etiology [10]. Whether this cTn rise reflects minor degrees of cardiac necrosis unrelated to coronary artery disease, or whether cTn is being released by some non-necrosis mechanism [20] cannot be determined from this study. The children with the larger biological
variation and those with results above the 99th percentile may simply be reflecting a minor episode of subclinical disease associated with cTn release.

Short term studies of the index of individuality have shown this to be low [21], indicating that monitoring serial changes in an individual are of greater value than using reference intervals. A longer-term study of 9 months duration, conducted in a population at high risk of cardiovascular disease found the index of individuality was still only 0.45 [22]. Our study conducted over an even longer time span (4 years), confirms this low index of individuality (0.36). CVI for our population was higher and as a consequence the RCVs were also higher (Table 4.2.4).

What is the significance of a cTn concentration above the 99th percentile in healthy children? While we will have to wait for many years to see the ultimate outcomes in these children, this lack of reproducibility of a high cTn concentration suggests that things other than primary cardiac disease may contribute. What do these data from children tell us about the 99th percentile in adults? Whilst in adults it is clear that the 99th percentile identifies a population at an increased risk of an adverse cardiac event [4-9], it is also the case that persons with cTn concentrations that are below the 99th percentile are also at risk, albeit lower than in those with cTn concentrations above the 99th percentile [23-25]. In contrast to previous studies in adults, our study is unique in being longitudinal. It may be that if longitudinal sampling was conducted in adult populations we may see a greater variability as well. We may be seeing a situation similar to C reactive protein where there is a substantial biological variability [26] and the CDC/AHA recommendation is that 2 measurements be made [27]. Using such a strategy might greatly amplify the predictive power of the 99th percentile by removing some of the background noise due to the biological variation associated with apparently minor illnesses.
REFERENCES


13. Lang RM, Bierig M, Devereux RB, Flachskampf FA, Foster E, Pellikka PA et al. Recommendations for chamber quantification: a report from the American Society of Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the...


CHAPTER 4.3

THE DISTRIBUTION OF CARDIAC TROPONIN I IN A POPULATION OF HEALTHY CHILDREN: LESSONS FOR ADULTS.

Part of this work was published in:

Koerbin G, Potter JM, Abhayaratna JM, Telford RD, Hickman PE.

Clin Chim Acta 2013;47:54-6
ABSTRACT: The objective of this research was to look at the distribution of hs-cTnI in a large cohort of healthy children.

As part of the LOOK study, blood was collected from a cohort of 450 healthy children on 3 separate occasions at ages 8, 10 and 12 years. Samples were stored at -80C after collection and assayed after 1 freeze-thaw cycle using a new pre-commercial release hs-cTnI assay from Abbott Diagnostics.

More than 98% of the 12 year old children had cTnI above the LoD of 1.0 ng/L. For the boys the central 95% of results was distributed in a Gaussian fashion. For the girls, the initial analysis was non-Gaussian, but after the elimination of 2 results, the pattern for girls was also Gaussian.

In healthy populations, troponin is present in a Gaussian distribution. Even minor illnesses can cause some troponin release, distorting this Gaussian distribution.
INTRODUCTION

We are continuing to learn about the cardiac troponins. Since their implementation into routine clinical use in the latter 1990s, they have become an important element in the diagnosis of myocardial injury and are part of the definition of myocardial infarction [1].

However, the situation with troponin is more complicated than at first sight. Troponin is not only increased in myocardial infarction, but in a variety of other non-cardiac conditions such as pulmonary embolism, sepsis, chronic renal failure, ischemic stroke and vigorous exercise [2]. More recently, with the advent of the high-sensitivity troponin assays (hs-cTn), it has become apparent that it is commonplace for even healthy persons to have detectable troponin in their blood [3,4]. Thus it appears that troponin release may be physiological as well as pathological. Bergmann [5] has shown there is a small turnover of cardiac myocytes each year and this may explain the background troponin in healthy persons.

We have recently had the opportunity to perform detailed longitudinal studies on a large cohort of healthy children and in this paper we report on the population distribution of cTnI using a new pre-commercial release assay from Abbott Diagnostics.

MATERIALS AND METHODS

This study was approved by the ACT Health Human Research Ethics Committee. Informed consent was obtained from the parents or guardians of all children studied.
STUDY PARTICIPANTS

The LOOK (Lifestyle Of Our Kids) study is a multidisciplinary study looking at the beneficial effects of exercise in healthy children. In 2005, a cohort of healthy 8 year old children was recruited from Canberra schools, assigned to intervention and control groups and followed for 4 years. Blood was collected from these children in 2005, 2007 and 2009 when the children were aged 8, 10 and 12 years old. As part of their health evaluation, echocardiography was performed on 3 separate occasions at ages 8, 10 and 12 years. 450 children who had multiple phlebotomies were included in this study. The details of this study and the sample handling procedures have been described in full elsewhere [6].

TROPONIN ANALYSES

Samples used in this study were stored at -80C and subject to only one freeze-thaw cycle before analysis. The long-term stability of cTnI when assayed using the Abbott Architect ci16200 analyser has been demonstrated [7]. The performance characteristics of this assay, made available to us in a pre-commercial form by the manufacturer (Abbott USA) were that the limit of blank was 0.5 ng/L, the limit of detection was 1.0 ng/L, the concentration corresponding to a 20% CV was 1.8 ng/L, the concentration corresponding to the 10% CV was 3.9 ng/L, and the concentration corresponding to the 99th percentile values in adult males was 14.0 ng/L and in adult females 11.1 ng/L [7].

As part of our studies we looked at the distribution of results for male and female children at each of the three collection periods when 8, 10 and 12 years old. The
proportion of children with cTnI concentrations above the LOD were 87%, 90% and >98% at the 3 ages [8]. In this chapter we report on the cTnI concentration distribution for 237 12 year old girls and 213 12 year old boys.

**DATA ANALYSIS**

All data was analysed using Analyze-It for Microsoft Excel. The Shapiro-Wilk test was used to assess whether the data was normally distributed and p>0.05 was interpreted as not significantly different to a normal distribution. The girls and boys data were subsequently screened for outlier exclusion using the criteria of Dixon [9]. One sample from the cohort of girls and no sample from the boys’ cohort met this exclusion criterion.

**RESULTS**

By the age of 12 years, 98% of children had cTnI above the limit of detection [8] and we were able to assess the central 95% population distribution. For 12 year old males and females, we log-transformed cTnI concentrations and looked at the population distribution of results.

For both the male and female children, when the total population was assessed, the distribution was significantly different to that of a Gaussian distribution (figure 4.3.1). If however, only the central 95% was assessed, for males the distribution was distributed in a Gaussian fashion (Figure 4.3.2) with p=0.2547 when testing for difference to a Gaussian Distribution using a Normality plot as shown in Figure 4.3.3.
Figure 4.3.1 Distribution of cTnI concentration in a population of 450 healthy 12 year old children. Dotted line represents an approximation of a normal distribution.

Figure 4.3.2 Distribution of cTnI concentration in the central 95% of a population of 213 healthy 12 year old males. Dotted line represents an approximation of a normal distribution.
Figure 4.3.3 Normality plot of data in Figure 4.3.2, showing no significant difference to a Gaussian distribution.

For females, the distribution was significantly different to a Gaussian distribution with p=0.0099 (figure 4.3.4). However, if 2 results were removed, one according to the criteria of Dixon [9] and the sample with the next highest troponin concentrations was also eliminated, then the female population also followed a Gaussian distribution with p=0.1281 when assessed with a Normality plot (figure 4.3.5). Using the more general Kolmogorov-Smirnov test for “goodness of fit”, both males and females fitted a Gaussian distribution without any samples being removed.
Figure 4.3.4 Non Gaussian distribution of cTnI concentration in the central 95% of a population of 237 healthy 12 year old females. Dotted line represents an approximation of a normal distribution.

Figure 4.3.5 Gaussian distribution of cTnI concentration in the central 95% of a population after 2 highest 12 year old female results excluded. Dotted line represents an approximation of a normal distribution.
DISCUSSION

Until recently, the presence of cTn in blood was considered to always reflect pathology and that myocardial necrosis had occurred. However, the advent of the new high sensitivity assays for troponin has shown that the majority of healthy persons have detectable troponin in their blood [3,4] and this has caused us to reconsider the significance of troponin in blood.

We have recently shown that in a cohort of healthy 12 year old children, nearly all had cTnI above the LoD [8]. This was a carefully assessed population which was free of any obvious disease and had been heavily studied to the extent that all children had echocardiography performed on 3 separate occasions.

We have previously reported on hs-cTnT distributions in the LOOK population [10]. This assay is less sensitive than the hs-cTnI assay and we scored results as either positive or negative. Using these criteria we found that the presence of hs-cTnT was transient and that the pattern of cTnT-positivity varied between different schools in different years and best fitted a transient infective agent as the cause.

In our current study we have a population of healthy male children in whom the central 95% conforms to a Gaussian distribution, and a population of healthy female children the central 95% of whom would fit a Gaussian distribution save for 2 children with high cTnI concentrations. In a recent analysis of hs-cTnI in this population we found that with longitudinal sampling, the occurrence of a high cTnI concentration on one occasion (ie above the 99th percentile) was not repeatable – a high cTnI concentration was transient [8]. We believe that the 2 female children with the high cTnI concentrations that prevented the female central 95% distribution from fitting a Gaussian distribution were displaying the effects of a transient minor illness.
Supporting this thesis is that the high results distorting the Gaussian curve – 19.10 and 6.48 ng/L – were 2.69 and 2.75 ng/L on a previous occasion. cTnI has a low Index of Individuality [8] and such large changes suggest an external effect in an apparently well child.

We conclude from these combined data that there is a background physiological release of troponin and that in a truly healthy population this is distributed in a Gaussian fashion. Venge has commented on healthy adult populations having a near-Gaussian distribution of cTnI concentrations [3] and other studies have also shown a similar distribution of cTnI in the blood of healthy persons [4]. Our data suggests that even mild subclinical disease can cause minor degrees of troponin release [10].

In conclusion, in this population of healthy children, the presence of any significant cardiac disease has been excluded by careful physical examination backed up by multiple echocardiographic studies. These data can be used as a model for consideration of cTn in adult populations. Where such populations are truly healthy the cTn concentrations should be distributed in a manner resembling a Gaussian distribution.
REFERENCES


CHAPTER 4.4

TRANSIENT TROPONIN ELEVATIONS IN THE BLOOD OF HEALTHY YOUNG CHILDREN

This work has been published in:

Potter JM, Koerbin G, Abhayaratna WP, Cunningham RD, Telford RD, Hickman PE.
Clin Chim Acta 2012;413:702–6
ABSTRACT: Whilst cardiac troponin is considered to be indicative of cardiac necrosis, the advent of new high sensitivity assays for troponin suggests that troponin may be present in the blood of healthy persons. We have examined a cohort of healthy children and measured cTnT in their blood.

In this community-based prospective study, we collected blood samples from a large cohort of healthy children at ages 8, 10 and 12 years and measured hs-TnT on these samples. 727 children had at least one blood sample collected and of these 28.6% had at least one sample in which troponin was detected. The number of samples with a positive troponin at each period of blood collection varied between 14.0% and 20.3%. Statistical analysis showed that the prevalence of positive cTnT varied between schools and the between school pattern was different in different years.

Low concentrations of troponin may be seen transiently in healthy children with no evidence of cardiac injury. This between-school by year variation is highly significant and is suggestive of a transient infective agent.
INTRODUCTION

Despite intensive use of troponin in clinical practice over the last 15 years, we are still learning about the significance of this analyte in a variety of clinical settings.

Current dogma is that the presence of cardiac troponin represents necrosis [1]. However, there are data that suggest that troponin release is not always accompanied by cardiomyocyte death. For example, vigorous exercise is frequently associated with a troponin rise without adverse sequelae [2,3]. Non-cardiac illnesses such as sepsis are associated with troponin rises and if the patient recovers from this illness there is no apparent increased likelihood of cardiac disease [4]. Carefully graded transient myocardial ischemia is associated with low-level troponin release [5,6]. Recent work suggests that cardiac myocytes may be renewed, albeit slowly. This implies a slow turnover of cardiomyocytes and troponin release may occur physiologically in this setting [7].

There is evidence from community-based studies that the presence of any troponin, even in asymptomatic individuals is associated with a worse prognosis [8,9], suggesting that the presence of troponin is a reflection of low-grade asymptomatic cardiac disease. However, studies using the recently released newer troponin assays, which can measure to an order of magnitude lower than previous assays, have shown the presence of low levels of troponin in comparatively young adults, with no suggestion of cardiac disease [10–13].

We have recently performed a longitudinal study looking at a cohort of healthy community living children taking part in a school based exercise program [14]. The cohort of approximately 700 children was assessed on 3 occasions at ages 8, 10 and 12 years [15]. As part of the study, high sensitivity cardiac troponin T (hs-cTnT) was
measured and echocardiography performed on all children. In this paper we report the outcomes of this study and discuss the significance of detectable troponin in this group.

MATERIALS AND METHODS

This study was approved by the Ethics Committees of the Australian Institute of Sport and ACT Health. Informed consent was obtained from the parents or guardians of all subjects.

STUDY PARTICIPANTS

The LOOK study has been described in detail elsewhere [14,15]. Briefly, the LOOK study is a multidisciplinary study that evaluated the effects of physical activity in healthy young children. In 2005 a cohort of 852 healthy young 8 year old children was recruited from schools in the Canberra region, assigned to intervention and control groups and followed over the next 4 years. A detailed assessment of health was undertaken even to the extent of all children undergoing echocardiographic studies. A small number of children with identified medical conditions were excluded. As part of this study blood samples were collected in 2005, 2007 and 2009. We have reported reference interval data on these children elsewhere [15]. After the initial analyses were performed, serum samples were stored at −80 C.
ECHOCARDIOGRAPHY

Cardiac structure and function were assessed by one of two experienced sonographers using trans-thoracic echocardiography (Vivid 7, General Electric Healthcare, Wisconsin, USA) according to a standardized protocol. Measurements were made online and recorded digitally with study number as their only identification. A cardiologist, blinded to the participant's clinical data, interpreted the echocardiogram after review off-line. Left ventricular (LV) ejection fraction and left atrial volume were quantified by the biplane disc summation method (Simpson's rule) using the two-dimensional echocardiography images from the apical four- and two-chamber views [16]. LV mass was quantified using the American Society of Echocardiography method [16]. LV mass and left atrial size were indexed for body size (height 2.7) in line with previous population-based studies [16]. Left ventricular diastolic function was assessed using Doppler assessment of mitral valve inflow tissue Doppler imaging of the lateral mitral annulus motion [17]. We have previously documented intra- and inter-rater reproducibility of these measures within our laboratory [17].

HIGH-SENSITIVITY TROPOIN T ASSAYS (hs-cTnT)

With the availability of the new hs-cTnT assay, we thawed and assayed cTnT on these samples. This was a single freeze–thaw on samples stored at −80 C. We have demonstrated the stability of hs-cTnT when stored frozen at this temperature [18].

STATISTICAL CONSIDERATIONS

The hs-cTnT assay has an analytical range from 3 to 10,000 ng/L [12]. For our analysis we have considered results as troponin present (hs-cTnT ≥3.0 ng/L) or
troponin absent (hs-cTnT <3.0 ng/L). A specific question of interest here is whether the proportion of positive results (rate) in individual schools remains constant between the three separate blood collections. In statistical terms we wish to test the hypothesis of a school by year interaction for our binary responses (hs-cTnT: coded 1 if $\geq 3$ and 0 if $<3$).

Given that children were measured on several occasions it is important that our statistical analysis not only account for clustering in our sampling design but for possible dependencies arising from the repeated measures structure. The above considerations lead to a random effects model where random terms include School; Year; “School by Year” interaction; and a variable for the individual child to account for the repeated measures structure. In this specification there are no fixed effects. The general statistical framework for these models is known as Generalised Linear Mixed Model or Hierarchical Generalised Linear Models [19] using the R statistical package. A parameter which quantifies the magnitude of the School by Year random term is known as a variance component. The statistical significance of this parameter can be assessed by comparing its estimate with its estimated standard error. Traditional likelihood based test statistics could not be used due to numerical instability in the fitting process.

RESULTS

There were no abnormalities of cardiac structure and function detected using conventional echocardiographic assessment.

Blood samples were available for assay of hs-cTnT in a total of 727 children across the three study periods (2005, 2007 and 2009). In 2005, 80/541 (14.8%) had
detectable cTnT, in 2007 92/454 (20.3%) had detectable cTnT and in 2009 68/487 (14.0%) had detectable cTnT. A total of 240 of 1482 samples had detectable cTnT.

Data relating to troponin positive results and the number of times a child was bled are described in Table 4.4.1.

Table 4.4.1: Proportion of cTnT-positive results relating to the number of times a child was bled.

<table>
<thead>
<tr>
<th>No. of samples assayed</th>
<th>No. of children</th>
<th>No. samples</th>
<th>Likelihood of positive results at any blood</th>
<th>Likelihood of positive result cumulative blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of episodes</td>
<td>All samples</td>
<td>collection</td>
</tr>
<tr>
<td>1</td>
<td>208</td>
<td>1 33</td>
<td>175</td>
<td>15.9%</td>
</tr>
<tr>
<td>2</td>
<td>286</td>
<td>1 68</td>
<td>209</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 4</td>
<td></td>
<td>13.3%</td>
</tr>
<tr>
<td>3</td>
<td>233</td>
<td>1 78</td>
<td>135</td>
<td>17.3%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 17</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 3</td>
<td></td>
<td>15.6%</td>
</tr>
</tbody>
</table>

Whilst the likelihood of a positive troponin being found at any particular blood sampling was relatively constant, the children who were bled 1, 2 or 3 times showed a progressively increasing likelihood of having a detectable troponin. A child bled once had a 15.9% likelihood of having a cTnT positive result, a child bled twice had a 25.2% chance of having at least one sample cTnT-positive and a child bled three times had a 42.1% chance of having a cTnT-positive result. In our analyses we have not separated the children by gender. The frequency distributions for each age group are shown in figure. 4.4.1.
Figure. 4.4.1. The troponin concentration in the blood of the same cohort of children, at ages 8, 10 and 12 years.

From these data we were able to calculate the 99th percentiles as 8.9 ng/L for the 8-year olds, 12.0 ng/L for the 10-year olds and 9.0 ng/L for the 12-year olds. This contrasts with 99th percentile values for adults reported as between 12.5 and 16.0 ng/L [10,12,13]. If we eliminated any children with any results for CK activity or any LFT result outside the reference interval, the 99th percentiles became 8.8 ng/L for the 8 year olds, 10.9 ng/L for the 10 year olds and 6.3 ng/L for the 12 year olds. This demonstrates the complexity of defining a truly healthy population for these studies.

A small number of children had measurable cTnT concentrations on more than one occasion. We assessed any possible association of the troponin rises with inflammation-associated analytes. CRP was unremarkable in most children, CK activity was in the upper quartile in approximately one-third of episodes compared
with only 1/17 cTnT negative episodes. In over half of these children higher CK was accompanied by ALT in the upper quartile; in cTnT negative children ALT and CK appeared to be elevated independently. Higher WCC and platelet counts were similar in both groups. This was different to the pattern seen in a separate analysis of results with cTnT>10 ng/L in which platelet counts tended to be elevated overall (data not shown).

There were 14 children with at least one cTnT result >10 ng/L. All 14 had more than one collection and measurement made. All but one of these 14 children had an isolated (single) cTnT positive result which was >10 ng/L, with all other cTnT results being below the LOD (<3 ng/L). In one child, cTnT was detected in all 3 samples (46.1, 3.17 and 3.46 ng/L). This child was a full participant in the 4 years of the study and had no abnormalities on echocardiography.

It is worth noting also that one of the highest cTnT concentrations (33 ng/L) was found in an apparently well child whose white cell count (WCC) was grossly elevated and in whom acute lymphocytic leukaemia was diagnosed that day. In figures 4.4.2 A,B and C we show the prevalence of positive cTnT by school. cTnT positivity varied markedly between schools in the same year and in the same school between years. For example in school #27, only 1 of 49 students was cTnT-positive in 2005 whilst in 2007 22 of 42 were cTnT-positive. Our statistical analysis showed that the between school by year variation in the prevalence of positive cTnT was unlikely to be due to chance (p<0.01).

The more often a child was bled, the more likely was that child to have a detectable troponin on at least one occasion.
Figure 4.4.2: Troponin positive results by school over different years (2005, 2007, 2009). The number on the x axis refers to the identifying number for each school. The dots (right hand scale) show the number of students at that school providing a blood sample, whilst the bars (left hand scale) show the proportion of cTnT positive results for an individual school.
DISCUSSION

This was a well-defined population of unambiguously healthy children. They were living in the community with no apparent health problems and indeed all had been confirmed as having normal cardiac structure and function by echocardiography. Finding detectable troponin in their blood was not unexpected as healthy adults clearly have troponin present. What was unexpected was that the presence of troponin was transient. In 163 children there was a transient presence of troponin — present in one year and absent at another collection. All cTnT-positive results were repeated and confirmed.

No child with a cTnT-positive result in either of the first 2 collections had health issues which precluded them from full participation in subsequent years of the study. Eight of the 14 children with cTnT >10 ng/L had at least one subsequent collection with cTnT being undetectable, so these increases appear to have been benign. Ten of the 14 students with a high cTnT had their samples collected before echocardiography was performed and no abnormalities were identified, suggesting that these transient cTnT increases were benign.

The pattern of cTnT-positivity varied between different schools in different years. This makes a local and non-infectious environmental toxin highly unlikely. Our statistical analysis showed this effect to be highly significant and not a chance event and makes a transient infective agent far more likely. Virus infection as a cause of troponin rise has been well documented, and is usually associated with severe myocarditis [20], though a benign course of viral myocarditis with transiently raised troponin has been described [21]. Our results suggest that (presumably viral) infection can cause transient minor increases in cTnT without major adverse sequelae. We found that 240 of 1482 samples had detectable cTnT using the new high sensitivity
assay, whereas only 5 samples from a total of 1482 would have had detectable cTnT using the previous generation assay [13].

These findings are important. Troponin release may occur in healthy persons when they are transiently unwell with minor illnesses, and with no lasting sequelae. The mechanisms that underlie the presence of circulating troponin in apparently healthy persons cannot be elucidated in this study. There is emerging evidence that low grade ischemia alone may cause troponin release without infarction occurring [5,6,22]. Whether systemic illness also causes a transient increase in circulating troponin levels because of low-grade myocardial inflammation is yet to be determined. With the new sensitive assays for troponin, it is likely we will find small transient increases in troponin to be a common occurrence with relatively minor illnesses, and that there is no cardiac prognostic significance attached to these transient increases. This situation is perhaps analogous to that seen with CRP as a cardiac risk indicator, where more than one estimation is recommended with the lower of the two concentrations considered to be more indicative of cardiac risk [23]. For assessing cardiac risk with troponin, it will be that concentration found when the individual is quite well which will indicate potential cardiac risk.

As we learn more about troponin, we are going “Back to the Future”. In the usual setting for troponin estimation, we will have to accept that the presence of troponin by itself is not absolutely diagnostic of the acute coronary syndrome. Instead, as with our approach with CK-MB, a careful clinical evaluation of patients will be required and where there is a low pre-test probability of an acute coronary syndrome, another cause for the troponin release will need to be sought. De Lemos [24] has indicated the need for a paradigm shift in how troponin results should be interpreted.
In conclusion, in healthy young children assessed on 3 separate occasions, we have found transient elevations of hs-TnT unrelated to any cardiac disease. Careful statistical analysis has shown clustering in each of the collection periods suggestive of an infective aetiology associated with the troponin rise.
REFERENCES


CHAPTER 4.5

THE EFFECT OF POPULATION SELECTION ON THE 99TH PERCENTILES FOR A HIGH SENSITIVITY CARDIAC TROPONIN I AND A HIGH SENSITIVITY CARDIAC TROPONIN T ASSAY

Part of this work has been publication in:
Koerbin G, Abhayaratna WP, Potter JM, Jaffe A, Apple FS, Ravalico T, Hickman PE.
**ABSTRACT:** This study uses objective laboratory and clinical criteria to more accurately determine the 99\textsuperscript{th} percentiles for troponin I and T.

We measured high-sensitivity cardiac troponin T and high-sensitivity cardiac troponin I in a large cohort of apparently healthy community subjects and calculated 99\textsuperscript{th} percentiles for different sexes and ages. Subjects with possible subclinical disease were eliminated based on objective laboratory criteria, eGFR and NT-proBNP, and clinical criteria, history and examination and echocardiogram.

For men and women of all ages, separately, more than 50\% of subjects were excluded using these criteria, with a lesser proportion of younger subjects being excluded. In men aged <75 years, the 99\textsuperscript{th} percentile for cTnI decreased by more than 50\% from 22.9 ng/L to 10.3 ng/L. In other age groups and for cTnT the decrease was smaller (\%) but still considerable.

For establishing cardiac troponin 99\textsuperscript{th} percentiles, simply using self-reporting of health is insufficient. Objective laboratory measures and clinical and echocardiographic assessment is essential to define a healthy population, especially in older persons.
INTRODUCTION

The cardiac troponin 99th percentile upper reference limit (URL) is an established component of the Third Universal Definition of Acute Myocardial Infarction (AMI) [1]. It is well established that in adult populations, asymptomatic persons with cTn above the 99th percentile are at an increased risk of major adverse cardiac events including death [2-4].

Defining the appropriate presumed healthy population to determine the 99th percentile is challenging. Different studies have published very different numbers even for the same cTn assay. For example, for the high sensitivity (hs)-cTnT assay, 99th percentiles have ranged from 12 ng/L [5] to 20 ng/L [6]. For sensitive cTnI assays, 99th percentiles have ranged from 34 ng/L [7] to 80 ng/L [8] for the Beckman assay, 7 ng/L [9] to 36 ng/L [6] for the Singulex assay, and from 13 ng/L [10] to 32 ng/L [11] for the Abbott assay. These studies are variable in the total number of subjects enrolled, the age of subjects, and by differences in gender composition. Most but not all have reported separate male and female 99th percentiles. As most laboratories have neither the interest nor the ability to perform large reference population studies, the 99th percentile they select from the literature or the manufacturer’s package insert has the potential to change the classification of their patients. There is still no universal agreement regarding how to define or select subjects for cTn reference interval studies. However, opinion is moving toward being more highly selective as recently described [12,13].

The purpose of the current study was to determine the 99th percentile URL for both hs-cTnI and hs-cTnT assays on a large cohort of apparently healthy adults, and then assess the effect of further selection (coning) using objective laboratory measures of health including serum creatinine concentration (using a calculated eGFR) and NT-
proBNP concentration, and objective clinical measures of health, namely a detailed clinical assessment and echocardiography.

MATERIALS AND METHODS

This study was approved by both the ACT Health Human Research Ethics Committee, and the Australian National University Ethics Committee and all subjects provided written formal consent.

STUDY POPULATION

Details of the Canberra Heart Study, a population-based study of randomly selected Canberra residents aged 60-85 years have been reported [14]. Subjects were originally recruited in 2002-2003. Those who were alive and not institutionalised since the baseline visit, had a follow-up visit during 2007-2009 when subjects were aged 65-92 years, with a response rate of 93%. Serum samples used in this study were collected at this time from all 707 subjects. A further younger cohort of 355 population-based subjects aged 48-52 years was also recruited to the study, over the same time period as the return visit of the older cohort. A total of 1062 subjects were recruited.

CLINICAL ASSESSMENT

A self-reported history of systemic hypertension, hypercholesterolemia, atrial fibrillation, coronary artery disease and heart failure was validated by a review of medical records. Hypertension was defined as a systolic blood pressure ≥140mmHg, diastolic blood pressure ≥90mmHg, or the use of medical therapy for hypertension
Diabetes mellitus was defined as a fasting glucose concentration ≥7.0 mmol/L or the use of insulin or oral hypoglycemic agents. Current smoking status and use of vasoactive medications was recorded.

**ECHOCARDIOGRAPHY**

Cardiac structure and function were assessed using transthoracic echocardiography (Vivid 7, GE Healthcare USA) according to current ASE guidelines [15]. Left ventricular ejection fraction (LVEF) was quantified by the biplane method of disks [15]. Abnormal LV diastolic function was graded into three categories using Doppler evaluation of mitral and pulmonary venous inflow and tissue Doppler imaging of lateral mitral annulus motion (abnormal relaxation filling pattern: mild LV diastolic dysfunction; pseudo-normal filling pattern: moderate LV diastolic dysfunction; restrictive filling pattern: severe LV diastolic dysfunction) [16]. Increased indexed LV mass was defined according to current ASE guidelines as > 95 g/m² for women and > 115 g/m² for men [15]. Valvular heart disease was defined as at least moderate stenosis or regurgitation of the mitral and/or aortic valves [17].

**SAMPLE COLLECTION AND HANDLING**

Samples were collected into gel separator tubes without anticoagulant, and processed within 4h, before freezing at -80°C. Samples used in this study were subjected to a single freeze-thaw cycle and centrifuged for 10 minutes at 10,000g for 10 minutes before assaying. The stability of both cTnI and cTnT under these conditions has been demonstrated [18,19].
LABORATORY MEASUREMENTS

cTnI was assayed on the Abbott ci16200 analyser using the pre-commercial Abbott ARCHITECT STAT hs-cTnI assay, with the following analytical characteristics: LoD at 1.0 ng/L, 20% CV at 1.8 ng/L and a 10% CV at 3.9 ng/L [20].

cTnT was measured using the adjusted hs-cTnT assay (lot number 16370401) on the Roche E411 analyzer (Roche Diagnostics, Sydney, Australia). Our initial evaluation of this assay was performed using lot numbers predating the downwards shift in subjects with low concentrations. In our hands this assay had an LoD of 5.0 ng/L, 20% CV at a concentration of 6.8 ng/L and a 10% CV at a concentration of 11.9 ng/L [21]. The 99th percentile URL of 14 ng/L [22] has not changed [23]. NT-proBNP was measured on the Roche E411 with total CV of 6.6% at 124 ng/L, 7.1% at 3,900 ng/L.

Creatinine (calibrators IDMS traceable) was measured on an Abbott ci16200 analyzer and the eGFR was calculated according to the modified CKD-EPI formula [24]. We used an eGFR of < 60 mL/min/1.73m² to identify persons with stage 3 kidney disease.

DATA MANIPULATION FOR PATIENT SELECTION

Of our population of 1062 subjects, 1027 had hs-cTnI assay analysis and 1001 were deemed useable with all laboratory and clinical parameters measured. Three further subjects were excluded using the outlier exclusion criteria of Dixon [25]. 1035 subjects had hs-cTnT assay analysis performed with 1006 deemed useable, and no additional subjects were excluded using the Dixon criteria.

We examined the effect on our calculated 99th percentile (the 1% upper tail of the 98% two tailed distribution) of progressively excluding (coning) subjects on the basis of objective laboratory and clinical measures of health as summarised below. The data
was log-transformed to look at the distribution of the total population and of the central 95% of that population.

OBJECTIVE LABORATORY MEASURES OF HEALTH

We progressively excluded subjects on the basis of

- abnormal renal function (eGFR) defined as an eGFR <60 mL/min/1.73m²
- and/or evidence of cardiac dysfunction as indicated by an increased concentration of NT-proBNP. We separately examined 2 separate criteria for NT-proBNP – those identified earlier as predictive of LVEF <50% (cutpoints of 151 ng/L for men <75 years, 426 ng/L for men >74 years, and 270 ng/L for women <75 years and 375 ng/L for women >74 years) [26], and those defined by the FDA as abnormal (125 ng/L for subjects <75 years old and 450 ng/L for subjects >75 years old). [27]

OBJECTIVE CLINICAL MEASURES OF HEALTH

Separately to the laboratory measures, we looked at the effects of coning using exclusion criteria

- History and medications - any history of heart failure, atrial fibrillation, clinically significant valvular heart disease, coronary artery disease and therapy with anti-hypertensive medications
- Abnormal echocardiography - left ventricular ejection fraction <50%, advanced diastolic dysfunction or left ventricular hypertrophy

Sequentially we looked at the effect of coning on the basis of:

- No coning of any subjects within our population
- Coning on the basis of abnormal eGFR alone
• Coning on the basis of abnormal NT-proBNP alone (Abhayaratna criteria)
• Coning on the basis of abnormal NT-proBNP alone (Baggish criteria)
• Coning on the basis of both eGFR and NT-proBNP (Abhayaratna criteria)
• Coning on the basis of both eGFR and NT-proBNP (Baggish criteria)
• Coning on the basis of an abnormal clinical history alone
• Coning on the basis of both an abnormal history and an abnormal ECG

Finally we looked at the effect of coning on the basis of both laboratory and clinical/echocardiographic criteria
• Coning on the basis of abnormal eGFR, NT-proBNP using the criteria of Abhayaratna, abnormal clinical history and abnormal echocardiogram and
• Coning on the basis of abnormal eGFR, NT-proBNP using the criteria of Baggish, abnormal clinical history and abnormal echocardiogram

This assessment is summarised in figure 4.5.1.
Figure 4.5.1: Study data analysis algorithm.

Sample analysis
Assay samples for hs-cTnT, hs-cTnI, NTproBNP, creatinine and calculate the eGFR using the CKD-EPI formula. Partition into 3 databases (all, male, female). Calculate the median, 99th and 97.5th percentile for hsTnT and hsTnI.

Partition 1, Remove Outliers
Perform outlier exclusion using the criteria of Dixon for hsTnT and hsTnI. Calculate #

Partition 2. Lab - eGFR
Perform exclusion on each database using an eGFR cut-off of 60 mL/min/1.73m². Calculate #

Partition 3a. Lab – NTproBNP (Abhayaratna)
Perform exclusion on each database using Abhayaratna criteria for NTproBNP. Calculate

Partition 4a. Lab - eGFR + NTproBNP (Abhayaratna)
Perform exclusion on Partition 2 database using criteria of Abhayaratna for NTproBNP and eGFR (CKD-EPI).

Partition 4b. Lab eGFR + NTproBNP (FDA)
Perform exclusion on Partition 2 database using the FDA criteria of for NTproBNP and eGFR (CKD-EPI). Calculate #

Partition 5. Clinical
Perform exclusion on each database using clinical criteria on partition 1 databases. Calculate #

Partition 6. Clinical and Echocardiography
Perform exclusion using echocardiography on partition 5 database. Calculate #

Partition 7a. Lab (Abhayaratna) + Clinical + Echocardiography
Perform exclusion on Partition 4a database using clinical criteria and echocardiography. Calculate #

Partition 7b. Lab (FDA) + Clinical + Echocardiography
Perform exclusion on Partition 4b database using clinical criteria and echocardiography. Calculate #
PLOTTING DATA FOR ASSESSMENT OF DISTRIBUTION

Statistical analysis was performed using Analyse-it v2.2 for Microsoft Excel. Non-parametric analysis was used throughout to determine median and interquartile ranges. Comparisons were performed using Mann-Whitney non-parametric analysis.

RESULTS

The clinical characteristics of our study population are shown in Table 4.5.1.

Table 4.5.1 Characteristics of the healthy controls, after coning based on biomarker, clinical and echocardiographic screening. The total number of subjects refers to those persons still included after coning as shown in Table 4.5.1, using the criteria of Abhayaratna [26]. The numbers quoted are medians and the figures in brackets are the 25th and 75th percentiles.

<table>
<thead>
<tr>
<th>Age 48-54y</th>
<th>Age 65-74y</th>
<th>Age 75+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men (n=123)</td>
<td>Men (n=74)</td>
<td>Men (n=29)</td>
</tr>
<tr>
<td>Age, years</td>
<td>51 (50, 52)</td>
<td>70 (69, 72)</td>
</tr>
<tr>
<td>History of congestive heart failure, %</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>History of atrial fibrillation, %</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>History of coronary artery disease, %</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>History of systemic hypertension, %</td>
<td>31</td>
<td>43</td>
</tr>
<tr>
<td>History of diabetes mellitus, %</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Estimated glomerular filtration rate, ml/min/1.73m²</td>
<td>105 (100, 113)</td>
<td>91 (86, 99)</td>
</tr>
<tr>
<td>NT-pro-B-type-natriuretic peptide, ng/L</td>
<td>29 (18, 47)</td>
<td>58 (41, 82)</td>
</tr>
<tr>
<td>High-sensitivity cardiac troponin I, ng/L</td>
<td>3 (2, 3)</td>
<td>3 (2, 4)</td>
</tr>
<tr>
<td>High-sensitivity cardiac troponin T, ng/L</td>
<td>1 (1, 1)</td>
<td>1 (1, 5)</td>
</tr>
<tr>
<td>Left ventricular ejection fraction, %</td>
<td>65 (62, 70)</td>
<td>66 (64, 70)</td>
</tr>
<tr>
<td>Left atrial volume index, ml/m²</td>
<td>25 (21, 27)</td>
<td>25 (22, 31)</td>
</tr>
<tr>
<td>Left ventricular mass index, g/m²</td>
<td>88 (74, 95)</td>
<td>83 (74, 99)</td>
</tr>
</tbody>
</table>

The effect of the coning procedure on the derived 99th percentile is shown for hs-cTnI in Table 4.5.2 and for hs-cTnT in Table 4.5.3. The upper part of each table shows the 99th
percentile and the lower part shows the number of subjects included under those conditions. After coning, the smallest number of subjects remaining in any partition was 123.

Table 4.5.2: The effect of coning with both laboratory and clinical indices on the 99\textsuperscript{th} percentile for the Abbott ARCHITECT hs-cTnI assay.

The top half of the table shows the concentration in ng/L at different ages and with different exclusions, whilst the bottom half shows the number of subjects assessed at each point. Each number is the troponin concentration (ng/L) followed by in brackets the number of subjects in the corresponding category.

<table>
<thead>
<tr>
<th>TnI (ng/L)</th>
<th>All (no exclusion)</th>
<th>Male (no exclusion)</th>
<th>Female (no exclusion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exclusion criteria</td>
<td>&lt;95</td>
<td>&lt;75</td>
<td>&lt;55</td>
</tr>
<tr>
<td>All (no exclusion)</td>
<td>32.2</td>
<td>20.4</td>
<td>12.8</td>
</tr>
<tr>
<td>All useable (no exclusion)</td>
<td>32.7</td>
<td>21.3</td>
<td>13.2</td>
</tr>
<tr>
<td>Dixon Exclusion</td>
<td>28.9</td>
<td>17.8</td>
<td>13.4</td>
</tr>
<tr>
<td>Laboratory exclusion criteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR</td>
<td>26.5</td>
<td>16.4</td>
<td>10.6</td>
</tr>
<tr>
<td>NTproBNP (Abhayaratna)</td>
<td>20.5</td>
<td>14.5</td>
<td>13.5</td>
</tr>
<tr>
<td>NTproBNP (FDA)</td>
<td>17.4</td>
<td>13.9</td>
<td>10.8</td>
</tr>
<tr>
<td>Both laboratory (Abhayaratna)</td>
<td>17.9</td>
<td>14.3</td>
<td>10.6</td>
</tr>
<tr>
<td>Both laboratory (FDA)</td>
<td>17.7</td>
<td>13.9</td>
<td>10.8</td>
</tr>
<tr>
<td>Clinical exclusion criteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical evaluation only</td>
<td>21.5</td>
<td>14.8</td>
<td>10.8</td>
</tr>
<tr>
<td>Clinical plus Echocardiography</td>
<td>14.6</td>
<td>13.6</td>
<td>10.3</td>
</tr>
<tr>
<td>Laboratory and Clinical exclusion criteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abhayaratna</td>
<td>13.9</td>
<td>12.3</td>
<td>10.3</td>
</tr>
<tr>
<td>FDA</td>
<td>14.3</td>
<td>11.1</td>
<td>10.4</td>
</tr>
<tr>
<td>n</td>
<td>&lt;95</td>
<td>&lt;75</td>
<td>&lt;55</td>
</tr>
<tr>
<td>All (no exclusion)</td>
<td>1027</td>
<td>705</td>
<td>334</td>
</tr>
<tr>
<td>All useable (no exclusion)</td>
<td>1001</td>
<td>683</td>
<td>321</td>
</tr>
<tr>
<td>Dixon Exclusion</td>
<td>998</td>
<td>682</td>
<td>315</td>
</tr>
<tr>
<td>eGFR</td>
<td>975</td>
<td>675</td>
<td>314</td>
</tr>
<tr>
<td>NTproBNP (Abhayaratna)</td>
<td>781</td>
<td>544</td>
<td>314</td>
</tr>
<tr>
<td>NTproBNP (FDA)</td>
<td>759</td>
<td>517</td>
<td>297</td>
</tr>
<tr>
<td>Both laboratory (Abhayaratna)</td>
<td>771</td>
<td>540</td>
<td>313</td>
</tr>
<tr>
<td>Both laboratory (FDA)</td>
<td>750</td>
<td>514</td>
<td>297</td>
</tr>
<tr>
<td>Clinical evaluation only</td>
<td>776</td>
<td>583</td>
<td>296</td>
</tr>
<tr>
<td>Clinical plus Echocardiography</td>
<td>564</td>
<td>455</td>
<td>259</td>
</tr>
<tr>
<td>Abhayaratna</td>
<td>532</td>
<td>438</td>
<td>258</td>
</tr>
<tr>
<td>FDA</td>
<td>484</td>
<td>387</td>
<td>247</td>
</tr>
</tbody>
</table>
Table 4.5.3: The effect of coning with both laboratory and clinical indices on the 99th percentile for the Roche hs-cTnT assay.

The top half of the table shows the concentration in ng/L at different ages and with different exclusions, whilst the bottom half shows the number of subjects assessed at each point. Each number is the troponin concentration (ng/L) followed by in brackets the number of subjects in the corresponding category.

<table>
<thead>
<tr>
<th>TnT (ng/L)</th>
<th>All</th>
<th>&lt;75</th>
<th>&lt;55</th>
<th>&lt;95</th>
<th>&lt;75</th>
<th>&lt;55</th>
<th>&lt;95</th>
<th>&lt;75</th>
<th>&lt;55</th>
</tr>
</thead>
<tbody>
<tr>
<td>All (no exclusion)</td>
<td>41.3</td>
<td>20.4</td>
<td>16.5</td>
<td>44.0</td>
<td>26.9</td>
<td>17.4</td>
<td>35.3</td>
<td>15.2</td>
<td>5.9</td>
</tr>
<tr>
<td>All useable (no exclusion)</td>
<td>42.2</td>
<td>21.1</td>
<td>16.8</td>
<td>44.2</td>
<td>27.0</td>
<td>17.5</td>
<td>35.5</td>
<td>15.7</td>
<td>6.4</td>
</tr>
<tr>
<td>Dixon Exclusion</td>
<td>42.2</td>
<td>21.1</td>
<td>16.8</td>
<td>44.2</td>
<td>27.0</td>
<td>17.5</td>
<td>35.5</td>
<td>15.7</td>
<td>6.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Laboratory exclusion criteria</th>
<th>eGFR</th>
<th>NTproBNP (Abhayaratna)</th>
<th>NTproBNP (FDA)</th>
<th>Both laboratory (Abhayaratna)</th>
<th>Both laboratory (FDA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFR</td>
<td>34.7</td>
<td>25.6</td>
<td>25.3</td>
<td>24.5</td>
<td>24.4</td>
</tr>
<tr>
<td>NTproBNP (Abhayaratna)</td>
<td>19.5</td>
<td>18.7</td>
<td>17.8</td>
<td>18.7</td>
<td>17.1</td>
</tr>
<tr>
<td>NTproBNP (FDA)</td>
<td>10.7</td>
<td>10.8</td>
<td>11.2</td>
<td>8.6</td>
<td>8.7</td>
</tr>
<tr>
<td>Both laboratory (Abhayaratna)</td>
<td>38.5</td>
<td>27.4</td>
<td>26.6</td>
<td>26.6</td>
<td>25.4</td>
</tr>
<tr>
<td>Both laboratory (FDA)</td>
<td>24.7</td>
<td>19.5</td>
<td>18.2</td>
<td>19.6</td>
<td>17.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clinical exclusion criteria</th>
<th>Clinical evaluation on ly</th>
<th>Clinical plus Echocardiography</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical evaluation only</td>
<td>28.9</td>
<td>22.2</td>
</tr>
<tr>
<td>Clinical plus Echocardiography</td>
<td>19.4</td>
<td>18.3</td>
</tr>
<tr>
<td>n</td>
<td>&lt;95</td>
<td>&lt;75</td>
</tr>
<tr>
<td>All (no exclusion)</td>
<td>1035</td>
<td>711</td>
</tr>
<tr>
<td>All useable (no exclusion)</td>
<td>1006</td>
<td>686</td>
</tr>
<tr>
<td>Dixon Exclusion</td>
<td>1006</td>
<td>686</td>
</tr>
<tr>
<td>Dixon Exclusion</td>
<td>1006</td>
<td>686</td>
</tr>
<tr>
<td>NTproBNP (Abhayaratna)</td>
<td>846</td>
<td>607</td>
</tr>
<tr>
<td>NTproBNP (FDA)</td>
<td>764</td>
<td>519</td>
</tr>
<tr>
<td>Both laboratory (Abhayaratna)</td>
<td>836</td>
<td>603</td>
</tr>
<tr>
<td>Both laboratory (FDA)</td>
<td>754</td>
<td>515</td>
</tr>
<tr>
<td>Clinical evaluation only</td>
<td>782</td>
<td>586</td>
</tr>
<tr>
<td>Clinical plus Echocardiography</td>
<td>566</td>
<td>456</td>
</tr>
<tr>
<td>Abhayaratna</td>
<td>533</td>
<td>435</td>
</tr>
<tr>
<td>FDA</td>
<td>490</td>
<td>388</td>
</tr>
</tbody>
</table>

We assessed the effects of coning by gender over 3 separate age ranges – <55 years, <75 years and <95 years. Over the 3 age groups assessed for hs-cTnI, coning caused the 99th percentile to decrease by 16-38% in women and by 29-56% for men. For hs-cTnT, coning caused the 99th percentile to decrease by 17-68% for women and by 42-56% for men. Neither of the laboratory parameters (eGFR and NT-proBNP) nor the clinical history plus echocardiography separately identified all subjects who were subsequently coned. Rather a
combination of laboratory plus clinical parameters removed the largest number of subjects with subclinical disease. These population distribution data are shown in graphical form in Figure 4.5.2A-F, showing only the group <75 years of age.

Figure 4.5.2A: All subjects <75 years old with Dixon outlier exclusion (15) only. All cTnI concentrations >35 ng/L are shown as one group of 36 ng/L.
Figure 4.5.2B: All subjects <75 years old with full exclusion criteria applied. All cTnI concentrations >35 ng/L are shown as one group of 36 ng/L.

Figure 4.5.2C: Male subjects <75 years old with Dixon outlier exclusion (15) only. All cTnI concentrations >35 ng/L are shown as one group of 36 ng/L.
Figure 4.5.2D: Male subjects <75 years old with full exclusion criteria applied. All cTnI concentrations >35 ng/L are shown as one group of 36 ng/L.

Figure 4.5.2E: Female subjects <75 years old with Dixon outlier exclusion (15) only. All cTnI concentrations >35 ng/L are shown as one group of 36 ng/L.
After coning, we log transformed the data and found the central 95% distribution of hs-cTnI was not significantly different to a Gaussian distribution in the 3 male age groups and 2 older female groups when assessed by the Shapiro-Wilk test (table 4.5.4). Despite this Gaussian distribution, the 99th percentile for the age group <95 years was substantially higher. We could not assess this for either sex with hs-cTnT because a significant number of subjects had results below the LoD.

Table 4.5.4: Shapiro – Wilk probability of a Gaussian distribution. $P \geq 0.05$ indicates a normal distribution.
Before coning, in all age groups and for both high sensitivity assays there were significant male-female differences in the 99\textsuperscript{th} percentile. After coning these differences were reduced, with only the youngest cohort (<55 years) still showing a marked sex difference in the 99\textsuperscript{th} percentile for both assays.

A number of population studies looking at the 99\textsuperscript{th} percentile using the current generation hs-cTnT assay from Roche and the hs-cTnI assay from Abbott have been performed. The results from these studies and their derived 99\textsuperscript{th} percentiles are shown in Table 4.5.5.

Table 4.5.5: Major population studies looking at 99\textsuperscript{th} percentiles for hs-cTnI and hs-cTnT

(a) Personal Communication from Rachid El Kouhen (Abbott Laboratories),
(b) Personal Communication from Stefan Blankenberg (University of Hamburg)
(c) Personal Communication from Per Venge (University of Uppsala)

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Specimen Type</th>
<th>Coning Strategy</th>
<th>Definition of “Normal”</th>
<th>n</th>
<th>99th percentile (ng/L)</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipowski (37)</td>
<td>All</td>
<td>BNP, HbA1c, eGFR</td>
<td>4593</td>
<td>26.2</td>
<td>34.2</td>
<td>15.6</td>
</tr>
<tr>
<td>(38), a</td>
<td>EDTA</td>
<td>BNP, HbA1c, eGFR</td>
<td>1531</td>
<td>27.8</td>
<td>35.1</td>
<td>16.7</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>BNP, HbA1c, eGFR</td>
<td>1529</td>
<td>22.3</td>
<td>28.3</td>
<td>14.7</td>
</tr>
<tr>
<td></td>
<td>Li Hep</td>
<td>BNP, HbA1c, eGFR</td>
<td>1531</td>
<td>26.9</td>
<td>34.5</td>
<td>14.3</td>
</tr>
<tr>
<td>Blankenberg (b)</td>
<td>Serum</td>
<td>No coning</td>
<td>4138</td>
<td>27.0</td>
<td>33.1</td>
<td>19.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NT-proBNP, eGFR</td>
<td>3799</td>
<td>21.5</td>
<td>25.9</td>
<td>13.9</td>
</tr>
<tr>
<td>Aw (39)</td>
<td>Serum</td>
<td>BNP, HbA1c, eGFR</td>
<td>1091</td>
<td>21.0</td>
<td>30.7</td>
<td>17.7</td>
</tr>
<tr>
<td>Apple (6)</td>
<td>Li Hep</td>
<td>Blood donors &amp; questionnaire</td>
<td>524</td>
<td>23.0</td>
<td>36.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Venge (c)</td>
<td>Li Hep</td>
<td>With outlier exclusion</td>
<td>417</td>
<td>25.0</td>
<td>24.2</td>
<td>15.2</td>
</tr>
<tr>
<td>Koerbin (21)</td>
<td>Serum</td>
<td>BNP, eGFR</td>
<td>497</td>
<td>13.9</td>
<td>14.6</td>
<td>11.3</td>
</tr>
</tbody>
</table>

**Abbott ARCHITECT hs-cTnI**

**Roche hs-cTnT**

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Specimen</th>
<th>Coning Strategy</th>
<th>Definition of “Normal”</th>
<th>n</th>
<th>99th percentile (ng/L)</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giannitsis (23)</td>
<td>Serum, plasma</td>
<td>Blood donors &amp; apparently healthy subjects</td>
<td>616</td>
<td>13.5</td>
<td>14.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Saenger (40)</td>
<td>Serum, plasma</td>
<td>Questionnaire</td>
<td>533</td>
<td>14.2</td>
<td>15.5</td>
<td>8.9</td>
</tr>
<tr>
<td>Mingels (10)</td>
<td>Serum</td>
<td>Cardiac biomarkers</td>
<td>479</td>
<td>16.0</td>
<td>18.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Apple (6)</td>
<td>Plasma</td>
<td>Blood donors and questionnaire</td>
<td>525</td>
<td>15.0</td>
<td>20.0</td>
<td>13.0</td>
</tr>
</tbody>
</table>
DISCUSSION

We have limited our table to studies with substantial numbers and where both male and female 99th percentiles were derived separately.

Ideally there should be little difference between reported 99th percentiles when using the same cTnI or cTnT assay. However, most reference populations studied vary to some extent by gender, ethnicity, age and the number of subjects enrolled, with resulting variability in reported 99th percentiles. In part, the variation between the different assays may be a reflection of ongoing problems with the standardisation of cTnI [28]. This is supported indirectly by comparison with cTnT, where only one assay is available. With that assay, the variability in reported 99th percentile is much smaller than for cTnI, ranging from 12 ng/L [5] to 20 ng/L [6]. However, at the extremes of reported 99th percentiles for cTnI, there are additional important causes that should be considered for the heterogeneity of reported cTnI 99th percentiles. This includes sample integrity, which can result in falsely low concentrations if storage conditions are sub-optimal. Previously we have reported the lowest 99th percentiles for the Abbott hs-cTnI assay; however, we carefully documented sample processing confirming the integrity of cTnI [20] when stored at -80C. Kavsak has independently reported similar stability of cTnI in long-term storage [18]. Thus our current and previously reported 99th percentiles would appear to be reliable. Further support for the integrity of our samples comes from the fact that our initial unconed data is similar to the other studies with the Abbott hs-cTnI assay as seen in table 4.4.5.

When a high 99th percentile is reported, two separate potential confounders should be considered, namely the effect of sample size and possibility of subclinical disease. First we consider sample size. While guidelines require 120 persons in a sample cohort to calculate the 90% confidence limit of the upper and lower reference limits when using non-parametric statistics [25], this is clearly insufficient when the 99th percentile is being considered. We
have presented data in children which suggests that there can be modest and transient increases in cTn concentrations [29]. In a small population sample this could distort the 99th percentile. Ideally a sample size of 350-500 persons would be necessary to avoid or at least minimize this potential artifactual distortion by minor illness [12,30].

Second, subclinical disease is always a potential concern. In the current study we have demonstrated that even in populations that are apparently healthy, simple coning of subjects using parameters indicating mild renal impairment and subclinical cardiac disease that is detectable by echocardiography results in a large fall in the apparent 99th percentiles for both men and women. These data are similar to those of McKie et al [13] and Collinson et al [12] who have shown the major impact of coning with laboratory parameters and echocardiography, to reduce reported 99th percentiles in an apparently healthy population. With the criteria used by Collinson they found similar 99th percentiles for hs-cTnT compared to previously published data, but their reported 99th percentiles for the Siemens and Beckman cTnI assays were still substantially higher than reported in the current study for the Abbott hs-cTnI assay. There also were occasional patients who appeared to be outliers despite the extensive screening suggesting that hs-cTn evaluations may in some situations detect co-morbidities that cannot be documented by other cardiovascular methods. Such a possibility has also been suggested by de Lemos [3].

Our current data confirms other reports using this Abbott hs-cTnI assay indicating that there are male-female differences in 99th percentiles. Our studies in children are of relevance here. In the LOOK study we observed that as children increased in age from 8 to 12 years, the proportion of children with hs-cTnI above the LoD increased from 87% to 98%. Over this time period the median left ventricular mass increased by 65% [31]. It may be that the male-female differences in 99th percentile are a reflection of different ventricular masses between the sexes. This concept is in keeping with the data suggesting the significant effect of
hypertrophy on cTn concentrations [32]. This may be one reason for the clinical evidence of relative under-treatment of women with acute coronary syndromes [33,34]. Use of a common 99th percentile for both sexes, which we do not endorse for high-sensitivity assays, would potentially exclude a significant proportion of women from appropriate treatment.

With hs-cTnI we found a Gaussian or near-Gaussian distribution to our data from males, in the 3 age groups studied and in 2 of the female groups. We have previously shown in a population of healthy male children, with all results above the limit of detection, that cTnI (Abbott pre-commercial hs-cTnI) is distributed in a Gaussian distribution [35]. That data, along with the data we present in the current study, indicates that in a truly healthy population where only “physiological” cardiac troponin is present, the population should be distributed in a Gaussian or near Gaussian manner.

If a Gaussian distribution is the underlying truly healthy distribution then consideration of reporting the central 95th percentile should be considered; i.e. the 97.5th percentile as the URL. In support of this is the fact that cTn has a low index of individuality [36] and changes in cardiac troponin are more important than movement outside a reference interval. This may be a particularly important consideration when hs-cTn is used in the evaluation of chronic disease.

In conclusion, even mild subclinical disease has a substantial effect upon the reported 99th percentile value in a reference population. Simply accepting persons who are self-reported as apparently healthy is insufficient, especially as high sensitivity assays become more commonplace in clinical practice. We have demonstrated that simple coning with two readily available laboratory biomarkers substantially reduces the 99th percentile. However, particularly in older persons, echocardiographic assessment resulted in a further substantial reduction in the 99th percentile. These coning methodologies need to be considered an essential component when assessing cardiac troponin 99th percentile values.
REFERENCES


15. Lang RM, Bierig M, Devereux RB, Flachskampf FA, Foster E, Pellikka PA et al. Recommendations for Chamber Quantification: A Report from the American Society of Echocardiography’s Guidelines and Standards Committee and the Chamber
Quantification Writing Group, Developed in Conjunction with the European Association of Echocardiography, a Branch of the European Society of Cardiology. J Am Soc Echocardiogr 2005;18:1440-63.


CHAPTER 5.1

PATHOLOGY AND TROPONIN: THE SIGNIFICANCE OF cTn IN THE ACS AND NON-ACS SETTING.

Older troponin assays were relatively insensitive and only detected quite high concentrations of troponin. If troponin was detected with these assays then the patient was at risk of an adverse cardiac event. Now that higher sensitivity assays can detect troponin at low concentrations, we are finding troponin present in apparently healthy person and the significance of detectable troponin may not always indicate a problem.

The release of cTn is specific to cardiac tissue, but not exclusive to MI. Therefore, increasing the analytical sensitivity of the assay also increases the number of patients who are detected as having an elevated cTn concentration, which could result from acute or chronic cardiovascular or extra-cardiac disease [1].

Troponin analysis is extremely valuable in the appropriate setting. Its widespread use in a variety of clinical scenarios however may lead to the detection of troponin elevation in the absence of thrombotic acute coronary syndromes. These elevations may arise from various causes, such as sepsis, congestive heart failure, renal failure and exercise. As assay sensitivity has increased, the list of non-ACS causes of an abnormally elevated cTn level (transient or prolonged) has expanded [2].

Cardiovascular disease accounts for more than 40% of all deaths in patients with end-stage renal disease [3]. In particular, the mortality rate of patients on maintenance dialysis following myocardial infarction (MI) is extremely high [4].

The third part of this thesis, investigates pathology and troponin - the significance of cTn in the ACS and non-ACS settings. To understand why detectable concentrations of troponin are
seen in the peripheral circulation and an understanding of the possible mechanisms of
troponin release are required. In chapter 5.1 I offer a hypothesis relating to the mechanism of
troponin release. Whilst NTproBNP continues to be a better prognostic indicator of all cause
mortality during the first 12 months on dialysis, in chapter 5.2, I describe, using observational
data, how improved sensitivity in cTnT assays has allowed cTnT to become a better
prognostic indicator of all cause mortality than conventional cTnT assay and NTproBNP for
patients with end stage renal disease if dialysis is continued for more than 1 year. I provide
information on the release of troponin into the peripheral circulation during recovery from
strenuous exercise in elite cyclists – suggesting cTn release where necrosis is absent in
chapter 5.3. Chapter 5.4 describes a cross-sectional study we undertook looking at troponin
concentrations and non cardiac illness in a general hospital and community population and
chapter 5.5 describes how the use of a new hs-cTnI assay with superior analytical
performance than conventional cTnI assays, can offer greater assistance to the clinician in
stratifying patients at greater of lesser risk of a major adverse cardiac event (MACE). In this
chapter we also suggest that the use of a multi-marker approach to identifying patients at risk
is potentially viable. These chapters offer an insight into the information that the improved
sensitivity of troponin assays can provide to the clinician in the ACS and non ACS setting.

REFERENCES

CHAPTER 5.2

CARDIAC TROPONIN MAY BE RELEASED BY ISCHEMIA ALONE, WITHOUT NECROSIS

This work has been published in:

Hickman PE, Potter JM, Aroney C, Koerbin G, Southcott E, Wu AHB, Roberts MS.

Clin Chim Acta. 2010;411:318-23
ABSTRACT

Whilst it is formally stated that cardiac troponin is only released when cardiac myocytes undergo necrosis, there are a number of clinical situations where troponin is present in the circulation, without any apparent cardiac injury. In these cases, troponin half-life in the circulation is usually substantially shorter than that seen when troponin is released following myocardial infarction with frank necrosis.

A mechanism has been described in liver, where large cytoplasmic molecules can pass from the intra- to extra-cellular space without cellular necrosis occurring. This occurs by the formation of membranous blebs which bud off from the plasma membrane of the cell. Blebs develop during cellular ischemia. If the ischemia is limited and re-oxygenation occurs, the blebs may be released into the circulation without rupture of the plasma membrane, resulting in a one-off release of cytoplasmic contents including macromolecules.

Evidence from cardiac studies is presented supporting the presence of membranous blebs in cardiac myocytes, enabling troponin to be released from cardiac cells due to ischemia alone, without necrosis.
INTRODUCTION

The introduction of cardiac troponin measurement to the clinical laboratory has been a signal advance in laboratory support for physicians investigating patients with possible myocardial disease. The presence of cardiac troponin was quickly included in the formal definition of myocardial infarction [1] and it is now recommended that troponin should be the only marker used for investigation of the acute coronary syndrome [2].

Although the possibility of troponin release due to ischemia without necrosis has been raised [3,4], more recently it has been stated unequivocally that troponin release only occurs in the presence of necrosis [5,6]. However, a recurring theme in the clinical literature has been the presence of troponin in the circulation, without any subsequent evidence of coronary artery disease or of cardiac myocyte necrosis. Whilst many of these conditions are related to major systemic disease and the precise causes are difficult to assess [7], there have been a number relating specifically to conditions affecting the myocardium and in particular, causing transient myocardial ischemia. This has been particularly noted with supraventricular tachycardia (SVT), where patients presented with SVT and detectable troponin, which settled quickly to undetectable after treatment, and there was no evidence of coronary artery disease on angiography [8,9].

In this paper, we review the available evidence and propose a mechanism whereby cardiac troponin can be released from myocardial cells by ischemia alone, without necrosis, and discuss the potential significance of this information.
THE CELLULAR LOCATION OF CARDIAC TROPNONIN

The cardiac troponins are predominantly myofibril bound with only approximately 5–8% of both troponin I and troponin T being unbound in the cytosol [10]. In any cardiac myocyte injury it will be this unbound pool of troponin which is released first.

CARDIAC TROPNONIN RELEASE DURING THE ACUTE CORONARY SYNDROME

During the acute coronary syndrome, there is an interruption of blood flow in the coronary circulation – most commonly involving thrombus formation on a ruptured plaque associated with transitory or prolonged occlusion of a coronary vessel, or distal embolization – leading to myocyte necrosis. The number of myocytes affected may vary from small through very large and the amount of damage caused is reflected by the rise in troponin concentration in the peripheral blood.

Although the true half-life of both troponins in the circulation is short – of the order of 2 h [11] – the clinical half-life of both troponins is substantially longer, due to the continued leaching of troponin from the remains of the necrotic cell. This clinical half-life of ≥20 h is the hallmark of cardiac myocyte necrosis [11].

There have been a number of reports of cases where troponin has been detected in the circulation in the absence of any apparent evidence of significant irreversible myocardial injury. A significant factor in many of these reports has been that where serial measurements have been made, the half-life of disappearance of troponin has been substantially shorter than is seen with myocardial infarction. This short half-life is of great importance in assessing whether troponin release is due to simple ischemia, frank necrosis, or a mixture of the two.
CLINICAL SITUATIONS ASSOCIATED WITH A SHORT HALF-LIFE OF TROPOVIN IN THE CIRCULATION

We describe 4 clinical cases from our own experience.

1. A 76 year old woman presented to her family practitioner with tiredness and anorexia. She developed marked tachycardia and hypotension whilst with the doctor and was admitted directly to hospital. At admission, an ECG showed atrial fibrillation with a rapid ventricular response and myocardial ischemia. The hypotension lasted for approximately 2 h. Troponin I at admission (Immuleite 2000) was undetectable but rose rapidly to a peak of 5.6 μg/L, then fell rapidly to undetectable with a half-life of disappearance of approximately 8 h. Coronary angiography showed no demonstrable vessel wall disease.

2. A 50 year old woman presented to her local hospital in a small country town with severe headache. Whilst being seen by the physician, she developed severe left-sided chest pain and developed a profuse sweat. Her blood pressure was 190/90 mm Hg and an ECG was non-diagnostic. She was immediately admitted to hospital. An initial measurement of troponin (Vitros 250) was negative, but the next morning had risen sharply to 22.7 μg/L. She was transferred to The Canberra Hospital with a provisional diagnosis of acute myocardial infarction, but ECG and coronary angiography on transfer were normal. Her blood pressure began to swing markedly and could not be controlled with medications. A CT abdomen revealed a large left adrenal mass with haemorrhage and an emergency adrenalectomy was performed. An adrenal gland weighing 550 g (usually approximately 10 g) was removed and confirmed to be a pheochromocytoma histologically. Her troponin concentration rapidly fell to low concentration with an initial half life of 6–8 h, though thereafter the decline was
slower, with a half life >20 h. Cardiological opinion was that she had not sustained a myocardial infarction.

3. A 66 year old woman was admitted to hospital with a fractured neck of femur. During admission procedures in the Emergency Department she experienced an episode of profound hypotension lasting for nearly 30 min. Her troponin I at admission (Abbott Ci8200) was 0.95 μg/L but rose within 2 h to 41.18 μg/L, then fell rapidly and at +23 h after the peak concentration had fallen to 8.30 μg/L. Thereafter the troponin declined more slowly and at +50 h the troponin concentration was 4.27 μg/L. There was no ECG evidence of myocardial infarction. The initial half-life of fall of TnI was approximately 9 h. The majority of the troponin released had a short half-life, but the tail of longer half-life troponin may represent myocardial infarction. During myocardial infarction, whilst some cardiomyocytes die, some will only become ischemic. Thus there will be 2 separate populations of cells releasing troponin and each of these populations will release troponin with a different half-life of clearance.

4. A 41 year old man was admitted with collapse after completing the Canberra Marathon. His CK peaked at 56,380 U/L. His troponin I (Abbott Ci8200) peaked at 0.917 μg/L, then fell rapidly with a half life of approximately 6–7 h to undetectable levels. He was released after 48 h with no evidence of cardiac injury. The troponin fall occurred as the CK rose. There is negligible cross-reactivity between skeletal muscle and cardiac troponin I in the Abbott assay. ECG and angiography were both negative for myocardial injury.
The fact that different troponin assays have been used gives potential for confusion. However, although numbers generated may vary from assay to assay, all assays are showing the same principle — that cardiac injury has occurred.

Apple and co-workers looked at half-lives of troponin clearance in patients with acute myocardial infarction and found a significantly shorter half-life of disappearance in patients with non-Q wave infarcts versus those with Q wave infarcts (6.8±5.6 h versus 20.4±10.7 h) [12]. It would be anticipated that STEMI infarcts would have predominant necrotic release of troponin, whereas many patients with NSTEMI may have a significant ischemic release of troponin.

Whilst the cardiac troponins appear to be absolutely specific for myocardial tissue, troponin release has been documented in a variety of conditions other than the acute coronary syndrome [7]. What is of particular interest are conditions where troponin released has a short half-life in the circulation. Perhaps the commonest of these is in association with vigorous and extended exercise. A meta-analysis of papers looking at exercise-associated troponin release [13] found that running by less well trained individuals was associated with the highest incidence of troponin release. Whilst most of these exercise studies have utilized single spot measurements of troponin, some have looked at two or more measures post-event and in nearly all cases, troponin half-life has been short [14–16].

Katus and colleagues reported on the release kinetics of troponin T after patients with myocardial infarction were reperfused [17]. They found that if patients were reperfused early, there was a sharp peak and sharp initial fall in troponin concentration and the troponin released was cytoplasmic in origin. Troponin released upon later reperfusion was cleared more slowly and had the characteristics of structurally bound troponin.
These studies all show that troponin can be released with markedly shorter half-lives of clearance from that characteristically found in association with acute myocardial infarction. The significance of this short half-life will be elaborated on below.

There is a substantial body of work involving the liver, which provides useful information indicating how cytoplasmic contents may be released into the circulation, without cellular necrosis occurring.

**LIVER STUDIES WHICH MIGHT EXPLAIN A MECHANISM FOR TROPONIN RELEASE BY ISCHEMIA ALONE**

**CLINICAL**

Ischemic hepatitis (an incorrect but clinical established terminology) describes the condition whereby the liver is rendered ischemic, usually due to a decrease in cardiac output, which in turn is often caused by myocardial infarction. The condition is characterized by a rapid and often very large increase in plasma concentration of hepatocyte intracellular enzymes. If cardiac output does not improve, patients die in a short timeframe, but if they recover their cardiac output, they survive and the plasma concentration of hepatocellular enzymes falls at the half-life of those enzymes. In this latter case, there does not appear to be any hepatic necrosis, but rather an increase in membrane permeability which abruptly ceases when re-oxygenation occurs [18]. CK concentrations were only mildly increased in some of these patients and the fall in AST concentration was 20–50 fold, demonstrating that liver was the source of the AST and not the myocardium.
EXPERIMENTAL STUDIES

Lemasters and co-workers have described the development of hepatocyte plasma membrane blebs and how they represent a means for intra-hepatocyte cytoplasmic contents to be released into the circulation, without cell necrosis occurring [19].

Blebs are bubbles in the plasma membrane which develop in response to ischemia. As ischemia is prolonged the blebs grow. Ultimately, the blebs will rupture and cell necrosis occurs. If however, re-oxygenation occurs before bleb rupture, then blebs are either resorbed or shed into the circulation releasing cytoplasmic contents, and the cell plasma membrane remains intact. Because the blebs are released into the circulation only at the time of re-oxygenation they represent a one-off release of cytoplasmic contents which is then cleared at the half-life of that substance. Larger organelles such as mitochondria are excluded from blebs.

If perfused rat liver is made ischemic and then re-oxygenated before hepatocyte necrosis occurs, cytosolic enzymes immediately appear in the effluent. Mitochondrial enzymes do not appear unless the period of ischemia is so prolonged that necrosis occurs. This release is paralleled by the development of membranous blebs [20]. A diagrammatic representation of what is happening is shown in figure 5.2.1
IS THERE ANY EVIDENCE FOR REVERSIBLE ENZYME OR TROPNIN RELEASE AND/OR BLEB DEVELOPMENT IN CARDIAC MYOCYTES?

There is evidence available that shows that cardiac myocytes both develop blebs and release cytoplasmic contents, without undergoing necrosis. Spieckerman and colleagues [21–24] (Figure 5.2.2) showed that cultured cardiac myocytes developed blebs during the development of anoxia, with the release of cytosolic enzymes without cell necrosis occurring.
Figure. 5.2.2. Microbleb formation of adult cultured cardiac myocytes. A: baseline. B: 30 min of anoxia. Reprinted from Schwartz et al [22], Am J Pathol 1984, 115: 349–361 with permission from the American Society for Investigative Pathology.
Katus and colleagues showed in perfused rat hearts that short duration ischemia followed by re-oxygenation led to a very short burst of cTnT release, but that if the period of ischemia was extended before re-oxygenation, troponin release was greater and more prolonged [25]. These data are consistent with bleb release of troponin during short duration ischemia and extended release of troponin as the period of ischemia was extended, and more cells became necrotic.

Hamm and colleagues identified patients with unstable angina who had detectable troponin at admission but which fell quickly to undetectable concentrations [26]. They suggested that in unstable angina reversible as well as irreversible cell injury may occur. The short-lived troponin in the circulation of these patients may reflect bleb formation and troponin release as a result of transient ischemia only.

Sabatine et al. studied patients undergoing stress testing, using the ultrasensitive Singulex TnI assay. They found that in patients, who experienced transient ischemic changes, low concentrations of troponin were detected and they raised the possibility that this may represent reversible myocardial injury [27].

**COULD BLEB DEVELOPMENT BE ARTIFACTUAL AND RELATED TO PREPARATION OF SINGLE CELLS?**

Whilst cell culture is an excellent way of viewing individual cells in detail, there is a concern that how cells behave in tissue culture may not reflect cell behaviour in an intact organ. The process of separating cells might cause membrane damage and the blebs that are seen may be artefacts of this process. However, even though cells in intact organs have less free
membrane surface from which blebs may develop, bleb formation in intact perfused organs has been well documented, in both liver [19,28] and heart [29–32].

**HOW CARDIAC TROPONIN IS RELEASED DURING ISCHEMIA WITHOUT NECROSIS**

During ischemia, blebs develop on the surface of cardiac myocytes. If the ischemia is prolonged the blebs rupture and cellular necrosis with prolonged troponin release follows. However, if the ischemia is corrected before any blebs rupture, then the blebs are either resorbed or shed into the circulation. If shed they will release cytoplasmic contents as a “one-off” event and this will be cleared with a short half-life (Figure 5.2.3).

During coronary artery occlusion, downstream myocytes will initially become hypoxic and ultimately necrotic. If reperfusion is established early, there is likely to be predominantly bleb release of troponin (short half-life) and as the time before reperfusion lengthens, the balance will switch to predominantly necrotic release of troponin (long half-life). The relative balance between ischemic release of troponin and necrotic release of troponin will be variable from case to case.

During clinical cases of troponin release without apparent coronary artery disease, such as during SVT, profound hypotension or extreme physical exertion, there will be predominantly ischemia-induced bleb formation and release of troponin with minimal necrosis-associated troponin release. In such cases the half-life of troponin clearance will be relatively short.
WHAT IS THE SIGNIFICANCE OF TROPNIN BEING RELEASED BY ISCHEMIA ALONE?

One important outcome from this model is that it explains a frequent puzzling clinical finding, namely the presence of cardiac troponin in the circulation, with either no necrosis, or insufficient cellular necrosis to explain the amount of troponin present. An ischemic mechanism for troponin release will correct the often incorrect interpretation that any troponin elevation is due to an acute coronary syndrome, which may lead to inappropriate
clinical management. The arrival of the new high sensitivity assays for both cTnT and cTnI
[33,34] suggest that very low concentrations of cTn may be present in the blood of healthy
individuals, possibly as a result of cardiomyocyte turnover [35]. Prototype troponin assays
with even higher sensitivity and precision have been developed that are able to detect
troponin in all healthy individuals [36].

Risk stratification of acute coronary syndromes, is based on the premise that bio-marker
positive acute coronary syndrome is due to atherothrombosis of a coronary artery with
myocyte necrosis. This necrosis carries a higher risk of recurrent adverse cardiac events, and
is commonly responsible for changes in patient management. It is often assumed that an
elevated troponin level equates to the acute coronary syndrome. However, there are many
clinical situations apart from the acute coronary syndrome which may cause a troponin rise
[7]. A clearly identified mechanism for a troponin rise other than by athero-thrombotic
mediated myocyte necrosis would encourage physicians to more carefully evaluate and
manage these different patient groups.

The current definition of acute myocardial infarction [37] is satisfied by a combination of a
rise in troponin concentration to above the 99th population percentile, accompanied by
symptoms of ischemia. This combination could be met with purely ischemic release of
troponin, with no myocardial necrosis. The current definition of myocardial infarction may
need to be revised.

We have presented evidence that supports the concept that troponin may be released as a
result of ischemia without necrosis. However, troponin is released in a variety of other
conditions besides ischemia and necrosis. For example troponin is released during sepsis,
without apparent irreversible cardiac injury [38] and it may be that circulating toxins also
cause bleb formation with associated troponin release. It is a real possibility that bleb
formation and troponin release are a final common pathway associated with many of the conditions reported to be associated with troponin release.

**LINKAGE OF BLEB FORMATION AND RELEASE OF CARDIAC TROPONIN WITH INTEGRIN STIMULATION**

The mechanism by which troponin may be released in reversible injury through bleb formation appears to be linked to integrin stimulation. The integrins are a family of adhesion molecules that have been implicated in the pathophysiology of atherosclerosis. Along with other adhesion molecules such as the selectins, the immunoglobulin super family, and the cadherins, the integrins participate in the extravasation of leukocytes and monocytes into the shoulder regions of coronary artery plaques [39]. From there, release of matrix metalloproteases and myeloperoxidase degrade the collagen cap making these lesions vulnerable to rupture. Recently, Hessel et al. linked the stimulation of integrin to the reversible release of cardiac troponin from cultured cardiomyocytes [40]. The pentapeptide Gly-Arg-Gly-Asp-Ser is a known agonist for integrin stimulation [41], and treatment of myocytes with this peptide resulted in a two to three-fold increase in cTnI release versus various controls. Reversible myocardial injury was inferred by the absence of lactate dehydrogenase (135 kDa) release in treated cells relative to controls.

The link of integrins to bleb formation was independently suggested by Larsen et al. [42] who performed live-cell morphology studies of calpain-knockout versus wild type using mouse embryonic fibroblasts. The calpains contribute to cytoskeletal remodelling as mediated by integrins. They showed that decreased levels of Rho GDP dissociation inhibitor, coflin 1, and membrane blebbing, and increased levels of tropomyosin in the knockout cells suggesting the calpain and the integrins are involved with bleb formation. Bleb formation is
also associated with apoptosis [43], an important aspect in patients with heart failure, and this may also be the mechanism for the release of troponin that is observed in these patients [44].
REFERENCES


223
CHAPTER 5.3

OVER TIME, HIGH-SENSITIVITY cTnT REPLACES NT-proBNP AS THE MOST POWERFUL PREDICTOR OF DEATH IN PATIENTS WITH DIALYSIS-DEPENDENT CHRONIC RENAL FAILURE

This work has been published in:

McGill D, Talaulikar G, Potter JM, Koerbin G, Hickman PE.

Clinica Chimica Acta 2010;414:936-9
**ABSTRACT:** Cardiac biomarkers are emerging as a potentially powerful prognostic tool for renal-dialysis patients. The optimal biomarker and/or combination of biomarkers for predicting mortality remain uncertain. This study evaluates the prognostic value of the new high-sensitivity troponin T (cTnT) assay compared to established biomarkers.

All patients had blood sampled for prospective assessment of the prognostic value of traditional risk markers including albumin and CRP, and cardiac biomarkers BNP, NT-proBNP, cTnT and cTnI. Patients were closely monitored clinically. Mortality and morbidity outcomes were documented for a national morbidity and mortality database. Stored samples were subsequently used to measure cTnT with a new high-sensitivity assay.

After a median of 30 months from blood collection, NT-proBNP was the most powerful predictor of all cause mortality, along with albumin. After a median of 46.7 months the new high-sensitive cTnT assay was the only cardiac biomarker predictive of all-cause mortality. cTnT was detectable in all dialysis patients using the hs-cTnT assay with a cut-point of 24.15 ng/L below which all patients survived.

The new hs-cTnT is the most powerful biomarker for prognostic classification for all-cause mortality of all the commonly used biomarkers for our renal-dialysis population. Our study also suggests that cardiac biomarkers have a different prognostic ability for different time frames with NT-proBNP being a better predictor for early mortality and troponin for later mortality.
INTRODUCTION

There is a high morbidity and mortality amongst patients undergoing dialysis. In 2007 in Australia there were 15.4 deaths per 100 patient years [1]. The most common cause of death amongst dialysis patients is cardiac death – predominantly myocardial infarction and cardiac arrest – accounting for approximately 40% of all deaths.

It was noted early on after the introduction of cardiac troponin measurements to the routine clinical laboratory, that many dialysis patients had detectable troponin T (cTnT) in their blood and that this was a predictor of poor outcome [2,3]. As assays have improved, more recently, troponin I has also been shown to be present in the blood of many dialysis patients and this has also been predictive of death [4].

Recently, a new formulation of the troponin T assay has been released, which can measure down to a substantially lower concentration than previously. Whilst the assay has been shown to improve diagnostic sensitivity for acute coronary syndromes [5], there is little outcome experience using this apparently very sensitive assay.

We have previously reported survival data in a cohort of dialysis patients at a median follow-up of 30 months, in which we compared the relative diagnostic efficacy of NT-proBNP and the 4th generation Roche cTnT assay [6]. In the current paper we have reviewed our data at a median of 46.7 months to evaluate the longer-term predictive ability of the commonly used prognostic biomarkers. In addition we included in our analysis results using the new 5th generation high sensitivity troponin T assay (hs-cTnT) to determine if the new assay offered any prognostic and/or discriminative advantages.
MATERIALS AND METHODS

This study was approved by the ACT Health Human Research Ethics Committee.

PATIENTS

A total of 143 dialysis patients (112 on haemodialysis and 31 on peritoneal dialysis) managed through the Canberra Hospital (Canberra, Australia) were enrolled in this study. Recruitment methods were described in detail in an earlier publication [6]. This report presents 3.9 year (median of 46.7 months) follow-up data on the same cohort with comparative predictive value of cardiac biomarkers using different assays for measurement. Blood samples were collected pre-dialysis and stored at −70 °C until assayed.

The ANZDATA Renal Registry [1] is very reliable and all patients were regularly reviewed with adverse events being methodically documented. We had 100% follow-up at a median of 46.7 months. At the time of this analysis, 55 of 143 patients had died (38.5%). The only outcome used in our analysis was all-cause mortality, because of the inaccuracy in the definition of many deaths and with no requirement for post-mortem studies.

SAMPLE INTEGRITY

Serum samples were stored at −70°C for a median of 46.7 months before the 5th generation hs-cTnT assay became available. We checked the integrity of samples by rerunning a random sample of 30 blood samples that had detectable cTnT using the previous 4th generation cTnT assay, using the same 4th generation assay. We found a very high correlation between the
two 4th generation assay measurements with an $r^2=0.979$, indicating excellent sample integrity.

**ASSAYS**

High-sensitivity cTnT was measured on a Roche E411 analyser, cTnI on an Abbott Ci8200, NT-proBNP on a Roche Elecsys 2010 and BNP on an Abbott Ci8200. Performance data for the latter 3 assays has been provided previously [6]. For the hs-cTnT the limit of detection was 3 ng/L. The inter-assay coefficient of variation was 20% at a concentration of 6.80 ng/L, 10% at a concentration of 11.86 ng/L and the population 99th percentile was 12.5 ng/L.

**STATISTICS**

All relevant clinical and laboratory data were entered onto a spreadsheet and all subsequent statistical analysis was with SPSS Version 16. The statistical approach has previously been described [6]. In summary, the main features included the use of natural log transformed biomarkers with skewed distributions (CRP, NT-proBNP, BNP and hs-cTnT); long-term survival outcomes in a Kaplan–Meier (K–M) model for relevant variables using the Mantel log-rank test to evaluate differences; for the relevant continuous variables the categories were identified using the outcome data (all-cause mortality) by determining a cut-off value from an ROC curve; and the Cox proportional hazard regression model was used to investigate the effect of variables selected because of a univariate or clinically expected relationship with survival.

To evaluate if there was an incremental yield of the new biomarker hs-cTnT over that of the currently used assay for cTnT, the Cox proportional hazard regression model was used to simultaneously investigate the predictive value of the two assay results for survival. The assay results were compared by categorizing them into cut-off values, the old assay by
detectable presence and the new assay with the cut-off point determined by the data-derived value from an ROC curve analysis.

Test accuracy assessments (inter- and intra-test) were with the coefficient of variation. A visual evaluation of categorized variables demonstrated that no K–M curve crossed for any of the covariates and all covariates had parallel log minus log regression curves indicating the proportional hazard model was valid. All statistical hypothesis tests were assessed with the 2-tailed tests with p<0.05.

RESULTS

Optimal risk predictors for all-cause mortality The survival analysis included variables with the strongest significant associations (p<0.005) with all-cause mortality (albumin, CRP, NT-proBNP, hs-cTnT, age, the presence or absence of cTnI and a history of cardiac co-morbidities), and one with a clinically expected association namely diabetes (Table 5.3.1). The variables that remained independently predictive of all-cause mortality were albumin (negative predictive association), natural log hs-cTnT, natural log CRP, and age.

The hazard for all-cause mortality in the final model for albumin was 0.893 (95% CI 0.843 to 0.947, p<0.001), for Ln CRP was 1.616 (95% CI 1.269 to 2.057, p<0.001), for Ln hs-cTnT was 1.404 (95% CI 1.001 to 1.968, p=0.049) and for age was 1.029 (95% CI 1.005 to 1.054, p=0.018). These hazard values are the predicted change in the hazard for a unit increase in the predictor. For example, the hazard reduction for the albumin being a unit higher (1 g/L) at the start of the study for a year is 10.7%. The hazard increase for having a unit higher at the beginning of the study for the natural log of hs-cTnT for one year is 1.404 times compared to a unit lower. Converting to units of ng/L from the natural log, 1 natural log unit is
approximately 2.72 ng/L. So a hazard increase of 1.404 times occurs each year for every increment of 2.72 ng/L increase in hs-cTnT.

Table 5.3.1: Descriptive statistics for high risk variables. n = number; ns = not significant; IQR = inter-quartile range. * Differences tested by the independent t-test; non-parametric and categorical variables tested as indicated by ^Mann–Whitney test, * Pearson χ² test and **Fisher's exact test.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean value at enrolment (or median or number as indicated)^a</th>
<th>Alive (SD)</th>
<th>Dead (SD)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
<td></td>
<td>55.58 (15.95)</td>
<td>67.00 (10.92)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time on dialysis in months</td>
<td></td>
<td>40.98 (40.21)</td>
<td>39.88 (29.39)</td>
<td>ns</td>
</tr>
<tr>
<td>Hours per session</td>
<td></td>
<td>4.28 (0.70)</td>
<td>4.01 (0.27)</td>
<td>0.02</td>
</tr>
<tr>
<td>Frequency of dialysis per week</td>
<td></td>
<td>2.94 (0.26)</td>
<td>3.00 (0.28)</td>
<td>ns</td>
</tr>
<tr>
<td>NT-proBNP (ng/L) median (IQR)</td>
<td></td>
<td>372 (125–945)</td>
<td>1030 (367–3773)</td>
<td>&lt;0.001^</td>
</tr>
<tr>
<td>BNP (ng/L) median (IQR)</td>
<td></td>
<td>97.2 (36.2–235.5)</td>
<td>146.0 (80.8–409.5)</td>
<td>0.015^</td>
</tr>
<tr>
<td>Albumin concentration (g/L)</td>
<td></td>
<td>43.02 (4.35)</td>
<td>38.53 (6.83)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Troponin T (μg/L) median (IQR)</td>
<td></td>
<td>0.023 (0–0.071)</td>
<td>0.069 (0.041–0.161)</td>
<td>&lt;0.001^</td>
</tr>
<tr>
<td>TnT measurable (n/%)</td>
<td></td>
<td>55/63%</td>
<td>53/96%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>New hs-TnT assay (ng/L) median (IQR)</td>
<td></td>
<td>39.46 (21.88–72.79)</td>
<td>80.56 (57.75–129.30)</td>
<td>&lt;0.001^</td>
</tr>
<tr>
<td>hs-TnT cut-off (n/%)</td>
<td></td>
<td>60/71%</td>
<td>54/100%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td></td>
<td>9.98 (16.19)</td>
<td>23.02 (29.09)</td>
<td>0.004</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td></td>
<td>0.97 (0.30)</td>
<td>0.95 (0.29)</td>
<td>ns</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td></td>
<td>4.12 (1.06)</td>
<td>3.75 (0.74)</td>
<td>0.019</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td></td>
<td>1.88 (0.96)</td>
<td>1.80 (1.16)</td>
<td>ns</td>
</tr>
<tr>
<td>Calcium concentration (mmol/L)</td>
<td></td>
<td>2.37 (0.16)</td>
<td>2.37 (0.14)</td>
<td>ns</td>
</tr>
<tr>
<td>Phosphate concentration (mmol/L)</td>
<td></td>
<td>1.73 (0.61)</td>
<td>1.54 (0.46)</td>
<td>0.045</td>
</tr>
<tr>
<td>Sodium concentration (mmol/L)</td>
<td></td>
<td>140.0 (3.40)</td>
<td>138.9 (3.08)</td>
<td>0.043</td>
</tr>
<tr>
<td>Diabetic (n)</td>
<td></td>
<td>19</td>
<td>19</td>
<td>ns</td>
</tr>
<tr>
<td>Insulin-dependent diabetic (n)</td>
<td></td>
<td>1</td>
<td>5</td>
<td>0.031**</td>
</tr>
<tr>
<td>Hypertension (n/%)</td>
<td></td>
<td>33/43%</td>
<td>22/42%</td>
<td>ns</td>
</tr>
<tr>
<td>Pre-existing cardiac condition (n/%)</td>
<td></td>
<td>21/27%</td>
<td>29/55%</td>
<td>0.001*</td>
</tr>
</tbody>
</table>
When the 4th generation cTnT assay was included in the model instead of the new hs-cTnT 5th generation assay, the old cTnT value also displaced NT-proBNP as a better predictor of all-cause mortality in renal patients after 3.9 years. The final model was 0.877 (95% CI 0.831 to 0.924, \( p < 0.001 \)), for LnCRP was 1.530 (95% CI 1.200 to 1.950, \( p = 0.001 \)), and non-detectable cTnT has a major hazard reduction but with a broad 95% CI, namely 0.174 (95% CI 0.042 to 0.725, \( p = 0.016 \)). The survival function for the detectable presence and absence of cTnT continued to be significantly different when adjusted for other predictors of all-cause mortality although mainly due to non-detection of cTnT to predict survival (Figure. 5.3.1).

Figure. 5.3.1. Survival predicted by the old cTnT assay adjusted for all the covariates entered into the Cox regression model.
PROGNOSTIC PERFORMANCE OF THE NEW (5TH GENERATION HIGH SENSITIVITY) VERSUS OLD (4TH GENERATION) cTnT ASSAYS

A simple bivariate Cox regression was used to identify if the new hs-cTnT assay results are a better predictor of mortality compared to the old assay method results. With this analysis the old assay was a categorical value (required because nearly 25% of samples had undetectable cTnT and hence cTnT was separated into quartiles). The new hs-cTnT assay could detect cTnT in all samples [7] and hence remains a continuous variable with the natural log transformed to a normal distribution. The new assay as a continuous variable is more predictive than the old assay as a categorical variable using the Cox proportional hazards model \{Chi-square=29.371, p<0.001; B (SE) = 0.812 (0.149); exp(B) 2.253 (95% CI 1.682 to 3.017), Wald statistic p<0.001\}.

Both the new hs-cTnT and old cTnT assay results were entered into the same model (forward stepwise conditional LR) as categorical variables to determine if the predictive advantage of the new assay was maintained for all-cause mortality. A cutoff point of 24.15 for the hs-cTnT assay was determined using the ROC curve analysis (Figure. 5.3.2).
The old cTnT assay was categorized as detected or not-detected. In these categories the new hs-cTnT but not the old assay remained predictive of all-cause mortality (Chi-square = 20.028, pb0.001; B (SE) = 3.111 (1.010); exp(B) 22.437 (95% CI 3.101 to 162.349), Wald statistic p=0.002). The area under the curve (AUC) for the new assay, under a non-parametric assumption was 0.760 (95% CI 0.683 to 0.838), and for the old assay the AUC was 0.746 (95% CI 0.667 to 0.826).

The cut-off point chosen from the ROC curve of 24.15 was the point at which the sensitivity changed from 100%, the cut-off value below which they are associated with zero mortality.

Figure. 5.3.2. ROC curve for standard cTnT and hs-cTnT (ng/L) for predicting all-cause mortality.
High sensitivity cTnT has the highest AUC statistic of all the main biomarkers most predictive of all-cause mortality (Table 5.3.2).

Figure 5.3.3. Kaplan–Meier curve for hs-cTnT cut-off point of 24.15 ng/L.

Table 5.3.2. Area under curve analysis for all cause mortality prediction

<table>
<thead>
<tr>
<th>Variable</th>
<th>AUC</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>hs-TnT (ng/L)</td>
<td>0.760</td>
<td>0.683 to 0.838</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>0.743</td>
<td>0.659 to 0.828</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.724</td>
<td>0.639 to 0.808</td>
</tr>
<tr>
<td>NT-proBNP (ng/L)</td>
<td>0.697</td>
<td>0.609 to 0.784</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>0.686</td>
<td>0.594 to 0.778</td>
</tr>
</tbody>
</table>
Since the use of continuous variables can improve the prognostic ability of that variable in regression models we reassessed the predictive value of all the same covariates for the previous analysis at a medium duration of 30 months follow-up [6]. Using the hs-cTnT as the representative cTnT measurement, the natural log of NT-proBNP still remained the most predictive of the cardiac biomarkers for all-cause mortality at that time of follow-up. The hazard for all-cause mortality in the final model for albumin was 0.812 (95% CI 0.759 to 0.870, p<0.001) and for the natural log of NT-proBNP the hazard was 1.511 (95% CI 1.093 to 2.089, p=0.012).

**DISCUSSION**

Patients on dialysis have a high cardiovascular mortality. Clinical assessment is relatively insensitive for identifying persons at risk of dying and biomarkers have proven useful in this role. Whilst a low albumin concentration has long been known to be a powerful predictor of mortality in dialysis patients [8,9] it is only recently that cardiac biomarkers have been shown to be powerful predictors of cardiac mortality [10].

cTnT is a strong index of poor outcome in patients with no cardiac symptoms. In a past analysis of data, we demonstrated that NT-proBNP was a better predictor of outcome than was cTnT [6] at a mean of 30 months of follow-up. In our most recent analysis we have found that cTnT (both old and new assays) proved to be better predictors than did NT-proBNP at 47 months of follow-up. This demonstration that different cardiac biomarkers may be predictive of outcome at different time frames is to our knowledge a novel finding. Cardiovascular disease in dialysis patients may be quite heterogeneous [11] and these different biomarkers may be identifying different subpopulations.
In our previous analysis of outcomes at a mean of 2.5 years, we found that NT-proBNP was more predictive of poor outcomes than was cTnT. The question arose — did this apparent association still hold if mortality data at 2.5 years was recalculated using the results only now available for the new hs-cTnT assay? We found that this relationship did persist and that our finding of different biomarkers being predictive at different times was substantiated.

The optimal cut-point for hs-cTnT for our population was 24.15 ng/L. Individuals with levels below this value (n=25) all remained alive during follow-up while 54 of 114 (47.4%) with values above this point died. This cut-point was more accurate in predicting prognosis than cTnT being present or absent for the old assay.

The new cTnT assay is a signal advance in the cardiac biomarker field. It can measure to a much lower concentration than previous assays, and in a study looking at a population of apparently healthy persons, detectable cTnT was found in the blood of nearly all subjects, distributed in apparently Gaussian fashion [12]. It appears that newly released highly sensitive troponin I assays also have the ability to detect troponin in the blood of healthy persons [13]. There is intriguing information available suggesting that normal myocardial cells can be turned over and renewed [14]. This new assay will require a rethink on what then presence of troponin in blood means.

There is little published data on the clinical applications of the new cTnT assay. There has been recent information showing that it has superior diagnostic performance to the older cTnT assay [5] but there is little outcome data using the new assay. We have demonstrated in this paper that the new hs-cTnT assay is highly predictive of outcomes in a renal-dialysis population and performs marginally better than the previous assay. The increase in the AUC is very small and may not be clinically relevant for overall prediction. However for our population the hs-cTnT assay has the best discriminatory capability for identifying those at high and low risk than any of the other commonly used biomarkers including the old assay.
The new high-sensitivity assay has 100% negative predictive accuracy for the chosen cut-point. Given these two features, hs-cTnT is the most powerful biomarker for prognostic classification for all-cause mortality of all the commonly used biomarkers for our renal-dialysis population.

The conclusions drawn from the study can only be considered exploratory. The study is limited by the small sample size of the study population. Additionally, we acknowledge the limits imposed by the absence of reliable echocardiographic data which could provide information to help understand better the differing predictive value of the two biomarkers at different time points.

CONCLUSION

The new high sensitivity troponin T assay is both a powerful predictor and discriminator of outcomes in this population of patients. The assay provides information to identify patients with a good prognosis more accurately than other cardiac and non-cardiac biomarkers. Our study also suggests that cardiac biomarkers and biomarkers in general differ in the predictive information they provide for different times of follow-up, indicating the need to delineate clearly and separately the time frame of the predictive utility for each biomarker.
REFERENCES

CHAPTER 5.4

CARDIAC ELECTRICAL CONDUCTION, AUTONOMIC ACTIVITY AND BIOMARKER RELEASE DURING RECOVERY FROM PROLONGED STRENUOUS EXERCISE IN TRAINED MALE CYCLISTS.

Part of this work has been published in:

Stewart GM, Kavanagh JJ, Koerbin G, Simmonds MJ, Sabapathy S

ABSTRACT: Although markers of myocyte injury, electrolyte disturbances and an autonomic imbalance have been reported following exercise, the effect of prolonged strenuous activity on cardiac electrical conduction is not well understood.

Electrocardiographic intervals were obtained from 8 highly-trained male cyclists before, during immediate recovery (15, 30, 45 and 60-min post) and 24-h after a prolonged bout of strenuous exercise. Time-domain, frequency-domain and non-linear analyses of the RR, PR and QT intervals were analysed to investigate the effect of prolonged strenuous exercise on autonomic modulation and cardiac electrical conduction during recovery. Cardiac troponin (cTn) was measured before, 1 and 24-h post exercise.

The root-mean-square of the successive differences of RR, PR and QT intervals were significantly reduced during immediate recovery (p < 0.05). Normalised low and high frequency power of RR intervals significantly increased and decreased, respectively, during immediate recovery (p < 0.002). Entropy of PR and QT intervals, and the QT variability index significantly increased during immediate recovery (p < 0.05). All measures, except mean QT and QTc intervals, returned to pre-exercise values after 24 hours of recovery. cTn concentrations were significantly elevated after 60-min of recovery (p = 0.001) and correlated with exercising heart rate ($R^2 = 0.89$, p < 0.001). Electrolyte concentrations were unchanged.

The results suggest suppressed parasympathetic and/or sustained sympathetic modulation of heart rate during immediate recovery, which occurred in parallel with perturbations in atrial and ventricular conduction dynamics. Troponin release was heart rate dependent, but did not correlate with alterations in conduction dynamics.
INTRODUCTION

Non-invasive measurements of cardiovascular dynamics are becoming increasingly popular for interpreting physiological manifestations of cardiac health and disease. Measurements of heart rate variability (HRV) can provide non-invasive insights into the fluctuations of sympathetic and parasympathetic nervous system activity [1], and as such has become a common tool for assessing autonomic cardiac modulation [2]. With acute exercise, HRV indices such as the root-mean-square of the successive RR differences (RMSSD), and total and high frequency spectral power of RR intervals decreases, which reflects parasympathetic withdrawal and increased sympathetic cardiac modulation [3,4]. Given that RMSSD and high frequency power also remain suppressed for up to 90 mins after a bout of prolonged high-intensity exercise [5], it is evident that sustained sympathetic nervous system outflow and dampened parasympathetic tone occur during recovery from prolonged strenuous exercise (PSE) [5-7].

Although a delayed parasympathetic reactivation has been reported following exercise, the direct influence of PSE on cardiac electrical conduction is not well understood. While HRV (variability of RR intervals) likely reflects dynamics in the spontaneous depolarization of the SA node, which initiates the cardiac cycle and controls heart rate, PR and QT interval variability may provide further insights into atrial and ventricular conduction dynamics. For example, elevated sympathetic activity reduces the PR interval [8], reflecting increased conduction velocity through the AV node and into the ventricles. Under the same elevated sympathetic conditions, or with reduced parasympathetic tone, the QT interval is prolonged [9] with increased complexity [10] and variability (when normalised to HRV) [11]; this suggests a direct autonomic influence on the ventricles. Given that cardiac depolarization and repolarization occur systematically to ensure optimal chamber filling and blood ejection,
independently examining RR, PR and QT intervals may provide a greater understanding of the autonomic influence on both the atria and ventricles during recovery from PSE.

In addition to the autonomic cardiac imbalance described above, PSE has also been implicated in transient functional myocardial alterations [12-14] and increases in cardiac-specific biomarkers (e.g. cardiac troponin) indicative of myocyte injury [15,16]. The notion that PSE may cause myocardial injury is incompletely understood, and given that cardiac dysfunction and biomarker release are transient, these measures may indicate acute stress rather than permanent myocardial damage [17]. Furthermore, electrolyte disturbances such as hypokalemia or hypomagnesemia have been reported following PSE and may contribute to exercise-induced perturbations in cardiac function and electrical conduction [18,19].

A recent review [20] suggested that non-invasive measurements of cardiac electrical conduction could provide feedback on cardiac recovery from exercise and aid in the investigation of exercise related arrhythmias. Indeed, QT interval dynamics have been used to assess sympathetic modulation of the ventricles during exercise [10,21] and as a predictor of sudden cardiac death in patient populations [22]. However, cardiac interval dynamics have not been extensively studied during recovery from PSE in healthy, athletic populations.

Studies investigating cardiac electrophysiology during recovery from PSE exercise have reported inconsistent results [18,19,23,24] and few have examined beat-to-beat dynamics of cardiac electrical conduction [10,21]. Therefore, the purpose of this study was to examine the impact of PSE on beat-to-beat cardiac interval dynamics and its relationship with electrolyte status and cardiac biomarker release in endurance trained athletes. Time-domain, frequency-domain and non-linear analyses of the time-series of electrocardiograph-derived cardiac intervals (RR, PR and QT) were examined to determine the effect of PSE on autonomic cardiac modulation and cardiac electrical conduction. It was hypothesised that immediately following PSE electrolyte disturbances, increased biomarker concentrations and an imbalance
between parasympathetic and sympathetic tone would exist and would occur in parallel to altered dynamics of cardiac electrical conduction. Furthermore, altered autonomic tone and cardiac electrical conduction would be transient and return to baseline levels 24 hours following PSE.

MATERIALS AND METHODS

Experimental procedures were approved by the Griffith University Human Research Ethics Committee and all subjects provided written and witnessed informed consent.

SUBJECTS

Eight highly-trained male cyclists (age: 18-29 yr) volunteered to participate in this study. Cyclists were considered highly-trained if they attained a peak oxygen uptake (VO₂peak) above 60 ml·kg⁻¹·min⁻¹ during an incremental cycling test to volitional fatigue. Pre-participation health screening was performed in accordance with guidelines published by the American College of Sports Medicine [25], and ensured that subjects were apparently healthy non-smokers, had no history of cardiopulmonary, metabolic or neuromuscular disorders, and were considered at low risk for atherosclerotic cardiovascular disease. Participants were not taking any medications and were actively competing in elite-level endurance cycling events (Australian state and national competitions) at the time of participation in this study.

STUDY DESIGN

All subjects visited the laboratory on three separate occasions. During the first visit, subjects underwent pre-participation health screening and then performed an incremental cycling test
to determine the gas exchange threshold (GET) and peak exercise values. On a subsequent visit, separated by at least 48 hours, subjects performed a constant-load exercise bout for 2-h at a power output corresponding to their individual GET, an intensity and duration similar to half-marathons or competitive endurance cycling and triathlon events [26]. Continuous resting electrocardiograph (ECG) recordings were obtained before exercise and for 60-min of recovery immediately after the cessation of exercise. Subjects returned to the laboratory 24-h after the exercise trial for an additional resting ECG recording. Blood samples were collected for subsequent analyses of electrolytes and high sensitivity cardiac troponin T (hs-cTnT) and high sensitivity cardiac troponin I (hs-cTnI), before exercise and after 60-min and 24-h of recovery. Cardiac intervals (RR, PR, QT) were extracted from 10-min segments of the ECG recordings at designated periods (pre exercise, 15-min, 30-min, 45-min, 60-min and 24-h post-exercise) for subsequent analysis. All subjects performed the experimental testing at the same time of day (i.e. 08:00 hr), and in a controlled laboratory environment (Exercise: 20°C, ~55% humidity; Resting ECG recordings: 24°C, ~55% humidity). Subjects were requested to refrain from any exercise or strenuous physical activity for 24 hours preceding each laboratory visit, and between the 2-h constant-load exercise bout and 24-h post-exercise sampling interval.

INCREMENTAL EXERCISE TEST

Incremental exercise tests were performed on an electronically-braked cycle ergometer (Excalibur Sport, Lode BV, Groningen, Netherlands). The test comprised 6 min of warm-up at 80 W, before the workload was increased by 20 W every 30 seconds until the subject reached volitional fatigue. Heart rate was measured using a 3-lead electrocardiogram (NORAV Medical, Kiryat Bialik, Israel). Oxygen uptake (VO₂), carbon dioxide output and minute expired ventilation were measured breath-by-breath using an automated metabolic
measurement system (MedGraphics CPX/D, Medical Graphics Corporation, St. Paul, MN, USA), and were subsequently averaged over 30-s intervals. Peak exercise values were calculated as the average of the highest two consecutive 30-s values attained during the test. The GET was determined using the modified V-slope method [27].

PROLONGED CONSTANT-LOAD EXERCISE TEST

Participants performed a 2-hour constant-load cycle test (Excalibur Sport, Lode BV, Groningen, Netherlands) at a power output equal to that attained at their individual GET. The exercise test was preceded by a 10-min warm-up at 80W, with the subject maintaining their preferred cadence throughout the entire exercise bout. Gas exchange and heart rate were measured (as described for the incremental test) for 5-min periods, every 15 min during exercise. Water was ingested ad libitum during exercise, and carbohydrate intake was administered at 1.5 g·kg⁻¹ body mass·hr⁻¹ in the form of Gatorade™. Following the 2-hour exercise bout, subjects performed a 3-min cool-down, and then 60 min of quiet, supine rest.

ELECTROCARDIOGRAPH RECORDINGS

Cardiac rhythm was monitored continuously throughout the pre-exercise and recovery periods using an electrocardiogram (NORAV Medical, Kiryat Bialik, Israel) with a 3-lead electrode configuration positioned to provide a Lead II view. ECG signals were recorded at 250Hz using a BIOPAC data acquisition system (MP100, BIOPAC Systems, Goleta, CA, USA) and Acknowledge 4.1 software (BIOPAC Systems, Goleta, CA, USA). The ECG recordings were acquired with the subject at rest in a supine position, and in a quiet, dimly-lit room to minimise external distractions. The pre-exercise and 24-hour post-exercise recovery periods comprised 15 min of rest followed by a 15-min continuous ECG recording, whereas
the recovery period immediately after the prolonged exercise test comprised a 60-min continuous ECG recording.

**DATA ANALYSIS**

The raw ECG signal was spline-interpolated to provide a re-sampling frequency of 1000Hz [28]. Beat-by-beat cardiac intervals (RR, PR, QT) were subsequently extracted from the raw ECG traces using specialised biological signals software (Acknowledge 4.1, BIOPAC Systems, Goleta, CA, USA). The software incorporates a waveform boundary detection algorithm [29], incorporating a modified Tompkins-based QRS detection algorithm. The waveform algorithm enables cycle-by-cycle extraction of the onset, peak, and end of the P wave, QRS complex, and T wave. A rate-corrected QT interval (QTc) was also calculated, given that QT interval is dependent upon heart rate. Cardiac intervals greater than 3.5 times the inter-quartile range from the median were considered outliers and removed prior to data analysis. Analysis bins (10 minute blocks) were delineated by extracting the cardiac intervals from the ECG waveform for the 5 minutes preceding and 5 minutes following the designated time points (pre exercise, 15-min, 30-min, 45-min, 60-min and 24-h post-exercise). For example, the analysis bin for 15-min post-exercise was delineated from 10-20 min post exercise. Figure 5.4.1 displays representative data of the interval time-series extracted for one subject.
Figure 5.4.1: Raw cardiac intervals from a subject’s electrocardiograph recordings during the pre-exercise and recovery periods. Data represents 10-min epoch of electrocardiograph intervals for each recording period. Note the reduced variability during the 15 min and 60 min recovery periods, which virtually return to pre-exercise levels by 24 hours recovery. RR: beat-to-beat RR intervals; QT: beat-to-beat QT Intervals; PR: beat-to-beat PR intervals.

Time-domain, frequency-domain and non-linear characteristics of cardiac intervals were calculated using custom designed Matlab software (version 7.7.0 The Mathworks Inc. R2008b). Time-domain parameters included the mean interval and root mean square of successive differences in intervals (RMSSD) for resting and recovery periods. Frequency-
domain parameters were derived from the power spectral density data of cardiac interval signals using auto-regression techniques. Low frequency (LF: 0.04-0.15 Hz) and high frequency (HF 0.15-0.4 Hz) parameters were normalised (nu) to total power. Non-linear cardiac interval parameters were examined using approximate entropy (ApEn). ApEn estimates the complexity of a signal by assessing the logarithmic likelihood that a sample of data will remain within a tolerance window defined as 20% of the standard deviation of the time-series (r = 0.2) in subsequent data increments of two data points within the time-series [30]. The complexity within the signal is subsequently graded on a 0-2 scale [31]. ApEn values that approach zero indicate increased complexity in the signal structure, whereas ApEn values that approach two indicate decreased complexity in the signal structure. The ratio of QT/RR ApEn was calculated [21], and a normalised QT variability index (QTvi) was also calculated [32]. Briefly, the mean RR interval (RRm), detrended RR variance (RRv), mean QT interval (QTm) and detrended QT variance (QTv) were derived and the QTvi was calculated:

\[
QTvi = \log_{10}\left[\frac{QTv/QTm^2}{RRV/RRm^2}\right]
\]

The QTvi is therefore the log ratio between the detrended QT and RR interval variability, normalised to the mean of each corresponding time-series.

**BLOOD SAMPLE COLLECTION AND BIOCHEMICAL ANALYSIS**

Blood samples were collected into serum separating tubes (SST) via venipuncture to determine routine biochemistry (sodium, potassium, urea, creatinine, calcium, magnesium, phosphate, albumin, ALT, GGT, ALP, CK and CRP) and troponin (hs-cTnT and hs-cTnl) concentrations at the designated time periods (pre-exercise, 60-min and 24-h post-exercise).
Blood samples were drawn after the ECG recordings to eliminate any affects of venipuncture on cardiac cycle dynamics. Serum was isolated from whole blood via centrifugation at 1,000g for 10 minutes and stored at -80°C for subsequent analysis. The samples underwent only 1 freeze-thaw cycle and were mixed and re-centrifuged at 10,000g for 10 minutes prior to analysis. Routine biochemical assays were performed on the Abbott Architect ci16200 (Abbott Diagnostics, IL, USA). High sensitivity cardiac troponin I analyses were performed using the pre-commercial Abbott ARCHITECT STAT hs-cTnI assay on the Abbott ci16200 analyser using. High sensitivity cardiac troponin T analyses were performed using the adjusted hs-cTnT assay (lot number 16370401) on the Roche E411 analyser (Roche Diagnostics, Sydney, Australia). All assays used were in commercial kit form supplied by the analyser manufacturer and performed in an ISO 15189 accredited laboratory. Performance characteristics The performance characteristics for the assays performed on the Abbott Architect over the analysis period showed coefficients of variation (CV’s) at the midpoint of the reference interval ranging from 0.8-8% (0.8-5.3% for kidney function tests, 1.1-3.4% for liver function tests, <8% for the cardiac function tests and CRP showed an imprecision of 2.9%. The performance characteristics for hs-cTnT, in our hands, showed a 20% CV at 6.8 ng/L and a 10% CV at a concentration of 11.9 ng/L with a limit of blank (LoB) 3.0 ng/L and limit of detection (LoD) 5 ng/L [33]. The imprecision using commercial control material with a concentration of 29 ng/L over the analysis period showed a CV of 4.9%. For the pre-commercial Abbott ARCHITECT STAT hs-cTnI assay, the analytical performance characteristics were LOB at 0.5 ng/L, LoD at 1.0 ng/L, 20% CV at 1.8 ng/L and a 10% CV at 3.9 ng/L [34]. The imprecision using commercial control material over the analysis period showed a CV of 2.9%. at a concentration of 20.0 ng/L.
STATISTICS

Statistical analysis was performed using SPSS 19.0 (SPSS Inc, Chicago, IL, USA). All data are presented as mean ± SEM. One-way analysis of variance with repeated measures were performed to determine any differences in electrolytes, troponin and cardiac interval parameters before exercise and during recovery.

When a significant main effect was detected, pair-wise comparisons using Bonferroni adjustments were used to further examine differences between time periods. Effect sizes were determined using partial eta squared ($\eta_p^2$). Significance was accepted at p < 0.05.

RESULTS

SUBJECT CHARACTERISTICS

All eight cyclists (age: 23 ± 4 yr; body mass: 73 ± 7.3 kg; VO$_{2\text{peak}}$: 64.8 ± 4.1 ml·kg$^{-1}$·min$^{-1}$; peak power output: 473 ± 37 W) completed the study.

The mean VO$_2$, power output and heart rate at the GET were 44.2 ± 4 ml·kg$^{-1}$·min$^{-1}$ (70 ± 5 % VO$_{2\text{peak}}$), 266 ± 27 W (56 ± 4 % peak power output) and 161 ± 10 beat·min$^{-1}$ (81 ± 2 % peak HR), respectively.

PROLONGED CONSTANT-LOAD CYCLE TEST

Mean VO$_2$ throughout the constant-load cycle test (52.5 ± 3.8 ml·kg$^{-1}$·min$^{-1}$) corresponded to 83 ± 5 % of VO$_{2\text{peak}}$ and mean HR (168 ± 8 beats·min$^{-1}$) equated to 84 ± 2 % of peak HR recorded during the incremental cycle test.
HEART RATE VARIABILITY

Mean heart rate variability (HRV) parameters are presented in Table 5.4.1.

All HRV parameters calculated from the RR time-series were significantly different during immediate recovery (15-, 30-, 45-, 60-min post exercise) when compared with pre-exercise, and returned to pre-exercise values after 24-hours of recovery (p < 0.002, $\eta^2_p$ range: 0.54-0.82). Time-domain parameters of the RR interval (mean RR interval and RMSSD) were reduced during immediate recovery compared to pre-exercise (p < 0.002). The frequency-domain parameters, total power and HFnu, were reduced, while LFnu was increased, during immediate recovery compared to pre-exercise (p < 0.02). The ApEn of the RR interval was reduced during the immediate recovery periods (p < 0.02).

Table 5.4.1. Heart rate variability parameters before and during recovery from prolonged strenuous exercise.

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>15-min</th>
<th>30-min</th>
<th>45-min</th>
<th>60-min</th>
<th>24-h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean R-R (ms)</td>
<td>1053 ± 43</td>
<td>823 ± 26*</td>
<td>830 ± 32*</td>
<td>830 ± 34*</td>
<td>837 ± 42*</td>
<td>1107 ± 68</td>
</tr>
<tr>
<td>RMSSD (ms)</td>
<td>73 ± 9</td>
<td>27 ± 3*</td>
<td>31.8 ± 5*</td>
<td>31.8 ± 6*</td>
<td>34 ± 6*</td>
<td>73 ± 13</td>
</tr>
<tr>
<td>Total Power (ms²)</td>
<td>4627 ± 1059</td>
<td>1295 ± 230*</td>
<td>1664 ± 376*</td>
<td>1725 ± 471*</td>
<td>1867 ± 468*</td>
<td>4752 ± 1189</td>
</tr>
<tr>
<td>LF nu</td>
<td>51.8 ± 5.4</td>
<td>73.5 ± 4.9*</td>
<td>72.8 ± 5.4*</td>
<td>73.6 ± 5.3*</td>
<td>71.8 ± 5.2*</td>
<td>54.7 ± 3.8</td>
</tr>
<tr>
<td>HF nu</td>
<td>48.3 ± 5.4</td>
<td>26.5 ± 4.9*</td>
<td>27.2 ± 5.4*</td>
<td>26.4 ± 5.3*</td>
<td>28.2 ± 5.2*</td>
<td>45.3 ± 3.8</td>
</tr>
<tr>
<td>LF:HF</td>
<td>1.26 ± 0.25</td>
<td>3.94 ± 0.96*</td>
<td>4.00 ± 1.03*</td>
<td>4.91 ± 1.62*</td>
<td>4.17 ± 1.36*</td>
<td>1.35 ± 0.26</td>
</tr>
<tr>
<td>ApEn</td>
<td>1.31 ± 0.01</td>
<td>1.16 ± 0.05*</td>
<td>1.15 ± 0.05*</td>
<td>1.14 ± 0.04*</td>
<td>1.15 ± 0.04*</td>
<td>1.28 ± 0.02</td>
</tr>
</tbody>
</table>
Heart rate variability parameters calculated from the RR interval time-series recorded before exercise (pre) and during recovery (15-, 30-, 45-, 60-min and 24-h) from exercise. Data are presented as mean ± SEM. RMSSD: root mean squares of successive differences; LF: low frequency component in normalised units (nu); HF: high frequency component in normalised units (nu); LF: HF: low frequency to high frequency ratio; ApEn: approximate entropy.

*Significantly different from pre-exercise (p < 0.05).

CARDIAC CYCLE DYNAMICS

All cardiac cycle parameters (Figure 5.3.2 and 5.3.3), calculated from the PR and QT interval time-series, were significantly altered during immediate recovery (15-, 30-, 45-, 60-min post exercise) when compared to pre-exercise (p < 0.02, η² range: 0.46-0.86). Mean QT intervals (Figure 5.4.2b) were reduced (p < 0.02), while PR intervals (Figure 5.4.2a) and QT intervals corrected for heart rate (QTc, Figure 5.3.3a) increased (p < 0.02) during immediate recovery. RMSSD for PR (Figure 5.4.2c) and QT intervals (Figure 5.4.2d) were reduced during immediate recovery (p < 0.05). ApEn of the PR intervals (Figure 5.4.2e) and QT intervals (Figure 5.4.2f), and the QT variability index (Figure 5.4.3b) were all increased during immediate recovery (p < 0.05). The ratio of QT/RR ApEn (Figure 5.4.3c) increased during immediate recovery (p < 0.02). All measures except for mean QT and QTc intervals returned to pre-exercise values following 24 hours of recovery.
Figure 5.4.2: Time-domain and non-linear parameters of cardiac intervals before exercise (Pre) and during recovery (15-, 30-, 45-, 60-min & 24h). Values presented as mean ± SEM.


*Significantly different from pre-exercise (p < 0.05).
Figure 5.4.3: The QT interval corrected for heart rate (QTc, a), QT interval variability index (QTvi, b) and ratio of the QT/RR interval approximate entropy (QT/RR ApEn, c) before exercise (Pre) and during recovery (15-, 30-, 45-, 60-min & 24h). Values presented as mean ± SEM. *Significantly different from pre-exercise (p < 0.05).
BIOCHEMICAL ANALYSIS

The troponin response to exercise for each cyclist is seen in figure 5.4.4 (hs-cTnT) and figure 5.4.5 (hs-cTnI). The median elevations in hs-cTnT and hs-cTnI (Figures 5.4.6 and 5.4.7) were observed at 60-min post-exercise when compared to pre-exercise (p < 0.01). One cyclist produced troponin I concentrations of >150 ng/L with no variation for all 3 samples (pre-exercise, 60 minute and 1 day post exercise). There was no opportunity to assess possible interference due to heterophilic antibodies or macrotroponin in these samples as they were exhausted and as such were removed from analysis. Exercise-induced hs-cTnT release was strongly correlated to average exercising heart rate (Pearson’s r = 0.943, p < 0.001, Figure 5.4.8).

Figure 5.4.4. Participant hs-cTnT response to exercise.

Each line represents and individual cyclist
Figure 5.4.5. Participant hs-cTnI response to exercise

Each line represents an individual cyclist
Figure 5.4.6: High sensitivity cardiac troponin T (hs-cTnT) concentrations before exercise (Pre-exercise) and during recovery (60 min and 24 hours). Values presented as median and inter-quartile range. 60 minute recovery concentrations are significantly different from pre-exercise ($p < 0.01$) and 24 hour post exercise ($p < 0.01$).
Figure 5.4.7: High sensitivity cardiac troponin I (hs-cTnI) concentrations before exercise (Pre-exercise) and during recovery (60 min and 24 hours). Values presented as mean and inter-quartile range. 60 minute recovery concentrations are significantly different from pre-exercise ($p < 0.01$) and 24 hour post exercise ($p < 0.01$).
Excluding troponin, there were no significant changes in the biochemical concentrations at 60-min post-exercise ($p > 0.05$, Table 5.4.2). All parameters were similar to pre-exercise following 24-hours of recovery ($p > 0.05$) except CRP. The 24 hour CRP concentration was however not different statistically from either the pre exercise or 60 minutes post exercise.

No significant associations between troponin or electrolyte concentrations, and cardiac cycle dynamics were observed.
Table 5.4.2. Biochemical analyte concentrations before and during recovery from prolonged strenuous exercise. Concentrations before exercise (pre) and during recovery (60-min and 24-h) from exercise. Data are presented as mean ± SEM

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Units</th>
<th>Pre</th>
<th>60-min</th>
<th>24-h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>mmol/L</td>
<td>140 ± 0.50</td>
<td>138 ± 0.36</td>
<td>140 ± 0.55</td>
</tr>
<tr>
<td>Potassium</td>
<td>mmol/L</td>
<td>4.2 ± 0.06</td>
<td>4.6 ± 0.15</td>
<td>4.3 ± 0.07</td>
</tr>
<tr>
<td>Urea</td>
<td>mmol/L</td>
<td>6.9 ± 0.8</td>
<td>7.3 ± 0.7</td>
<td>7.6 ± 0.7</td>
</tr>
<tr>
<td>Creatinine</td>
<td>umol/L</td>
<td>77 ± 2.4</td>
<td>85 ± 4.9</td>
<td>78 ± 3.2</td>
</tr>
<tr>
<td>CRP</td>
<td>ug/L</td>
<td>0.38 ± 0.08</td>
<td>0.38 ± 0.06</td>
<td>1.17 ± 0.34</td>
</tr>
<tr>
<td>Calcium</td>
<td>mmol/L</td>
<td>2.40 ± 0.03</td>
<td>2.42 ± 0.02</td>
<td>2.40 ± 0.02</td>
</tr>
<tr>
<td>Magnesium</td>
<td>mmol/L</td>
<td>0.85 ± 0.01</td>
<td>0.81 ± 0.02</td>
<td>0.83 ± 0.02</td>
</tr>
<tr>
<td>Phosphate</td>
<td>mmol/L</td>
<td>1.21 ± 0.03</td>
<td>1.42 ± 0.03</td>
<td>1.24 ± 0.07</td>
</tr>
<tr>
<td>Albumin</td>
<td>g/L</td>
<td>43 ± 0.6</td>
<td>44 ± 0.7</td>
<td>42 ± 1.1</td>
</tr>
<tr>
<td>ALT</td>
<td>U/L</td>
<td>18 ± 1.2</td>
<td>19 ± 1.2</td>
<td>18 ± 1.2</td>
</tr>
<tr>
<td>GGT</td>
<td>U/L</td>
<td>14 ± 1.3</td>
<td>14 ± 1.3</td>
<td>13 ± 1.1</td>
</tr>
<tr>
<td>ALP</td>
<td>U/L</td>
<td>76 ± 21</td>
<td>76 ± 22</td>
<td>75 ± 23</td>
</tr>
<tr>
<td>CK</td>
<td>U/L</td>
<td>120 ± 11</td>
<td>140 ± 13</td>
<td>131 ± 15</td>
</tr>
<tr>
<td>TnT</td>
<td>ng/L</td>
<td>4.6 ± 1.9</td>
<td>26.9 ± 15</td>
<td>6.5 ± 2.9</td>
</tr>
<tr>
<td>TnI</td>
<td>ng/L</td>
<td>2.5 ± 1.4</td>
<td>27.3 ± 19</td>
<td>5.0 ± 2.7</td>
</tr>
</tbody>
</table>
DISCUSSION

While perturbations in standard ECG-derived cardiac intervals have previously been observed during recovery from exercise to our knowledge no previous study has examined beat-to-beat cardiac interval dynamics during recovery from PSE that mimics routine competition in highly-trained endurance athletes. Furthermore, this study is the first to examine post-exercise cardiac interval dynamics concurrently with non-invasive measures of autonomic tone and biomarkers of myocyte injury. Overall, we found that PSE provoked a reversible cardiac autonomic imbalance as evidenced by changes in RR interval profiles. Furthermore cardiac interval dynamics (PR and QT interval parameters) were lengthened and less variable during the 60-min of immediate recovery from exercise. Rate-related increases in hs-cTnT concentration were observed during immediate recovery, however no associations were observed between hs-cTnT and perturbations in cardiac cycle dynamics. No significant changes were observed in electrolyte concentrations. Collectively, these findings suggest a suppressed parasympathetic and/or sustained sympathetic modulation of heart rate during the 60-min of immediate recovery, which occurred in parallel with alterations in atrial and ventricular conduction dynamics.

In the present study the lower RMSSD, total power and HFnu of RR intervals during immediate recovery reflects reduced parasympathetic cardiac modulation, while the increased LF:HF ratio suggests sustained sympathetic outflow, during 60-min of passive recovery from PSE. These findings are supported by studies utilising pharmacological autonomic blockade to determine if an autonomic imbalance manifests following exercise [35,36]. Our HRV observations are consistent with previous studies [5,6,37]; however, the magnitude and duration of post-exercise cardiac autonomic imbalance varies from 5 to 90 min between studies. The varied
duration and intensity of exercise protocols used in studies, in addition to differences in subject characteristics such as physical fitness, age, and sex may contribute to the differing results reported in the literature [5,37,38].

Interestingly, despite HRV parameters returning to baseline following 24h of recovery, QT and QTc intervals did not return to pre-exercise values. A post-exercise autonomic imbalance may therefore have varying effects on the SA node, AV node and the ventricles. The QTvi and QT/RR ApEn ratio (RR interval complexity decreased while QT interval complexity increased) increased during recovery, indicating a sustained sympathetic drive to the ventricles following PSE [10,11,21].

The PR interval, commonly used as an index of atrioventricular (AV) conduction time [39], was lengthened and less variable during the immediate 60-min of recovery following PSE. Paradoxically, sympathetic activity is known to facilitate AV conduction (i.e., shorten the PR interval) [8]. The QTc interval was also lengthened compared to pre-exercise, indicating that when controlled for heart rate cardiac electrical conduction was slowed following PSE. Given that parasympathetic withdrawal has a mediating effect on the QT-RR relationship following exercise [40], the increased QTc interval post-exercise may reflect an independent autonomic influence on the SA node and the ventricles; that is, the QT interval may be lengthened for a given heart rate in the presence of an autonomic imbalance [40]. Therefore, a delayed parasympathetic reactivation following exercise may cause a prolongation of depolarization and repolarization of the ventricles [41,42]. It is pertinent to note that the QT-RR relationship may be highly individual and careful interpretation is required; however, heart rate correction of the QT interval is reliable over a narrow band of resting heart rates (50 – 70 beats·min⁻¹) [43], which is consistent with the heart rate range assessed in this study.
Although this study and others [10,21,40] demonstrate that perturbations in cardiac
electrical activity during recovery from exercise largely reflect autonomic influences
on the myocardium, other mechanisms may have impacted on the current results.
Indeed, β-receptor desensitisation, which has been observed following PSE [44], may
present as a mechanism which could affect heart rate and cardiac conduction [44,45].
Alterations in cellular electrolytes (e.g. potassium, calcium, magnesium) are known to
influence myocyte action potentials and may be associated with adverse changes in
the ECG including widening of the QRS complex and AV conduction disturbances
[46,47]. For example, post-exercise changes in cardiac repolarization coincident with
hypokalemia and hypomagnesemia have been reported [48]. However, these studies
did not examine beat-to-beat cardiac interval dynamics or ECG-derived indicators of
autonomic tone. In the current study perturbations in atrial and ventricular conduction
dynamics (QT/RR ApEn, QTvi and, RMSSD and ApEn of the PR and QT intervals)
were observed despite no significant changes in electrolyte concentrations. Although
mild electrolyte changes were observed in some subjects, no associations between
electrolyte disturbances and cardiac cycle dynamics were observed. The potential
effects of these putative mediators on cardiac cycle dynamics remain to be fully
elucidated.

Several studies have demonstrated transient elevations in cTnT after PSE, suggesting
that extreme exercise may result in myocardial injury [49-51]. However, the
mechanisms responsible for exercise-induced cTnT release are not well understood;
whether this reflects permanent myocardial damage or temporary myocardial
‘stunning’ has been debated [17,52].

There are a number of potential pathobiological mechanisms for troponin release from
myocytes without the cell membrane being necrotic which may account for the
exercise induced release of troponin seen in this study. They include apoptosis with preserved membrane integrity associated where activation of caspases that mediate the cleavage of structural proteins leads to troponin release [53]. The formation and active secretion of membranous blebs during ischemia may also allow troponin to pass from intracellular to extracellular spaces without necrosis occurring. Once the cell is reperfused, these blebs would be reabsorbed or released into the circulation without rupture of the plasma membrane. It has been shown using cultured cardiac myocytes that the development of blebs during periods of anoxia, release cytosolic enzymes without the cell undergoing necrosis [54].

The release of cardiac troponins may also be the result of leakage from reversibly damaged myocardial membranes as intact non-degraded protein chains or by release of proteolytic troponin degradation products. It is presumed that troponins are degraded by matrix metalloproteinases (MMP) activated by integrin mediated myocardial stretch [55]. It has been shown that only 15 minutes of mild ischaemia can cause development of troponin I degradation products [56].

Studies using recently developed hs-cTnT assays, which lower the minimum detectable troponin level, have established that cardiac biomarker release during endurance exercise is a widespread phenomenon [19,51]. However, no studies have examined associations between hs-cTnT and cardiac cycle dynamics during recovery from PSE. In the present study a strong correlation was observed between exercising heart rate and hs-cTnT release suggesting exercise-induced cardiac biomarker release is rate-dependent. Although elevations in cardiac troponin were coincident with perturbations in cardiac cycle dynamics no significant associations were observed. Future research should examine the rate-dependent hs-cTnT and hs-cTnI relationship with functional cardiac measures.
The current study has implications for cardiac autonomic recovery from PSE and the occurrence of post-exercise arrhythmias. The QTvi represents ventricular repolarization lability [57], whereby an elevated QTvi (≥ -0.47) in patient populations is associated with ventricular arrhythmias and a high risk of sudden cardiac death [22,57]. While the elevated post-exercise QTvi (-1.24 ± 0.15) in the present study was well below that found in patient populations, one subject did record a peak post-exercise QTvi of -0.64. To what extent an elevated QTvi in healthy populations relates to the risk of sudden cardiac death is currently unknown. Future investigations should determine whether healthy populations unaccustomed to exercise render similar results to those found in highly-trained individuals, and if the capacity to perform prolonged high-intensity exercise impacts upon cardiac autonomic recovery.

The examined cardiac intervals were extracted from supine resting ECG recordings. Establishing HRV parameters and cardiac conduction dynamics during exercise would be valuable; however, ECG interval extraction is notoriously challenging during exercise, particularly at high intensities, and could negate the assumption of ‘stationarity’. Furthermore, the autonomic imbalance identified in the current study likely extended beyond 60-min; however, measures were not taken between 60-min and 24-hours post exercise. The time-course of post-exercise alterations in autonomic modulation and cardiac cycle dynamics should be investigated to clarify the duration of a cardiac autonomic imbalance that manifests following PSE.

**CONCLUSION**

Endurance trained athletes who perform an extended period of high-intensity exercise experience a prolonged delay in the reactivation of parasympathetic cardiac control and sustained sympathetic cardiac modulation and, concurrently, a rate-dependent
increase of cardiac troponin following exercise. Our findings indicate that the changes in cardiac interval dynamics are not constrained purely to parameters of heart rate variability, but that atrial and ventricular conduction dynamics are also transiently altered. Thus time-domain, frequency-domain and non-linear analyses of the PR and QT cardiac intervals may provide a means of monitoring atrial and ventricular conduction dynamics following PSE.
REFERENCES


CHAPTER 5.5

CARDIAC TROPONIN AND NON-CARDIAC ILLNESS: HIGH SENSITIVITY CARDIAC TROPONINS IN A CROSS-SECTIONAL STUDY IN A GENERAL HOSPITAL AND A COMMUNITY POPULATION

Part of this work has been submitted for publication in:

Potter JM, Simpson A, Koerbin G, Kerrigan J, Southcott E, Hickman PE.

Clinica Chimica Acta
**ABSTRACT**: New highly-sensitive assays for troponin can detect troponin in the blood of most healthy persons. No information is available relating to troponin concentrations in persons with non-cardiac illnesses. Presented here is a cross-sectional study looking at all persons who had samples referred to a general hospital-based pathology laboratory which deals primarily with acute hospital patients, but also with community family practices over a 24 hour period.

Over 800 persons were assessed. A large number of persons had detectable troponin in their blood. The highest concentrations were seen in persons in Intensive Care, Coronary Care and the Emergency Department. Many more persons had results above the 99th percentile than in a healthy population. The death rate was significantly higher (p<0.05) in persons without a history of pre-existing cardiac disease than in those with a history of cardiac disease.

With the new highly sensitive assays for cardiac troponin, many persons have detectable troponin in their blood and many have high concentrations even in the absence of overt cardiac illness. A high troponin concentration in itself does not provide diagnostic support to the physician of cardiac disease and must be interpreted in the clinical setting of the individual patient.
INTRODUCTION

The cardiac troponins are now well established as the first line investigation for the laboratory assessment of persons with presumed acute coronary syndromes [1]. However, the situation has become complex with the recent availability of highly sensitive assays for troponin which have shown that low concentrations of troponin are present in the blood of nearly all healthy persons, even children [2].

It has long been known that troponin concentration may rise in non-cardiac illnesses such as sepsis [3]. To date, there have been no systematic studies reported on troponin concentrations in non-cardiac illness, using the new high sensitivity assays for cardiac troponin (hs-cTn).

ACT Pathology provides pathology services to the Canberra region of approximately 500,000 persons, and this includes 2 general hospitals with approximately 750 acute general hospital beds, as well as to family practices in the Canberra region. We have performed a study in which we collected all samples referred to our laboratory for any investigations over a 24h period and measured the concentrations of cardiac troponins T and I in these samples, using high sensitivity assays. cTnI is our routine assay, so more detailed analysis was performed with regard to cTnI.

MATERIALS AND METHODS

This study was approved by the ACT Health Human Research Ethics Committee. Individual patient consent was not requested and this approval without obtaining participant consent was based on the recommendations of Section 2.3.6 of the National Statement on Ethical Conduct in Human Research (2007), particularly part b
“the benefits of the research justify any risk or harm associated with not seeking consent”, and part c “it is impracticable to obtain consent” [4].

PATIENT SAMPLES

ACT Pathology provides in-patient pathology services to the Canberra and Calvary tertiary referral hospitals, to out-patients associated with these hospitals and to family practitioners caring for non-hospital patients. We collected all samples referred to our service over a 24h period from all sources. If more than one sample was received from the same individual during the course of the study, only the first sample was utilised. After requested pathology was performed, cardiac troponin analysis was undertaken. This analysis was completed within 6 hours of collection. The remainder of the sample was separated and the aliquot of plasma frozen at -20C in case further analysis was required. If there were any significant differences between measured cTnI and cTnT (more than 3x the lower concentration), an aliquot of the stored samples was thawed, centrifuged at 10,000g for 10 minutes before repeat analysis was performed.

LABORATORY ANALYSES

Hs-cTnI was assayed on the Abbott Architect ci16200 analyser (Abbott Diagnostics, Sydney, Australia) using the pre-commercial Abbott ARCHITECT STAT hs-cTnI assay, with a limit of detection (LoD) of 1.0 ng/L, 20% coefficient of variation (CV) at 1.8 ng/L and a 10% CV at 3.9 ng/L [5]. Abbott report that the 99th percentile upper reference limit (URL) is 34.2 ng/L for males and 15.6 ng/L for females [6]. Our
routine sample type is lithium-heparin and this has a small negative bias compared to serum for hs-cTnI at concentrations <10 ng/L, well below the 99th percentile [5].

Hs-cTnT was measured using the adjusted hs-cTnT assay (lot number 16370401) on the Roche E411 analyzer (Roche Diagnostics, Sydney, Australia). Our initial evaluation of this assay was performed using lot numbers predating the downwards shift in subjects with low concentrations. In our hands this assay had an LoD of 5.0 ng/L, 20% CV at a concentration of 6.8 ng/L and a 10% CV at a concentration of 11.9 ng/L [7]. It is reported that the 99th percentile URL of 14.0 ng/L [8] has not changed with the recalibration of this assay [9].

**DATA HANDLING**

After analyses were completed, results were sorted by location of patient, as a guide to severity and type of illness. We reviewed the case notes of all subjects with cTnI concentrations above the relevant 99th percentile. Statistical analysis was undertaken using Analyse-it for excel.

**RESULTS**

After accounting for multiple samples from the one patient and in a small number of cases where there was insufficient sample for analysis, a total of 818 analyses were performed for hs-cTnI and 805 for hs-cTnT. All available non-duplicate samples were assayed and there was no selection or bias in terms of the samples assayed. Tables 5.5.1 and 5.5.2 show the results for males and females separately for hs-cTnI and hs-cTnT respectively according to patient location at the time of sample collection.
For all patient groups surveyed, males had a higher median concentration of both cTnI and cTnT than females. For cTnI, more than 75% of results were above the LoD, save for maternity (predominantly antenatal) and community female patients (also including antenatal) where approximately 50% had results above the LoD. Overall 93% of males had concentrations above the LoD and 70% of females. For cTnT, a much smaller number of persons had results above the LoD. Overall, 71% of males and 47% of females had concentrations of cTnT above the LoD.

Concentrations of both cTnT and cTnI varied greatly between different locations. Not surprisingly, the highest concentrations for both men and women, for both cTnT and cTnI were highest in patients in Intensive Care, Coronary Care and the Emergency Department.

Given the importance of early identification of acute coronary syndrome, samples arising from Emergency Department testing are of particular interest. As shown in Table 5.5.3, in this cross sectional review, there were 97 patients presenting, of whom troponin concentrations were requested in 21 (23%). Amongst those who had troponin requested the concentration of cTnI exceeded the gender-specific 99th percentile in two men and five women, and for cTnT in 6 males and 6 females. Where troponin was not requested there was a slightly lower degree of troponin results above the respective 99th percentiles. The derivation and absolute definition of a cut point of the 99th percentile particularly for cTnI is currently under debate, however application of a suggested lower concentration to be adopted by laboratories in Australia (agreed by consensus to be <26 ng/L male and <16 ng/L female) made no significant difference to interpretation.
From our total population, 41% of men and 24% of women had cTnT concentrations above the 99th percentile. Only 15% of men and 16% of women had cTnI concentrations above the 99th percentile. Because cTnI is our routine assay we limited our review of patient case notes to those patients with a cTnI above the 99th percentile. Table 5.5.4 summarises the clinical outcomes for these patients.
Table 5.5.1: cTnI concentration measured with a high-sensitivity assay, in a hospital and community practice population. All samples received over a 24h period were assayed. The limit of detection (LoD) for this assay is 1.0 ng/L, the 99th percentile for males is 34.2 ng/L and for women 15.6 ng/L.

<table>
<thead>
<tr>
<th>Location</th>
<th>n</th>
<th>Median (ng/L)</th>
<th>Q1 (ng/L)</th>
<th>Q3 (ng/L)</th>
<th>Range (ng/L)</th>
<th>&gt;LoD (n)</th>
<th>&gt;LoD (%)</th>
<th>&gt;99th centile (n)</th>
<th>&gt;99th centile (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hs=cTnI (MALE)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emergency Department</td>
<td>47</td>
<td>4.1</td>
<td>2.2</td>
<td>14.3</td>
<td>&lt;1.0 - 25560</td>
<td>45</td>
<td>96%</td>
<td>7</td>
<td>15%</td>
</tr>
<tr>
<td>Medical In-patient</td>
<td>67</td>
<td>10</td>
<td>3.3</td>
<td>24.9</td>
<td>&lt;1.0 - 1255</td>
<td>66</td>
<td>99%</td>
<td>14</td>
<td>21%</td>
</tr>
<tr>
<td>Surgical In-patient</td>
<td>61</td>
<td>8.8</td>
<td>4.1</td>
<td>28</td>
<td>&lt;1.0 - 1050</td>
<td>59</td>
<td>97%</td>
<td>13</td>
<td>21%</td>
</tr>
<tr>
<td>Intensive Care</td>
<td>24</td>
<td>45.2</td>
<td>5.9</td>
<td>2104</td>
<td>1.5 - 29500</td>
<td>24</td>
<td>100%</td>
<td>13</td>
<td>54%</td>
</tr>
<tr>
<td>Coronary Care</td>
<td>19</td>
<td>30.8</td>
<td>5.5</td>
<td>90.8</td>
<td>&lt;1.0 - 579</td>
<td>18</td>
<td>95%</td>
<td>9</td>
<td>47%</td>
</tr>
<tr>
<td>Oncology (In and Outpatient)</td>
<td>48</td>
<td>4.7</td>
<td>2.2</td>
<td>10</td>
<td>&lt;1.0 - 144</td>
<td>46</td>
<td>96%</td>
<td>3</td>
<td>6%</td>
</tr>
<tr>
<td>Maternity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outpatients &amp; Community</td>
<td>126</td>
<td>2.1</td>
<td>1.3</td>
<td>4.5</td>
<td>&lt;1.0 - 49</td>
<td>106</td>
<td>84%</td>
<td>1</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td>392</td>
<td>4.9</td>
<td>1.9</td>
<td>16.4</td>
<td>&lt;1.0 - 29500</td>
<td>364</td>
<td>93%</td>
<td>60</td>
<td>15%</td>
</tr>
<tr>
<td><strong>Hs-cTnI (FEMALE)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emergency Department</td>
<td>50</td>
<td>3</td>
<td>1</td>
<td>33.3</td>
<td>&lt;1.0 - 1027</td>
<td>38</td>
<td>76%</td>
<td>14</td>
<td>28%</td>
</tr>
<tr>
<td>Medical Inpatient</td>
<td>53</td>
<td>5.5</td>
<td>1.2</td>
<td>20</td>
<td>&lt;1.0 - 332</td>
<td>43</td>
<td>81%</td>
<td>17</td>
<td>32%</td>
</tr>
<tr>
<td>Surgical Inpatient</td>
<td>61</td>
<td>4.9</td>
<td>2.4</td>
<td>17.4</td>
<td>&lt;1.0 - 496</td>
<td>54</td>
<td>89%</td>
<td>17</td>
<td>28%</td>
</tr>
<tr>
<td>Intensive Care</td>
<td>14</td>
<td>7.8</td>
<td>4.2</td>
<td>17.2</td>
<td>1.5 - 1950</td>
<td>14</td>
<td>100%</td>
<td>4</td>
<td>29%</td>
</tr>
<tr>
<td>Coronary Care</td>
<td>12</td>
<td>24.2</td>
<td>7.1</td>
<td>55.3</td>
<td>1.0 - 2615</td>
<td>12</td>
<td>100%</td>
<td>7</td>
<td>58%</td>
</tr>
<tr>
<td>Oncology (In and Outpatient)</td>
<td>40</td>
<td>2.4</td>
<td>1.4</td>
<td>6.6</td>
<td>&lt;1.0 - 144</td>
<td>32</td>
<td>80%</td>
<td>4</td>
<td>10%</td>
</tr>
<tr>
<td>Maternity</td>
<td>23</td>
<td>1.1</td>
<td>&lt;1.0</td>
<td>1.9</td>
<td>&lt;1.0 - 16</td>
<td>12</td>
<td>52%</td>
<td>3</td>
<td>13%</td>
</tr>
<tr>
<td>Outpatients &amp; Community</td>
<td>173</td>
<td>1.1</td>
<td>&lt;1.0</td>
<td>2.5</td>
<td>&lt;1.0 - 34</td>
<td>94</td>
<td>54%</td>
<td>1</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td>426</td>
<td>2.2</td>
<td>&lt;1.0</td>
<td>6.7</td>
<td>&lt;1.0 - 2615</td>
<td>299</td>
<td>70%</td>
<td>67</td>
<td>16%</td>
</tr>
</tbody>
</table>
Table 5.5.2: cTnT concentration measured with a high-sensitivity assay, in a hospital and community practice population. All samples received over a 24h period were assayed. The limit of detection (LoD) for this assay is 5.0 ng/L, and the 99\textsuperscript{th} percentile for both males and females is 14.0 ng/L.

<table>
<thead>
<tr>
<th>Location</th>
<th>n</th>
<th>Median (ng/L)</th>
<th>Q1 (ng/L)</th>
<th>Q3 (ng/L)</th>
<th>Range (ng/L)</th>
<th>&gt;LoD (n)</th>
<th>&gt;LoD (5 ng/L)</th>
<th>&gt;99th centile (n)</th>
<th>&gt;99th centile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emergency Medicine</td>
<td>47</td>
<td>8.9</td>
<td>3.4</td>
<td>22.4</td>
<td>&lt;3 - 10000</td>
<td>30</td>
<td>64%</td>
<td>17</td>
<td>36%</td>
</tr>
<tr>
<td>Medical In-patient</td>
<td>65</td>
<td>23</td>
<td>9.9</td>
<td>52.1</td>
<td>&lt;3 - 412</td>
<td>54</td>
<td>83%</td>
<td>36</td>
<td>55%</td>
</tr>
<tr>
<td>Surgical In-patient</td>
<td>59</td>
<td>17</td>
<td>10.3</td>
<td>36.3</td>
<td>&lt;3 - 262</td>
<td>51</td>
<td>86%</td>
<td>34</td>
<td>58%</td>
</tr>
<tr>
<td>Intensive Care</td>
<td>25</td>
<td>28.7</td>
<td>15.8</td>
<td>409</td>
<td>&lt;3 - 4580</td>
<td>24</td>
<td>96%</td>
<td>20</td>
<td>80%</td>
</tr>
<tr>
<td>Coronary Care</td>
<td>19</td>
<td>33.4</td>
<td>8</td>
<td>77.8</td>
<td>&lt;3 - 196</td>
<td>17</td>
<td>89%</td>
<td>13</td>
<td>68%</td>
</tr>
<tr>
<td>Oncology (In and Outpatient)</td>
<td>48</td>
<td>11.6</td>
<td>6.6</td>
<td>19.2</td>
<td>&lt;3 - 96</td>
<td>40</td>
<td>83%</td>
<td>24</td>
<td>50%</td>
</tr>
<tr>
<td>Maternity</td>
<td>0</td>
<td>&lt;3</td>
<td></td>
<td></td>
<td>&lt;3 - 69</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outpatients &amp; Community</td>
<td>124</td>
<td>4.7</td>
<td>&lt;3</td>
<td>9</td>
<td>&lt;3 - 63</td>
<td>60</td>
<td>48%</td>
<td>16</td>
<td>13%</td>
</tr>
<tr>
<td>Total</td>
<td>387</td>
<td>10.6</td>
<td>4.4</td>
<td>28.7</td>
<td>&lt;3 - 10000</td>
<td>276</td>
<td>71%</td>
<td>160</td>
<td>41%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Location</th>
<th>n</th>
<th>Median (ng/L)</th>
<th>Q1 (ng/L)</th>
<th>Q3 (ng/L)</th>
<th>Range (ng/L)</th>
<th>&gt;LoD (n)</th>
<th>&gt;LoD (5 ng/L)</th>
<th>&gt;99th centile (n)</th>
<th>&gt;99th centile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emergency Medicine</td>
<td>48</td>
<td>4.2</td>
<td>&lt;3</td>
<td>26.3</td>
<td>&lt;3 - 260</td>
<td>24</td>
<td>50%</td>
<td>15</td>
<td>31%</td>
</tr>
<tr>
<td>Medical Inpatient</td>
<td>53</td>
<td>13.7</td>
<td>4.8</td>
<td>29</td>
<td>&lt;3 - 320</td>
<td>38</td>
<td>72%</td>
<td>25</td>
<td>47%</td>
</tr>
<tr>
<td>Surgical Inpatient</td>
<td>59</td>
<td>14.8</td>
<td>8.4</td>
<td>24.6</td>
<td>&lt;3 - 223</td>
<td>48</td>
<td>81%</td>
<td>30</td>
<td>51%</td>
</tr>
<tr>
<td>Intensive Care</td>
<td>14</td>
<td>7.7</td>
<td>4.9</td>
<td>17.6</td>
<td>&lt;3 - 869</td>
<td>10</td>
<td>71%</td>
<td>5</td>
<td>36%</td>
</tr>
<tr>
<td>Coronary Care</td>
<td>12</td>
<td>31.2</td>
<td>22.2</td>
<td>61.9</td>
<td>&lt;3 - 746</td>
<td>10</td>
<td>83%</td>
<td>9</td>
<td>75%</td>
</tr>
<tr>
<td>Oncology (In and Outpatient)</td>
<td>39</td>
<td>5.5</td>
<td>&lt;3</td>
<td>10.8</td>
<td>&lt;3 - 96</td>
<td>22</td>
<td>56%</td>
<td>6</td>
<td>15%</td>
</tr>
<tr>
<td>Maternity</td>
<td>23</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>&lt;3 - 52</td>
<td>3</td>
<td>13%</td>
<td>1</td>
<td>4%</td>
</tr>
<tr>
<td>Outpatients &amp; Community</td>
<td>170</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>5.2</td>
<td>&lt;3 - 65</td>
<td>43</td>
<td>25%</td>
<td>8</td>
<td>5%</td>
</tr>
<tr>
<td>Total</td>
<td>418</td>
<td>4.3</td>
<td>&lt;3</td>
<td>13.5</td>
<td>&lt;3 - 869</td>
<td>198</td>
<td>47%</td>
<td>99</td>
<td>24%</td>
</tr>
</tbody>
</table>
Table 5.5.3. Summary of Emergency Department requests

<table>
<thead>
<tr>
<th></th>
<th>cTnI</th>
<th>cTnT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;99th percentile</td>
<td>&gt;99th percentile</td>
</tr>
<tr>
<td>Troponin requested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
<td>2/12 (17%)</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>5/9 (56%)</td>
</tr>
<tr>
<td>Troponin not requested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>35</td>
<td>5/35 (14%)</td>
</tr>
<tr>
<td>Female</td>
<td>41</td>
<td>9/41 (15%)</td>
</tr>
</tbody>
</table>

Fourteen of 128 patients surveyed died during their current hospital admission. In our review we assessed whether patients had a significant history of cardiac disease (current cardiac diagnosis, past medical history of cardiac disease or co-morbidity with high risk of cardiac disease) or not. We found a significantly higher morbidity (chi-square 4.19, p<0.05) in persons without a history of cardiovascular disease. There was a significantly greater death rate in those without a previous history of heart disease, compared to those with. (Chi-square 4.19, p<0.05).

Table 5.5.4: Clinical assessment and mortality in all patients with cTnI above the 99th percentile

<table>
<thead>
<tr>
<th>Clinical categories in patients with cTnI &gt;99th percentile</th>
<th>Number of subjects</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current diagnosis cardiac related</td>
<td>44</td>
<td>3</td>
</tr>
<tr>
<td>Past medical history of cardiac disease</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>Co-morbidity with high risk of cardiac disease</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Malignancy – current or previous Rx</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>No history or risk factors as above</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Deaths</td>
<td>14</td>
<td>7</td>
</tr>
</tbody>
</table>
DISCUSSION

With the advent of the new high sensitivity cardiac troponin assays into routine clinical practice, it is evident that most healthy persons, even children, have low concentrations of circulating troponin present in their blood [2,10]. There has been a tendency, particularly amongst junior medical staff, to equate the simple presence of troponin as being indicative of the acute coronary syndrome. This background of “physiological” troponin will reinforce the requirement of a careful clinical assessment before calling for active cardiac management.

Data are starting to accumulate on the use of the new troponin assays in investigation of persons with cardiac disease [11-13]. It has long been known that the cardiac troponins may be increased in persons who are unwell with non-cardiac diseases [3]. To date there is no systematic information available on non-cardiac illness and the high sensitivity troponin assays.

The 99th percentile is used as an arbitrary cutpoint to identify persons at risk of adverse outcome in possible acute coronary syndromes [1]. We have demonstrated that many studies determining the 99th percentile contain persons with subclinical cardiac disease [14]. Given that it is increasingly recognised that there are numerous reasons for a raised troponin over and above primary cardiac disease per se, it is not surprising that a high incidence of raised troponins would be identified in this setting. Troponin in the setting of non-cardiac illness seems to reflect the total body burden of disease. Our data as shown in Table 4 indicate that many of these persons with increased cTnI have underlying cardiac disease, regardless of their presenting complaint. Interestingly, we found a higher in-hospital death rate in persons without current or a past history of cardiac disease. Whether this is real, and perhaps reflects some protective effect due to ischaemic pre-conditioning [15] or simply a chance association due to the small number of deaths observed in our study cannot be determined.
In this 24 hour overview, cTnI was detected in the majority of male and female samples. However, the number noted (93% of males and 70% of females) is likely to be understated as we have shown that at concentrations of cTnI<10 ng/L, there is a negative bias for Li-heparin plasma samples compared to serum. However, this bias disappears at concentrations >10 ng/L [5]. Our data for patient numbers above the 99th percentile are not affected, as at these higher concentrations there is no significant difference between different sample types.

We found that substantially more patients had results above the 99th percentile with the hs-cTnT assay (32.2%) than for the hs-cTnI assay (15.5%). Evidence is accumulating that many studies defining the 99th percentile may contain persons with subclinical cardiac disease and it appears that the 99th percentiles for cTnI in particular are too high [14].

In conclusion, we have performed a study looking at the concentrations of both cardiac troponins T and I in all patients referred to a pathology service over a 24 hour period. Our survey included hospital in-patients, hospital out-patients and persons attending family practitioners in the community. Troponin concentrations were higher in the sickest patients, regardless of their primary diagnosis, further supporting the concept that in settings other than the acute coronary syndrome, the cardiac troponin concentration reflects the severity of illness or burden of disease. It also emphasises the importance of interpretation of results in the context of the clinical setting.
REFERENCES

CHAPTER 5.6

A DATA MINING APPROACH FOR THE PROGNOSTIC EFFICACY OF TROPONIN I AND OTHER BIOMARKERS FOR PREDICTING A CORONARY EVENT WITHIN 30 DAYS OF AN EMERGENCY DEPARTMENT PRESENTATION.

ABSTRACT: Research to date using high-sensitivity troponin assays has mainly focused on early exclusion, or rule-out of acute myocardial infarction (MI). Use of data mining techniques to find useful patterns or previously undiscovered information in pathology laboratory databases may assist in the stratification of patient at risk of a Major Adverse Cardiac Event (MACE). This approach has become an increasingly popular tool for healthcare organizations to make decisions based on the analysis of their huge repositories of generated clinical data. This study uses that tool to investigate risk stratification in a group of emergency room patients presenting with chest pain. Cardiac troponin analysis was undertaken using the pre-commercial high sensitivity TnI assay from Abbott Diagnostics and analysed in conjunction with routine laboratory assays ordered in the course of clinical care. Data mining was undertaken using the statistical computing language, R. Troponin concentration decision points of 26 ng/L for non gender specific analysis, 14.0, 26.0 and 34.2 ng/L for males and 11.1 and 15.6 ng/L for females provide similar performance in risk stratification for a MACE. An algorithm using the presentation, 2 hour post presentation and their corresponding delta troponin concentrations provide an improved ability to stratify patients. The new high sensitivity cTnI assay shows superior performance in identifying patients at risk or MACE. Analysis demonstrates that it is also viable to develop a multi biomarker algorithm to aid clinicians in patient risk stratification.
INTRODUCTION

Cardiac troponin is now clearly established as the superior biomarker for evaluation of patients with possible acute myocardial infarction [1-3]. As the new high sensitivity assays for both troponin T (cTnT) and troponin I (cTnI) can measure to an order of magnitude lower than previously possible, clinicians have become concerned that with the advent of these new assays, that for the diagnosis of AMI, many patients may require unnecessary investigations because of detectable or elevated troponin concentrations in the peripheral circulation [4,5]. For optimum use of these high sensitivity assays, evidence is required to guide their integration into clinical practice. [6]. Research to date using high sensitivity troponin assays has mainly focused on early exclusion, or rule-out of acute myocardial infarction [2,3], but has not focused on the low risk group and defined the optimal methods of integrating assays into emergency department clinical practice pathways [4].

A recent study undertaken by Cullen [4] aimed to validate the newly available Abbott Diagnostics high sensitivity troponin (hs-cTnI) assay with an accelerated diagnostic pathway (ADP) for patients with possible acute coronary syndrome. Two multicentre emergency-department cohorts were investigated and the patient population was identified as low-risk for serious 30-day major adverse events.

Outcome studies using a priori methods are difficult to develop, undertake and report and are out of the scope of many clinical services and clinical laboratories. Hospital and health service had extensive repositories of information stored in databases such as medical and pathology records. Finding useful patterns or previously undiscovered information in these databases is referred to as data mining [7]. Data mining can be used to predict disease or prognosis as well as defining optimum patient management [8]. Data mining is a step in the knowledge discovery in databases (KDD) process that consists of applying data analysis and
algorithms to the data under analysis that produce patterns or models [9]. Data mining approaches have become an increasingly popular tool for healthcare organizations to make decisions based on the analysis of their huge repositories of generated clinical data.

**DECISION TREE**

A decision tree is a graphic representation of obtained knowledge in the form of a tree or flow chart, where each non-leaf node denotes a test on an attribute, and each branch indicates an output of the test [8,10]. It uses a combination of mathematical and computational techniques to aid description and classification, and to extract knowledge from a data set [10,11]. Because nodes and branches are organized hierarchically, they are easy to understand and interpret.

**RANDOM FOREST**

A random forest is a combination of tree predictors where the values of a random vector determine each tree. This vector is sampled independently and has the same distribution for all trees in that particular forest. To improve accuracy and lower generalised error, the randomness injected has to minimize the correlation between two different members of the forest averaged over the distribution, while maintaining strength.

If we consider the $k$th tree in a particular forest, a random vector $\Theta_k$ is generated, independent of the past random vectors $\Theta_1, \ldots, \Theta_{k-1}$ but with the same distribution. A tree is grown using this random vector, resulting in a classifier $h(x, \Theta_k)$ where $x$ is an input vector. Tree construction determines the nature and dimension of $\Theta$. After the generation of a large number of trees, the most popular class is determined; this is the random forest [12].
The simplest random forest with random features is formed by selecting at random, at each node, a small group of input variables. The tree is grown using classification and regression trees (CART) methodology to maximum size [12].

**SUPPORT VECTOR MACHINE (SVM)**

A Support Vector Machine is a high-dimension machine leaning method used to model and predict responses in linear and non-linear data [13]. SVM’s are learning machines constructed through an algorithm that uses an optimization criterion. The simplicity of the algorithm along with state of the art performance for many learning problems (classification, regression, and detection) as well as simple input without high cost computationally, has contributed to their popular use [13].

**PRINCIPAL COMPONENTS ANALYSIS (PCA)**

Principal components analysis transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. This analysis reduces the dimensions of a complex data set and can be used to visualize complex data. The first principal component accounts for the majority of the variability in the data. Each subsequent component accounts for decreasing proportions of the remaining variability [14,15].

**CLASSICAL MULTIDIMENSIONAL SCALING (MDS)**

Classical multidimensional scaling is similar to principal component analysis but it takes a dissimilarity matrix as input. A dissimilarity matrix shows the distance between every possible pair of objects. It is a means of visualizing the level of similarity of individual cases
of a dataset. A multidimensional scaling algorithm aims to place each object in \( N \)-
dimensional space where \( N \) is small, typically 2, such that the between-object distances are
preserved as well as possible. Each object is then assigned coordinates in each of the \( N \)
dimensions \([15,16]\).

Developing predictions using multiple biochemical or clinical markers is difficult. We used
these common predictive data mining methods in our analysis of the biochemical data. The
use of data mining applications such as SVM’s decision tree analysis, random forest, PCA
and MDS allows the ability to transform a large amount of complex data into patterns and
trends that may be used to enable, in this case, the clinician to identify more easily or earlier
patients at risk of MACE. The data used was obtained from patients recruited at a single site
(Brisbane) and included the cTnI concentration using the conventional cTnI assay from
Beckman Coulter (AccuTnI), obtained in 983/2544 subjects from the Cullen study \([4]\) and
the TnI concentrations using the pre-commercial high sensitivity TnI (hs-cTnI) assay from
Abbott Diagnostics in 875/2544 subjects. The results, from this modelling, were then
compared with the diagnostic classifications determined by Cullen \([4]\) of these patients into
MACE within 30 days or no cardiac event.

MATERIALS AND METHODS

PARTICIPANTS

The accelerated diagnostic protocol (ADP) was investigated as two sub-studies; the original
ADAPT study using sensitive troponin assays \([17]\) and the modified ADAPT study
incorporating the high sensitivity assay results. Patients that were recruited in Brisbane,
Australia were used in this study. These adult patients had at least five minutes of possible
cardiac symptoms in accordance with the American Heart Association case definitions [18]. Patient exclusion criteria included pregnancy, terminal illness, and patients under the age of 18 or where the patient was transferred from another hospital and in whom follow-up was considered impossible.

In this current data mining study we reviewed the results and outcomes from 983 participants (598 males, 385 females) using a conventional cTnI assay and 785 participants (469 males, 316 females), using a pre commercial hsTnI assay, from the original cohort of 2544 participants.

**PROCEDURES**

The primary endpoint was major adverse cardiac event within 30 days after initial presentation (including initial hospital attendance). The criteria for a MACE included death (excluding clearly non-cardiac), cardiac arrest, acute myocardial infarction, an emergency revascularization procedure, cardiogenic shock, ventricular arrhythmia needing intervention, and high-degree atrio-ventricular block needing intervention. The presence of a 30-day MACE was adjudicated independently. Adjudication of all cardiac endpoints was performed by two cardiologists with a third cardiologist in cases of disagreement. Cardiologists were masked to results of the index biomarkers under investigation, but had knowledge of the clinical record, ECG, and serial conventional cTnI results, from routine care.

In accordance with international guidelines, blood was drawn on presentation and at least 6 h later or as long as clinically indicated for troponin results that were used to determine the presence of myocardial necrosis [1]. These samples were analysed at the recruitment site laboratories and were the only troponin values used in patient management.
The diagnosis of acute myocardial infarction was based on evidence of myocardial necrosis together with clinical evidence of myocardial ischemia (ischemic symptoms, ECG changes or imaging evidence) in accordance with current guidelines [1].

In addition to sampling for routine clinical care, blood was drawn on presentation and two hours later for the ADAPT cohorts. Samples were immediately centrifuged and assayed using the Beckman Coulter AccuTnI assay (Beckman Coulter, Australia) troponin I assay. The AccuTnI assay has a LoD of 10 ng/L, 20% CV at 43 ng/L and a 10% CV at 104 ng/L [19]. Serum was then stored frozen at -70°C, within two hours of separation, prior to high-sensitivity TnI analysis using the Abbott ARCHITECT STAT hs-cTnI assay (Abbott Laboratories, Abbott Park, IL) with the following analytical characteristics: LoD at 1.0 ng/L, 20% CV at 1.8 ng/L and a 10% CV at 3.9 ng/L [20]. Samples were thawed, mixed, and centrifuged for 30 minutes at 3,000 RCF and 4°C prior to analysis. The hsTnI assay has a combined 99th percentile concentration of 26.2ng/L with a male and female 99th percentile of 34.2 ng/L and 15.6 ng/L respectively with a corresponding co-efficient of variation (CV) of <5% and a limit of detection of 1.2ng/L [21,22]. Long-term stability of cTnI has been demonstrated previously by Kavsak [23]. Imprecision (CV) using the manufacturer’s QC ranged from 3.5% at 19.90ng/L to 2.20% at 14600ng/L (n=31,33) for the hs-cTnI assay.

DATA MINING

Decision tree (R-rpart library, 4.1-3), PCA (R-PCA, pcaPP 1.9-49), classical MDS (R-MDS, MDSGUI 0.1.1), random forest (R-random forest, randomForest,4.6-7), and SVM (R-SVM library, e1017) were analysed using the statistical computing language package R version 2.1.15[10]. Data was partitioned as all participants and gender specific participants, with the outcome assessment as no cardiovascular disease, or MACE. Patients who did not have a
MACE but were identified having a stable cardiac condition were also assessed. cTnI was used as the primary indicator at presentation (time 0), at 2 hours post presentation and 6 hours post presentation (conventional assay only). The delta change in troponin concentration at 2 hours and 6 hours (conventional assay only) from the concentration at presentation was also used as a primary marker. Not all biochemical markers were available for all time points.

RESULTS

Of the 983 subjects studied using the Beckman assay, 348 (230 males, 118 females) had a confirmed cardiac event, and 635 no cardiac event. Decision tree analysis identified a cut point in troponin at \( \geq 55 \) resulting in an observed cardiac event rate of 91.1%. We applied decision tree analysis to those 983 subjects with and without gender separation and calculated the sensitivity, specificity, PPV and NPV using the troponin concentration determined by that decision tree analysis. These results are shown in Table 5.6.1.

Random forest analysis showed that the hs-cTnI assay concentration at 2 hours post presentation and at presentation have a much higher significance than all other biomarkers analysed in determining outcome (MACE). Patient age and the 6 hour post presentation conventional cTnI assay concentration have a marginally greater significance than other biochemical and haematological parameters and gender appears to have minimal impact. Figure 5.6.1 shows the random forest analysis.
Table 5.6.1 Combined and gender specific sensitivity, specificity, PPV and NPV values (%) obtained when comparing patients with no cardiac condition and those who had a confirmed MACE using decision tree defined conventional cTnI concentration decision points.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Combined Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At presentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥55 ng/L</td>
<td>26.4</td>
<td>98.5</td>
<td>91.1</td>
<td>71</td>
</tr>
<tr>
<td>2 hours post presentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥55 ng/L</td>
<td>30.5</td>
<td>83.1</td>
<td>93.8</td>
<td>60.7</td>
</tr>
<tr>
<td><strong>Delta presentation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- 2 hours post</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥35 ng/L</td>
<td>18.7</td>
<td>100</td>
<td>100</td>
<td>69.1</td>
</tr>
<tr>
<td><strong>Gender Specific</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 hours post presentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(≥55 ng/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>30.9</td>
<td>96.7</td>
<td>94.7</td>
<td>69.6</td>
</tr>
<tr>
<td>Females</td>
<td>34.7</td>
<td>97.8</td>
<td>85.4</td>
<td>77.4</td>
</tr>
</tbody>
</table>

Figure 5.6.1. Random Forest analysis of variable importance for discriminating patients with MACE versus patients with non cardiac condition.

X-axis: shows increasing degree of significance; y-axis shows the analyte
Partitioning of the 785 subjects studied using the Abbott hs-cTnI assay was undertaken resulting in 60 subjects (41 males, 19 females) with a confirmed cardiac event (MACE) which included cardiac arrest, cardiogenic shock, STEMI, NSTEMI, ventricular arrhythmia, high AV block and unstable angina. 186 subjects (119 males, 67 females) had a confirmed stable cardiac condition which included heart failure, stable coronary artery disease or other non specified cardiovascular problems. There were 13 patients in this group, 9 males and 4 females, who required revascularisation. 539 (309 males, 230 females) had no cardiac event or condition. We calculated event rates (table 5.6.2) for those subjects without gender separation and stratifying using the 99th percentile non gender specific troponin concentration recommended by the manufacturer [22], ≥26 ng/L, as the decision point at presentation (Time 0) and at 2 hours post presentation.

We also applied partitioning using the manufacturer’s recommended gender 99th percentile concentrations [22] of 15.6 ng/L for females and 34.2 ng/L for males. We also applied partitioning to the male cohort using the decision point of 26 ng/L as agreed and instituted by consensus in Australian and New Zealand laboratories (personal communication). Further 99th percentile decision points as demonstrated by Koerbin [20] of 11.1ng/L for females and 14.0 ng/L for males were also analysed.
Table 5.6.2. Event rates (%) for patients with identified MACE, stable cardiac conditions and no cardiac condition shown at the manufacturer [22] and literature [20] identified hs-cTnI concentration decision points.

<table>
<thead>
<tr>
<th>Combined</th>
<th>MACE</th>
<th>Non Cardiac</th>
<th>Stable Cardiac Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>At presentation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥26.0 ng/L</td>
<td>93.0</td>
<td>1.9</td>
<td>16.2</td>
</tr>
<tr>
<td>&lt;26.0 ng/L</td>
<td>7.0</td>
<td>98.1</td>
<td>83.8</td>
</tr>
<tr>
<td><strong>2 hours post presentation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥26.0 ng/L</td>
<td>86.7</td>
<td>1.5</td>
<td>18.3</td>
</tr>
<tr>
<td>&lt;26.0 ng/L</td>
<td>13.3</td>
<td>98.5</td>
<td>81.7</td>
</tr>
</tbody>
</table>

**Females**

| At presentation | | | |
| ≥15.6 ng/L | 68.4 | 17.8 | 68.7 |
| <15.6 ng/L | 31.6 | 82.1 | 31.3 |
| ≥11.1 ng/L | 78.9 | 13.0 | 70.1 |
| <11.1 ng/L | 21.1 | 77.0 | 29.9 |

**Males**

| At presentation | | | |
| ≥34.2 ng/L | 75.6 | 6.8 | 17.6 |
| <34.2 ng/L | 24.6 | 93.2 | 82.4 |
| ≥26.0 ng/L | 80.4 | 9.4 | 18.5 |
| <26.0 ng/L | 19.6 | 90.6 | 81.5 |
| ≥14.0 ng/L | 85.3 | 13.9 | 21.0 |
| <14.0 ng/L | 14.7 | 86.1 | 79.0 |

The combined and gender specific population sensitivity, specificity and predictive values for patients where there was a confirmed cardiac for the various manufacturer or literature determined troponin concentration decision points is shown in table 5.6.3.
Table 5.6.3. Combined and gender specific sensitivity, specificity, PPV and NPV values (%) obtained for those patients who had a confirmed MACE using manufacturer [22] defined hs-cTnI concentration decision points: combined gender, 26.0 ng/L; male, 34.2 ng/L and female 15.6 ng/L and literature [20] decision points of male, 14.0 ng/L and female 11.1 ng/L .

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Combined</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At presentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥26.0 ng/L</td>
<td>93.3</td>
<td>98.1</td>
<td>84.8</td>
<td>99.2</td>
</tr>
<tr>
<td>2 hours post presentation</td>
<td>86.7</td>
<td>98.5</td>
<td>86.7</td>
<td>98.5</td>
</tr>
<tr>
<td><strong>Males at presentation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥14.0 ng/L</td>
<td>85.3</td>
<td>86.1</td>
<td>44.9</td>
<td>97.8</td>
</tr>
<tr>
<td>≥26.0 ng/L</td>
<td>80.5</td>
<td>90.6</td>
<td>53.2</td>
<td>97.2</td>
</tr>
<tr>
<td>≥34.2 ng/L</td>
<td>75.6</td>
<td>93.2</td>
<td>59.6</td>
<td>96.6</td>
</tr>
<tr>
<td><strong>Females at presentation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥11.1 ng/L</td>
<td>78.9</td>
<td>77</td>
<td>22.1</td>
<td>97.8</td>
</tr>
<tr>
<td>≥15.6 ng/L</td>
<td>68.4</td>
<td>82.2</td>
<td>24.0</td>
<td>96.9</td>
</tr>
</tbody>
</table>

The combined and gender specific population sensitivity, specificity and predictive values for patients with a confirmed stable cardiac condition at the various manufacturer or literature determined troponin concentration decision points is shown in table 5.6.4.
Table 5.6.4. Combined and gender specific sensitivity, specificity, PPV and NPV values (%) obtained for those patients with a stable cardiac condition using manufacturer [22] defined hs-cTnI concentration decision points: combined gender, 26.0 ng/L; male, 34.2 ng/L and female 15.6 ng/L and literature [20] decision points of male, 14.0 ng/L and female 11.1 ng/L.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At presentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥26.0 ng/L</td>
<td>18.4</td>
<td>98.1</td>
<td>77.3</td>
<td>77.8</td>
</tr>
<tr>
<td>2 hours post presentation</td>
<td>16.1</td>
<td>98.5</td>
<td>78.9</td>
<td>76.9</td>
</tr>
<tr>
<td>Males at presentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥14.0 ng/L</td>
<td>21.1</td>
<td>86.1</td>
<td>36.8</td>
<td>73.9</td>
</tr>
<tr>
<td>≥26.0 ng/L</td>
<td>18.5</td>
<td>90.6</td>
<td>43.1</td>
<td>74.3</td>
</tr>
<tr>
<td>≥34.2 ng/L</td>
<td>17.6</td>
<td>93.2</td>
<td>50.0</td>
<td>74.6</td>
</tr>
<tr>
<td>Females at presentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥11.1 ng/L</td>
<td>70.1</td>
<td>77.0</td>
<td>47.0</td>
<td>89.8</td>
</tr>
<tr>
<td>≥15.6 ng/L</td>
<td>68.7</td>
<td>82.1</td>
<td>52.9</td>
<td>90.0</td>
</tr>
</tbody>
</table>

Using an absolute change determined by decision tree analysis of 100 ng/L in troponin I concentration between presentation and the 2 hour post presentation, in those patients where we have a known MACE and those with no cardiac condition we obtain a sensitivity of 51.7%, specificity and positive predictive value of 100% and negative predictive value of 94.9%. Of the 186 patients with a stable cardiac condition only 3 had a delta troponin of >100 ng/L and these patients all required revascularisation.

We then allowed decision tree analysis to identify non gender specific cut points at time of presentation and the change in troponin concentration from presentation to 2 hours post presentation. The troponin concentrations identified were 11.0 ng/L at time of presentation;
the delta was 17.5 ng/L. The decision trees for these are shown in figures 5.6.2 and 5.6.3.

Table 5.6.5 shows the sensitivity, specificity, PPV and NPV values obtained. The non gender specific troponin concentration determined by decision tree analysis on presentation of 11.0ng/L gave similar sensitivity, specificity, and PPV to the combined gender troponin concentration of 26.0 ng/L recommended by the manufacturer. The PPV was slightly worse. The delta cTn concentration of 17.5 ng/L showed improved specificity, PPV and NPV over the 2 hour troponin concentration of 26.0 ng/L suggesting that it may be a more useful parameter than the single 2 hour post presentation analysis.

Figure 5.6.2. Decision tree: cTnI at presentation.

Figure 5.6.3. Decision tree: delta cTnI (from presentation to 2 hours post presentation)
Table 5.6.5. Combined gender sensitivity, specificity, PPV and NPV values (%) obtained for those patients with MACE and stable cardiac condition using decision tree defined cutpoints: at presentation, 11.0 ng/L; 2 hour delta, 17.5 ng/L.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MACE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At presentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥11.0 ng/L</td>
<td>93.3</td>
<td>97.1</td>
<td>69.2</td>
<td>99.2</td>
</tr>
<tr>
<td>Delta (presentation - 2 hours post)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥17.5 ng/L</td>
<td>75.0</td>
<td>99.6</td>
<td>95.7</td>
<td>97.2</td>
</tr>
<tr>
<td><strong>Stable Cardiac Condition</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At presentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥11.0 ng/L</td>
<td>30.4</td>
<td>97.1</td>
<td>69.5</td>
<td>79.9</td>
</tr>
<tr>
<td>Delta (presentation - 2 hours post)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥17.5 ng/L</td>
<td>5.3</td>
<td>99.6</td>
<td>83.3</td>
<td>75.3</td>
</tr>
<tr>
<td><strong>Combined</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At presentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥11.0 ng/L</td>
<td>45.9</td>
<td>95.3</td>
<td>81.9</td>
<td>79.4</td>
</tr>
<tr>
<td>Delta (presentation - 2 hours post)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥17.5 ng/L</td>
<td>22.3</td>
<td>99.6</td>
<td>96.5</td>
<td>73.8</td>
</tr>
</tbody>
</table>

Combinations of biomarkers are frequently used to diagnose medical conditions, determine prognosis and guide therapies. The determination of a MACE is no different with most clinicians using troponin as a biochemical marker in combination with patient history and other parameters such as TIMI score and ECG. To date a combination of biochemical markers has not routinely been used. As seen in the random forest analysis, troponin concentration at presentation and 2 hours post presentation shown a greater ability to differentiate patients who have had a MACE from those with no cardiac event. Using PCA, an algorithm that included the troponin concentration at presentation, at 2 hours post presentation and the delta troponin between these two time periods was generated. This
algorithm uses 26.1% of the presentation troponin, 80% of the 2 hour troponin and 53.9% of the delta troponin.

If we sum these 3 parameter (0.261 x presentation cTnI, 0.800 x 2 hour presentation cTnI and 0.539 x delta cTnI) determine the median and interquartile ranges for each of the categories of patients, MACE, stable cardiac condition and no cardiac condition we see very good discrimination between the MACE and non cardiac cohorts. The discrimination between the stable cardiac and the non cardiac cohort is less well defined. (Figure 5.6.4.)

![Log Troponin algorithm](image)

**Figure 5.6.4.** Median and inter quartile troponin concentration range (ng/L), shown on a log scale, for patients with observed MACE, stable cardiac conditions and no cardiac events. Dot = median troponin concentration; Bar = inter quartile range.
The receiver operator curve (ROC) for patients who had a confirmed MACE within 30 days of presentation and for those patients with a stable cardiac condition is demonstrated in figure 5.6.5. Using this curve a decision point of 32.3 units provides sensitivity of 98.9% (95%CI 97.6-99.6), specificity of 95.0% (95%CI 86.1-99.0), and a likelihood ratio of a 19.78 identifying those patients with a MACE from those patients with no cardiac condition. Using the curve of those patients with a stable cardiac condition showed a significantly poorer discrimination. A decision point of 5.0 units provided the best sensitivity, 77.2% (95%CI 73.5-80.7) and specificity, 54.8% (95%CI 47.4-62.1), with a likelihood ratio of 1.71.

Figure 5.6.5. ROC curve using PCA determined troponin algorithm. The cohort of patients with a stable cardiac condition is represented in blue. The MACE cohort is represented in green. AUC: MACE = 0.99, Stable cardiac condition = 0.73
A further random forest analysis identified that the log of the 2 hour troponin concentration, platelet count and white cell count as factors to be considered. The abbreviated random forest analysis is shown in figure 5.6.6.

Figure 5.6.6. Abbreviated random forest analysis of variable importance for discriminating patients with stable cardiac condition from those patients with MACE or no cardiac condition. The key predictors, log hs-cTnI, white cell count (WBC) and platelets (Plt) are shown only. x-axis: shows increasing degree of significance; y-axis shows the analyte.

To attempt to further differentiate those patients with a stable cardiac condition from the cohort of patients with a MACE, R software was employed to develop an SVM (Figure 5.6.7.) where a troponin concentration decision point of 29 ng/L or equal to 1.45 Log_{10} was obtained. As the platelet count increases, the stable cohort cTnI concentration at 2 hour post presentation also increases. A 10-fold cross validation produced an accuracy of 86.2%.
Figure 5.6.7. SVM classification plot of patients with a stable cardiac condition and those with MACE at 2 hours post presentation. x-axis cut point for the categories MACE (1, blue area) versus stable cardiac condition (3, pink area) The red components represent individual stable cardiac patient data, the black components represent the MACE patient data. X = support vector, o = data point. The SVM has been normalised for age and with a constant white cell count of 10.0*10^9/cells/L.
DISCUSSION

Limitations associated with this study were that the clinical outcomes of the patients used were adjudicated using the 0 and >6 hour troponin values based on sensitive (and not highly-sensitive) troponin assay results. This will affect the final diagnosis of those patients with a diagnosis of NSTEMI and unstable angina.

The study does however demonstrate the superior efficiency of the new hs-cTnI assay from Abbott Diagnostics over the conventional cTnI assay from Beckman Coulter in identifying patients at risk of a MACE within 30 days of presentation in an emergency room setting. This efficiency is demonstrated with hs-cTnI assay have superior sensitivity and specificity to the conventional cTnI assay. It also highlights the difficulty of stratifying risk in patients who have a known stable cardiac condition.

The descriptive statistics using a hs-cTnI decision point of 26 ng/L, shows equivalent analysis at both presentation and 2 hours post presentation to the outcomes of the study by Cullen [4] where they reported a sensitivity of 82.1-91.9%, specificity of 91.8-93.1%, PPV of 67.5-70.5% and NPV of 96.3-98.5% with patients who had a confirmed MACE and a cTnI concentration > 26.2 ng/L at either presentation or 2 hours post presentation using the Abbott pre-commercial high sensitivity assay. The results obtained here suggests that either the presentation or 2 hour cTnI concentration has equivalent clinical utility, but, use of the at presentation cTnI concentration may allow earlier intervention improving patient outcome.

We have demonstrated that for males, the presentation troponin concentrations of ≥14.0, ≥26.0 and ≥34.2 ng/L have similar performance in their ability to predict or rule out a 30 day MACE. For females the decision points at presentation of ≥11.1 and ≥15.6 ng/L also provide similar performance.
This study demonstrates the difficulty in using only biochemical markers such as cTnI concentrations as decision points in identifying those patients with a stable cardiac condition at risk of MACE. Neither the decision tree selected or the manufacturer or agreed troponin concentration cutpoints provided adequate discrimination to reliably use troponin alone. For females the concentrations selected do provide a reasonably sensitivity and specificity but show less useful predictive values. The results indicate that for males, a troponin concentration on its own has limited use.

Using PCA analysis identified an algorithm using troponin concentrations at presentation and 2 hours post presentation in conjunction with their delta concentration that shows equivalent or slightly better sensitivity and specificity that a single troponin concentration at either presentation or 2 hours post presentation, in aiding the stratification of risk of a MACE. The superior sensitivity and specificity that this algorithm offers needs further assessment to confirm its usefulness. A further prospective confirmatory study would be required.

**CONCLUSION**

This study, using decision tree analysis, demonstrates that the new Abbott hs-cTnI assay has superior performance compared with the conventional Beckman Coulter TnI assay in aiding the clinician in identifying patients with chest pain at risk of a MACE within 30 days of presentation to an emergency department. PCA analysis identified that the use of a multi-marker approach to identify risk is viable and further investigations to confirm its utility. Additional prospective studies using this suggested multi-marker approach and in combination with other potential markers is warranted.
REFERENCES


17. Than M, Cullen L, Aldous S, Parsonage WA, Reid CM, Greenslade J et al. 2-Hour Accelerated Diagnostic Protocol to Assess Patients With Chest Pain Symptoms Using


CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

Although there are guidelines for use of cTn in myocardial infarction there continues to be discussion regarding the use of troponin [1] in the diagnosis and prognosis of myocardial events. Evidence has been presented that some of the assumptions made in developing these guidelines may now require revision. In particular, questions such as does the presence of cTn always indicate necrosis? What is the significance of elevated cardiac troponin in the non-ACS setting and how should we use troponin in investigating the acute coronary syndrome (ACS) should be asked.

The aims of this thesis were to investigate these assumptions regarding cardiac troponin (cTn) in the circulation and its use in laboratory medicine.

A process can only be understood when that process can be well measured [2]. The availability of troponin assays with improved analytical sensitivity is allowing us to ask those questions regarding the pathways from myocardial health to myocardial damage.

There are differences in analytical performance of cardiac troponin assays [1]. The new high sensitivity assays provide improved precision by decreasing the analytical threshold of the assay, however there has been a fear that this improvement has come at the expense of decreased diagnostic specificity. The studies outlined in chapter 3 of this thesis, offer guidelines in determining the analytical characteristics of troponin assay and demonstrate that these new assays meet the most stringent criteria of Apple’s scorecard grading system that considers both precision and analytical sensitivity [3].

Studies offered in chapter 4 of this thesis demonstrate detectable concentrations of cardiac troponin in the general population including children. The detection of elevated troponin concentrations in asymptomatic and symptomatic patients not due to MI has started to
confuse physicians. As may be expected, with this improved analytical sensitivity and precision, the lower diagnostic threshold of troponin will increase the diagnostic sensitivity for myocardial injury but may decrease the diagnostic specificity for MI. Chapter 4 provides a study demonstrating the appropriate population selection for determining decision points such as the 99th percentile URL, highlighting the need for both laboratory and clinical parameters for appropriate partitioning. This chapter also supports the opinion that elevations in troponin concentrations are not always associated with myocyte necrosis, demonstrated by transient increases in troponin concentration in children with seasonal illness. These transient elevations are also demonstrated in a study in chapter 5 where elite athletes undertaking strenuous exercise showed 10 fold increases in troponin concentration one hour into recovery after a constant-load exercise bout for 2 hours with a return to baseline concentrations within 24 hours of cessation of that exercise.

A number of possibilities have been suggested for release of structural proteins from the myocardium. As well as myocardial necrosis, they include normal turnover of myocardial cells, apoptosis, cellular release of troponin degradation products and increased cellular wall permeability. In chapter 5 of this thesis, the formation and release of membranous blebs is offered as an additional hypothesis.

The effect on the clinical performance of the improved Abbott Diagnostics hs-cTnI assay is demonstrated with the early detection and stratification of patients with MACE from those with no cardiac disease is demonstrated using the data obtained by Cullen [4] and applying statistical analysis. This study demonstrated, using a variety of different troponin concentrations decision points at presentation and 2 hours post presentation, that this assay has a superior ability to identify those patients with MACE than conventional cTn assays. The development of an algorithm using a combination of presentation, 2 hours post presentation and the delta change concentrations between those two time points is viable and
has possibilities to aid in patient stratification improving early rule in and rule out of suspected MI and a more accurate and sensitive diagnosis of MI. The use of such algorithms requires scrutiny and a high level of professional experience for proper interpretation.

The improved analytical performance of troponin assays demonstrated in this thesis is also highlighted in the improved identification of ESRD patients for all cause mortality.

PRACTICAL RECOMMENDATIONS PROVIDED BY THE STUDIES IN THIS THESIS

- Laboratories should adopt the recommended guidelines for validation and verification of troponin assays as offered in this thesis.
- General health as assessed by questionnaire alone is insufficient in selecting a population for the determination of clinical decision points. In addition laboratory measures as well as clinical measures, in particular echocardiography should be used.
- Gender specific decision points should be considered in laboratories using high sensitivity troponin assays
- Development of a multi marker algorithm is viable to aid in the stratification of patients at risk of MACE, however these algorithms will need to be compared against hsTnI alone.

THE FUTURE

Troponin assays now have the sensitivity to detect troponin in nearly all persons. That troponin is in physiological concentration in a healthy population affords the opportunity to review the recommended 99th percentile decision point for MI.
The concept and use of the 99th percentile URL for cTn as a decision point was introduced with the redefinition of myocardial infarction in 2000. At this time few assays could detect troponin at that level and none could meet the criteria of an assay CV of 10% at this concentration. This concept is flawed in that it uses an arbitrary cut point inserted into a continuum of disease. Evidence in this thesis suggests that biological variation of troponin is small and significant changes could occur within the 99th percentile and that transient non-cardiac illness, age, male gender and subclinical cardiovascular disease may all elevate the 99th percentile artifactually. Subclinical cardiovascular diseases may also elevate the 97.5th percentile artifactually albeit less so. Additionally lack of assay harmonisation and problems with population selection, make the 99th percentile an unreliable boundary to use in assessing patients with presumed ACS. If we were to do away with the 99th percentile, what would we put in its place?

Gaussian or near Gaussian distributions have been noted in adult and paediatric populations. It appears that truly healthy populations have an underlying concentration of troponin that reflects physiology rather than pathology and that assessment of health or otherwise in a population should assess how closely that population lies to a Gaussian distribution. If Gaussian, then perhaps the standard procedure for setting reference intervals, namely taking the central 95% should be adopted [5]. This will result in 2.5% having an abnormally high troponin and will cause some anxiety. This anxiety results from many years where troponin concentrations have been used inappropriately as an absolute diagnostic test, rather than an aid to diagnosis.

In an appropriate clinical setting, looking for a delta change in troponin may be the best way to define the presence of an acute coronary syndrome. Whether this delta change is an absolute increment or a percentage change, and over what time period the delta change should be calculated, remains to be clarified [5].
Further studies are needed using serial sampling before it can be concluded that gender-specific cutpoints are needed to optimize the clinical care in patients with acute ischaemic heart disease. Jaffe [6] has suggested that use of these gender specific cutpoints would improve specificity in men and sensitivity in women. Kavsak [7] demonstrated no difference in the median troponin I concentrations between male and female hospital mortality. This suggests that gender-specific cutpoints may not be significant in more diverse groups of acutely ill patients. However this also begs the question regarding the utility of gender specific cutpoints in the chronically ill patients. Further studies are required to investigate the utility of gender specific cutpoints for cardiac troponin.

The discussion between cardiologists, emergency physicians, pathologists and scientists on the most appropriate decision point should now commence.

That we are now able to reliably detect troponin in the peripheral circulation of the general population also provides a vehicle to look into mechanisms of possible associations of troponin antibodies with cellular damage [8]. As Popp has commented, “It would be fascinating if cTn is proved to be an actor in this process of ongoing myocardial injury and not simply a messenger of that injury” [2].
REFERENCES